UPPSALA
UNIVERSITET

Digital Comprehensive Summaries of Uppsala Dissertations from the Faculty of Medicine 1788

Exploring the interactions of antibiotic combinations against multidrug-resistant Gram-negative bacteria

ANNA OLSSON

ACTA
UNIVERSITATIS
UPSALIENSIS
UPPSALA
2021

ISSN 1651-6206
ISBN 978-91-513-1340-5
URN urn:nbn:se:uu:diva-457615

Dissertation presented at Uppsala University to be publicly examined in Hubben, föreläsningssal 2, plan 2, Dag Hammarskjölds Väg 38, Uppsala, Friday, 17 December 2021 at 13:00 for the degree of Doctor of Philosophy. The examination will be conducted in English. Faculty examiner: Assistant Professor Joseph Meletiadis (Attikon University General Hospital, Medical School, National and KapodistrianUniversity of Athens).

Abstract

Olsson, A. 2021. Exploring the interactions of antibiotic combinations against multidrug-resistant Gram-negative bacteria. *Digital Comprehensive Summaries of Uppsala Dissertations from the Faculty of Medicine* 1788. 70 pp. Uppsala: Uppsala University. ISBN 978-91-513-1340-5.

Antimicrobial resistance is emerging and available treatment options are lacking. Antibiotics is a cornerstone in modern medicine where medical procedures such as surgery, care of premature babies or treatment of cancer is dependent on efficient drugs. The need for novel treatment alternatives is increasing as bacteria continue to develop new resistance mechanisms.

The main goal of this thesis was to screen for antimicrobial combinations efficient against Gram-negative bacteria. The complex membrane structure of Gram-negative bacteria is very protective against antimicrobial activity making many antibiotics ineffective. Polymyxin B was therefore used as a main component in the combinations evaluated due to its membrane disruptive mode of action. Previously neglected or disused antibiotics was used in combination with polymyxin B as a part of a Joint Programming Initiative on Antimicrobial Resistance (JPIAMR) called CO-ACTION. The prevailing theory of polymyxin B combinations is that the membrane disruptive effect will facilitate entry of a second antibiotic and reduce efflux activity. In contrast, the combination will not be as efficient in the presence of bacterial enzymatic activity as the resistance mechanism is not affected by changes in the membrane composition. To increase knowledge on underlying mechanisms behind the success of antimicrobial combinations an extensive genetic analysis was performed.

Several promising polymyxin B combinations were found which could offer a treatment option in caring for severely ill patients for which few alternatives exist. Associations between genetic background and efficient bacterial killing was also established. The chance of synergistic effect by the combination was increased if the antibiotic used in combination with polymyxin B could normally not enter the bacterial cell or in presence of resistance mechanisms increasing efflux activity. This thesis highlights the fact that only phenotypical antimicrobial susceptibility testing would not be used in forecasting the success of antimicrobial combinations. Information on antimicrobial susceptibility in combination with knowledge on resistance mechanisms present and how it influences the antibiotics used in combination is equally important.

With this work increased knowledge on genetic background of resistance mechanisms and bacterial killing by polymyxin B combinations and was provided. Antimicrobial combinations offer an interesting feature when no other treatment alternatives are available. The lack of diagnostics in forecasting the success of combination therapy in a clinical microbiology lab is of concern. The knowledge obtained in this work contributes to the general knowledge on antimicrobial combinations and provides an example of how to evaluate their effect.

Keywords: Gram-negative bacteria combination therapy antimicrobial resistence

Anna Olsson, Department of Medical Sciences, Infection medicine, Akademiska sjukhuset, Ingång 30, våning 1, Uppsala University, SE-751 85 Uppsala, Sweden.

ISSN 1651-6206
ISBN 978-91-513-1340-5
URN urn:nbn:se:uu:diva-457615 (http://urn.kb.se/resolve?urn=urn:nbn:se:uu:diva-457615)

“Nothing in life is to be feared, it is only to be understood.
Now is the time to understand more, so that we may fear less.”

Marie Curie

List of Papers

This thesis is based on the following papers, which are referred to in the text by their Roman numerals.

I **Olsson**, **A**., Wistrand-Yuen, P., Nielsen, E I., Friberg, L E., Sandegren, L., Lagerbäck, P., Tängdén, T. (2020) Efficacy of antibiotic combinations against multidrug-resistant *Pseudomonas aeruginosa* in automated time-lapse microscopy and static time-kill experiments. *Antimicrob Agents Chemother*. 2020;64 (6):e02111-19.

II Wistrand-Yuen, P., **Olsson**, **A**., Skarp, K-P., Friberg, L E., Nielsen, E I., Lagerbäck, P., Tängdén, T. (2020) Evaluation of polymyxin B in combination with 13 other antibiotics against carbapenemase-producing *Klebsiella pneumoniae* in time-lapse microscopy and time-kill experiments. *Clin Microbiol Infect.* 2020; 26 (9):1214-1221.

III **Olsson**, **A**., Hong, M., Al-Farsi, H., Giske, C G., Lagerbäck, P., Tängdén, T. (2021) Interactions of polymyxin B in combination with aztreonam, minocycline, meropenem and rifampicin against *Escherichia coli* producing NDM and OXA-48-group carbapenemases. *Antimicrob Agents Chemother. 2021 Sep 13;AAC0106521*.

IV **Olsson**, **A**., Malmberg, C., Zhao, C., Lagerbäck, P., Tängdén, T. *In vitro* pharmacodynamics of polymyxin B in combination with minocycline against carbapenemase-producing *Klebsiella pneumoniae*. *Manuscript in preparation.*

Contents

Abbreviations

CMS	Colistimethate sodium
CRE	Carbapenem-resistant *Enterobacterales*
ESBL-E	Extended-Spectrum β-lactamase Producing *Enterobacterale*s
EUCAST	The European Committee on Antimicrobial Susceptibility Testing
HGT	Horizontal gene transfer
IDSA	Infectious Diseases Society of America
KPC	*Klebsiella pneumoniae* carbapenemases
LPS	Lipopolysaccharides
MDR	Multi-drug resistant
MH-II	Mueller-Hinton II (cation-adjusted)
MIC	Minimum Inhibitory Concentration
NDM	New Delhi metallo-β-lactamase
OXA	Oxacillinase
PBP	Penicillin Binding Protein
STRAMA	the Swedish strategic programme against antibiotic resistance
UTI	Urinary tract infections

Introduction

Antimicrobial resistance is an increasing threat to healthcare globally (1). A great part of modern medicine relies on the availability of efficient antimicrobial therapy. Clinical procedures such as transplantations, surgery and the care of prematurely born babies or cancer patients are threatened as treatment alternatives are lacking.

Alexander Fleming discovered the effect of penicillin in 1928 and made way for modern medicine as we know it today. Ten years later, Howard Florey and Norman Heatley continued the studies on *Penicillium notatum* at the Sir William Dunn School of Pathology in Oxford, UK (2). Large-scale production was made possible by American pharmaceutical companies in 1942 and the accessibility to penicillin made a huge impact on recovery for soldiers fighting in World War II.

In an article published in the New York Times in 1945, Fleming pointed out that antimicrobial resistance would emerge as it is a natural phenomenon and a part of bacterial evolution biology (3). However, the past eighty years of overuse and misuse have accelerated the speed at which resistance arises. The reason is multifactorial and varies with location as the worldwide access to healthcare, reliable diagnostics, sufficient antimicrobial therapy and clean water is lacking (4).

As antimicrobial resistance is emerging, the search for new treatment alternatives is crucial. Since 1987, and the development of lipopeptides, there has been a discovery void of new antimicrobial classes. Instead, different combinations of already developed drugs have been introduced in the clinical setting. The situation is alarming in Gram-negative bacteria, e.g., *Escherichia coli*, *Klebsiella pneumoniae* and *Pseudomonas aeruginosa*. In 2018, WHO launched a priority list to guide future development and research initiatives (5). Carbapenem- or third-generation cephalosporin-resistant *Enterobacterales* (e.g. *E. coli* and *K. pneumoniae)* and carbapenem-resistant *P. aeruginosa* are included in the first priority group and will be further discussed below.

Gram-negative bacteria

The bacterial membrane of Gram-negative bacteria consists of an impenetrable construction leaving many antibiotics ineffective (6,7). A diagnostic method called *Gram-staining* has allowed for separation between Gram-positive and Gram-negative bacteria since 1884. Hans Christian Gram stained the bacteria with a crystal violet-iodine complex followed by counterstaining with safranin leaving Gram-positive bacteria purple and Gram-negative bacteria pink (8). Gram-positive bacteria are stained by the crystal violet-iodine complex while Gram-negative bacteria cannot retain it and are instead stained by safranin which can reach the cell nuclei.

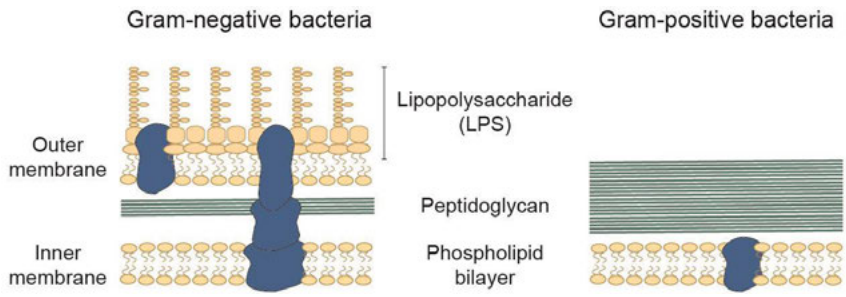


Figure 1: Differences in the bacterial cell wall. The cell wall of Gram-negative bacteria is composed of an outer and inner membrane, while Gram-positive bacteria are surrounded by one phospholipid bilayer. The outer leaflet of the Gram-negative outer membrane is composed of glycolipids, principally lipopolysaccharides (LPS). A peptidoglycan layer is found in both bacteria but it is much thicker in Gram-positive bacteria. The typical placement of membrane proteins are also shown in blue.

The bacterial cell wall

The Gram-negative cell wall protecting the bacterial cytoplasm consists of a double-membrane with a thin peptidoglycan layer in between (Figure 1) (6,7,9,10). The inner membrane consists of a bilayer of phospholipids, while the outer membrane is made up by one leaflet of phospholipids and one leaflet of glycolipids, mainly lipopolysaccharides (LPS). Some Gram-negatives such as *Acinetobacter baumanii* can be found without a LPS structure (11). The cell wall is traversed by different transport systems responsible for uptake and release of metabolites or other compounds. These transport systems are closely related to the resistance–nodulation–division (RND) family of multidrug efflux pumps, which are highly involved in resistance phenotypes including several antibiotic classes (12,13). Another important structure of the bacterial call wall are porins which are generally

small proteins generating hydrophilic channels that span across the outer membrane facilitating entry of metabolites and hydrophilic antibiotics (14). Deficient porins decrease permeability and can sometimes result in antimicrobial resistance.

The peptidoglycan layer

The peptidoglycan layer is important for cell shape and structure but also for the bacterial ability to resist osmotic challenges (15). Transpeptidases, glycosyltransferases and D-carboxypeptidases belongs to the group of penicillin-binding proteins (PBPs) which catalyzes the synthesis of the peptidoglycan layer (Figure 2). The PBPs are the target for β-lactam antibiotics which are the most important class of antibiotics in treatment of Gram-negative bacteria (16).

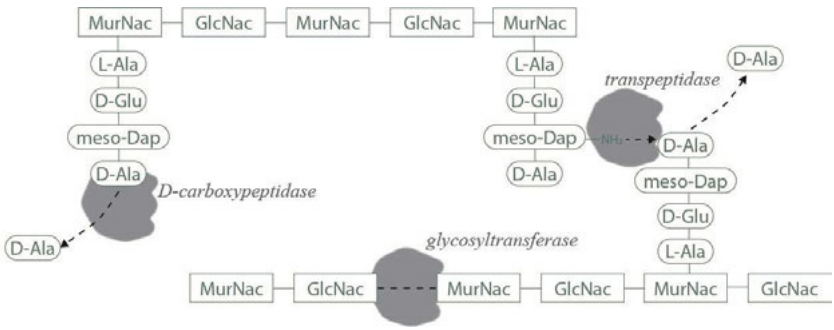


Figure 2: The composition and synthesis of the peptidoglycan layer. The peptidoglycan layer is composed by a rigid mesh of polysaccharide chains (green) cross-linked by peptide bonds. Several enzymes (in italics) catalyze important steps in the peptidoglycan biosynthesis. The crosslinking is catalyzed by transpeptidases generating a peptide bond between meso-Dap and the D-Ala in the fourth position in the pentapeptide. Glycosyltransferases catalyze the polymerization of the GlcNac-MurNac chain and D-carboxypeptidases limit further peptidoglycan crosslinking by removal of the terminal D-ala. Transpeptidases, glycosyltransferases and D-carboxypeptidases are all examples of PBPs.
Abbreviations: meso-Dap, meso-diaminopimeilic acid; D-Ala, D-alanine; GlcNac, N-acetylglucosamine; MurNac, N-acetylmuramic acid; penicillin-binding proteins, PBPs.

The peptidoglycan layer in Gram-positive bacteria is very strong and contains several layers connected in three dimensions (Figure 1). In contrast, the peptidoglycan in Gram-negatives is usually one molecule thick and the outer membrane is connected to the peptidoglycan layer via lipoproteins (6). The peptidoglycan is composed of a rigid mesh of linear chains made up by N-acetylmuramic acid (MurNac) and N-acetylglucosamine (GlcNac) which is cross-linked by peptides. The precursor MurNac is synthesized

and extended with a pentapeptide (L-Ala, D-Glu, meso-Dap, D-Ala and D-Ala) in the cytoplasm. The pentapeptide sequence can vary between bacterial species but the terminal D-Ala-D-Ala moiety is conserved (16). The MurNac pentapeptide is attached to the inner leaflet of the inner membrane before linkage with GlcNac which is catalyzed by a glycosyltransferase. This generates the disaccharide building block (GlcNac-MurNac + pentapeptide) which is then transported across the inner membrane and later attached to existing peptidoglycan chains in the periplasmic space. The crosslinking of new building blocks with existing peptidoglycan chains is catalyzed by transpeptidases which generates a peptide bond between the amino acid of the third peptide and the D-ala at the fourth position of the building block (the D-ala in position five is released) (Figure 2). The extension of the peptidoglycan is limited by D-carboxypeptidases removal of unreacted D-ala in the fifth position.

Lipopolysaccharides (LPS)

Lipopolysaccharides are crucial for the impermeable feature of the Gram-negative outer membrane and serve as the major structural component (Figure 1). Three structural domains constitute the LPS glycolipid: lipid A, the core oligosaccharide and the O-antigen (Figure 3) (9). Lipid A is the hydrophobic portion of the molecule and is a disaccharide with numerous fatty acids attached that forms the outer leaflet of the outer membrane. Lipid A is phosphorylated which adds up to the negatively charged feature of the LPS molecule. Linked to the glucosamine of lipid A is the core oligosaccharide which usually contains 3-deoxy-D-manno-oct-2-ulosonic acid (Kdo) residues, heptoses and various hexoses (9,17). The most distal part of the LPS is the O-antigen which is composed of repeating oligosaccharides.

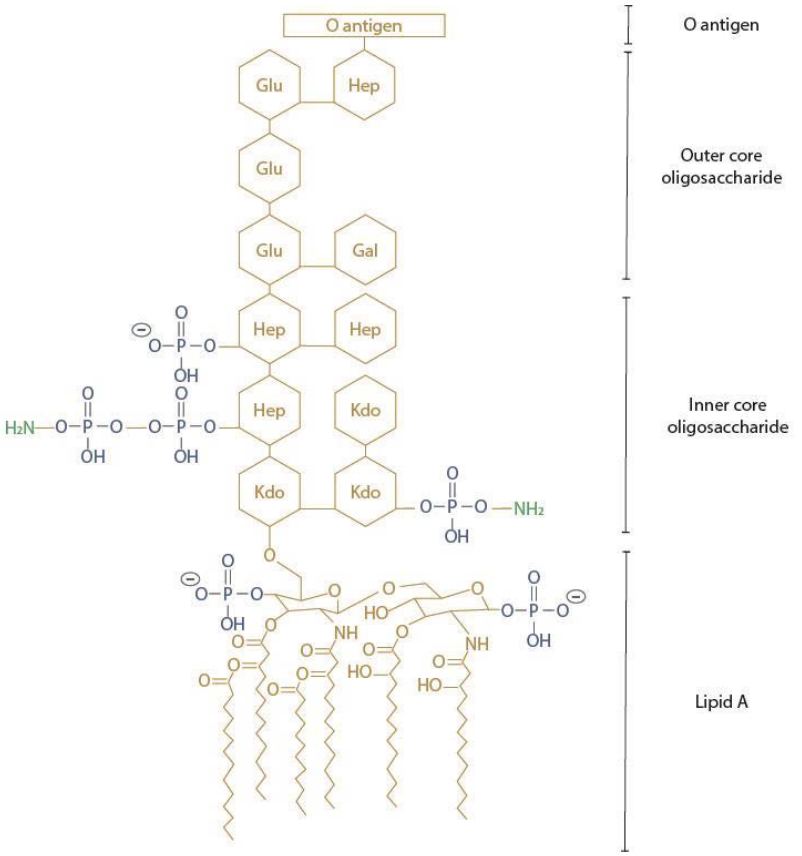


Figure 3: The structure of the lipopolysaccharide (LPS). The O antigen, the core oligosaccharide and lipid A composing the LPS structure is displayed using *E. coli* core type K-12 as an example. Important structures such as phosphates (blue) and amides (green) are also shown.
Abbreviations: Gal, galactose; Glu, glucose; Hep, heptose; Kdo, 3-deoxy-D-manno-oct-2-ulosonic acid.

Cation (Mg^{2+} and Ca^{2+}) linkages between phosphate groups and hydrophobic interactions between LPS and membrane proteins establishes strong interactions (6,9). Another important feature of the barrier function is the ability of LPS to pack densely. The cations prevent repulsion between the negatively charged phosphate groups within the LPS structures. These interactions can be disrupted by antibiotics such as polymyxins, which make the bacterial cell wall more permeable.

The overall structure of LPS is conserved but there are differences at species and strain level (9,17–19). The lipid A moiety and the inner part of the core oligo saccharide is conserved at species level, while the outer part can

vary between strains of the same species. For example, five core types of *E. coli* are known: R1-4 and K-12 (9,18). The O-antigen is the most diverse part of LPS and is not even synthesized in some Gram-negative bacteria. LPS molecules that consist of lipid A and a core oligosaccharide are referred to as smooth LPS while rough LPS lacks an O-antigen. Further, the composition of the LPS molecule is modified as bacteria adapt to different environments (9).

Capsule

Some bacteria, e.g., *P. aeruginosa, K. pneumoniae* and *E. coli* strains produce a capsule, which surrounds the outer membrane and consists of various polysaccharides (6,7,20). The role of the capsule is mainly to protect bacteria against the host´s immune response phagocytosis. The capsule structure varies and different types are especially important for certain clinical conditions. As an example, the K1 capsule of *E. coli* is associated with urinary tract infections (UTIs), sepsis and meningitis (21). Also, all *K. pneumoniae* strains produces a capsule but hypervirulent strains are often associated with increased capsule production expressed by chromosomal or plasmid-encoded genes (22). Hair like structures, named fimbriae, can be found on the surface of the capsule (6). These are important for adhesion to cell layers or medical devises and play an important role in increased virulence (21,22).

Biofilm formation

Many bacteria are able to form biofilm which is a complex mesh-like composition consisting of multiple bacteria and polysaccharides attached to a surface (6). Bacterial cells within the structure are tolerant to antibiotic treatment. The biofilm formation of *P. aeruginosa* is notable as the formation obstruct antibiotic treatment of cystic fibrosis (23). Recurrent infections are also common due to occasional bacterial shedding from the biofilm. Biofilms are often formed on medical devices such as catheters and is a common source of UTIs. Biofilm formation has been associated with UTIs caused by *E. coli* and *K. pneumoniae* with antibiotic resistance phenotypes (24,25).

High priority Gram-negative bacteria included in this work

Enterobacterales

This order includes many clinically important bacteria which are ubiquitous and found in soil, water and the intestinal flora of human and animals. Bacteria within the *Enterobacterales* order cause a wide variety of diseases and this work will mainly focus on two members of the *Enterobacteriace*ae family: *E. coli* and *K. pneumoniae*.

E. coli is found in the gastrointestinal tract were it can cause opportunistic infections and is the major cause of sepsis in Europe (26). Fecal contamination of food or water by different pathogenic *E. coli* can also result in infection and gastroenteritis. The symptoms are varying, from watery to bloody diarrhea or fever. The severity of the disease is determined by the site of the infection, fimbriae production or toxin-production (6). *E. coli* can also cause extraintestinal infections and is the predominant pathogen in UTIs. Food poisoning and mild UTIs are normally self-limiting but antibiotic resistance and difficult-to-treat infections are emerging. Also, neonatal meningitis or sepsis can be caused by *E. coli* strains, but the initial source of the infection is usually the intestines or the urinary tract.

K. pneumoniae is found on the skin, in the gastrointestinal tract and in the urinary tract where it causes opportunistic infections. Common *K. pneumoniae* infections are UTIs, hospital-acquired pneumonia, wound infections and sepsis. Opportunistic infections are associated with immunocompromised patients but hypervirulent strains are becoming an increasing threat to healthy and immune sufficient people. Hypervirulent strains are associated with virulence factors such as increased capsule production, LPS variations or expression of fimbriae (22,27).

Pseudomonadales

P. aeruginosa belongs to the order *Pseudomonadales* and the family *Pseudomonas* and is frequently found in the environment: soil, vegetation and water. *P. aeruginosa* can also colonize the respiratory tract and the skin but infections are primarily opportunistic and commonly seen in patients with compromised immune system or post treatment by broad-spectrum antibiotics (6). *P. aeruginosa* is one of the major causes of healthcare-associated infections in Europe and often associated with cystic fibrosis (23,26). Biofilm production is a common reason for difficult-to-treat and recurrent *P. aeruginosa* infections.

Treatment of Gram-negative bacteria

β-lactam antibiotics

The most important class of antibiotics in the treatment of Gram-negative bacteria is β-lactam antibiotics. These antibiotics interfere with peptidoglycan elongation and crosslinking by binding bacterial PBPs. In general, β-lactam antibiotics are very potent drugs with limited side effects. At present, four main classes of β-lactam antibiotics are in clinical use: penicillins, cephalosporins, carbapenems and monobactams (6,28). They all share a structure (the amide group and carboxylate group or a sulfonic acid) resembling the D-Ala-D-Ala moiety of PBPs and the characteristic β-lactam ring (Figure 4).

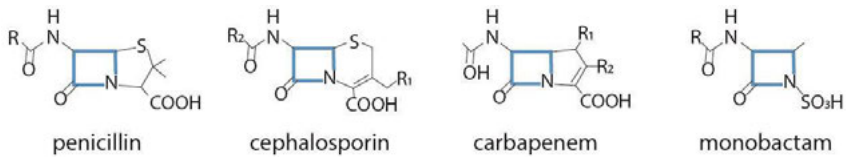


Figure 4: The chemical structures of different β-lactam antibiotic classes. Important structures for binding bacterial PBPs are the carbonyl group (=O) and the carboxylate group (-COOH) or a sulfonic acid (-SO3H) composing the PBP-binding site. "R_1" and "R_1" symbolizes other parts of the molecule not included in the functional group. The characteristic β-lactam ring, which is cleaved by bacterial β-lactamases to inactivate β-lactam antibiotics is shown in blue.

Since the introduction of penicillin in the 1940's, the emergence of β-lactam resistance has pushed the development of novel β-lactams. As a result, the first cephalosporin was introduced to the market in 1948 followed by carbapenems in 1976 and monbactams in 1979. Today, cephalosporins are the first-line choice in the treatment of Gram-negative bacteria and multiple generations of cephalosporin antibiotics offers various treatment alternatives. In parallel to the development of new β-lactam antibiotics the bacteria continue to find new ways of acquiring resistance.

The most common resistance mechanism against β-lactams is production of bacterial enzymes causing antibiotic inactivation. Bacterial β-lactamases performs enzymatic cleavage of the β-lactam ring to inactivate the antibiotic (Figure 4). These enzymes can be grouped according to the Ambler-classification based on specific sequence motifs and they cause resistance against penicillins, cephalosporins and monobactams such as aztreonam (Table 1). These enzymes are known as Extended Spectrum β-lactamases (ESBL) and as several enzymes are usually coexisting, the

number of treatment alternatives are few. The rise and spread of β-lactamases forces clinicians to move away from cephalosporins and utilize carbapenems to a greater extent. Carbapenems are still effective against ESBL-producing *Enterobacterales* as they are resilient to such enzymatic inactivation. Unfortunately, emergence of carbapenem inactivating enzymes, carbapenemases, is also increasing. In 2018, the highest carbapenem resistance levels in Europe were found in Greece, Italy and Rumania. The European Antimicrobial Resistance Surveillance Network (EARS-Net) reported carbapenemase resistance in <5 % of the *E. coli* isolates and >10 % of *K. pneumoniae* isolates. For collected *P. aeruginosa* isolates 16.5 % were carbapenem-resistant. Of note, carbapenemase-producing bacteria are often associated with a multidrug-resistance (MDR) phenotypes covering at least three antibiotic classes e.g., β-lactams, fluoroquinolones and aminoglycosides (29). Cefiderocol, a recently developed cephalosporin antibiotic linked to a siderophore, uses the bacterial iron uptake to facilitate entry into Gram-negative bacteria (30). This agent is efficient against Gram-negative bacteria such as *Enterobacteriales* and *P. aeruginosa* and is not hydrolyzed by carbapenemases.

Table 1: Ambler classification of β-lactamases.

Ambler classification	Catalytic site	ESBL	Carbapenemase
Class A	Serine	TEM, SHV, CTX-M	KPC
Class B	Zinc		NDM, VIM, IMP
Class C	Serine	AmpC	
Class D	Serine	OXA	OXA-48

β-lactam and β-lactamase inhibitor combinations

In an attempt to inhibit enzymatic inactivation of β-lactams, several β-lactamase inhibitors have been introduced to the market (28,31). These inhibitors prevent the mode of action of β-lactamases and restore the function of the β-lactam antibiotic used in combination. The lack of inhibitors with effect against all carbapenemases is of great concern. This is especially true for Ambler Class B β-lactamases for which no inhibitors are available. However, aztreonam offers an interesting feature as it is unaffected by Class B β-lactamases. To this day, no inhibitor can alone inhibit the mode of action of all known β-lactamases (Figure 5).

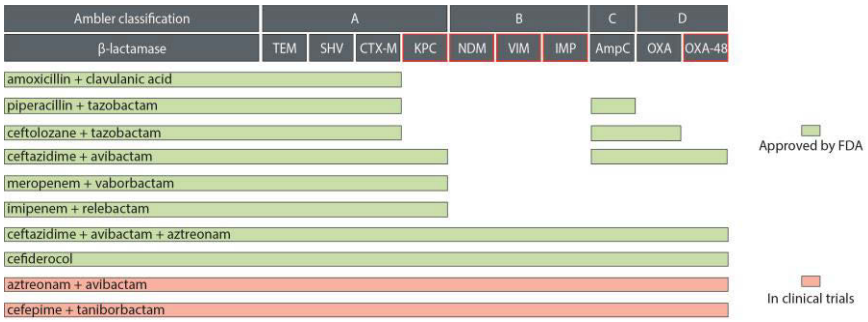


Figure 5: β-lactam-β-lactamase inhibitor combinations, cefiderocol and β-lactamase range. Most carbapenemases (indicated by a red square) are not inhibited by β-lactamase inhibitors. The cephalosporin cefiderocol is not hydrolyzed by any carbapenemases. Drugs or combinations approved for clinical use by the U.S. Food and Drug Administration (FDA) are shown in green and combinations currently in clinical trials are shown in red.

Clavulanic acid was the first β-lactamase inhibitor approved by the U.S. Food and Drug Administration (FDA) back in 1984. The inhibitor binds serine residues and can inhibit class A β-lactamases except KPC. The introduction of tazobactam increased the inhibition range to class C β-lactamases. Using tazobactam with the cephalosporin ceftolozane increased the range even further. It was not until the development of avibactam that the inhibition range also included KPC and OXA-48. The success of avibactam resulted in development of several diazabicyclooctanes (DBOs) such as relebactam. Further, boronate-based compounds such as vaborbactam have also shown activity against all class A β-lactamases. The FDA-approved ceftazidime and avibactam combination has been used together with aztreonam to cover the by Class B β-lactamases. Clinical trials on aztreonam and avibactam or the cefepime and taniborbactam combination might reveal additional alternatives for treatment of class B β-lactamase producing bacteria.

Aminoglycosides

Aminoglycosides are administered intravenously and are potent broad spectrum antibiotics which were widely used clinically before the introduction of less toxic alternatives such as cephalosporins and carbapenems (32). The emergence of resistance against first-choice antibiotics and MDR phenotypes has renewed the interest in aminoglycosides. Aminoglycosides enter the bacterial cell by binding to negatively charged sites in the outer membrane which disrupts the membrane and increases permeability. With

high affinity for the A-site on the 16S ribosomal RNA of the 30S ribosome the protein synthesis is disturbed and mistranslation promoted (33).

Carbapenem-producing *Enterobacterales* isolates usually remains susceptible to aminoglycosides such as gentamicin and amikacin (34,35). Even so, aminoglycosides are considered an alternative-treatment and first-line choices are prioritized if effective. Long term aminoglycoside treatment is sometimes used in cystic fibrosis patients and is associated with side-effects such as kidney damages and hearing impairment (36). Reduced exposure to aminoglycosides has been associated with reduced toxicity (32).

Single-dose aminoglycoside is an alternative treatment option for ESBL–producing *Enterobacterales*, carbapenem-resistant *Enterobacterales*, and difficult-to-treat *Pseudomonas aeruginosa* (37). If susceptible, single-dose aminoglycoside is the recommended treatment option for presumed or confirmed ESBL–producing *Enterobacterales* causing cystitis (35,37). A single-dose aminoglycoside is generally effective against cystitis as it is almost exclusively eliminated via the renal route generating a high concentration in the urine. Daily treatment using aminoglycosides can be an alternative for treatment of complicated UTIs if the degree of nephrotoxicity is deemed tolerable.

Fosfomycin

Fosfomycin interferes with the bacterial peptidoglycan synthesis (38,39). More specifically, fosfomycin enters the bacterial cell through membrane transporters/channels and targets the UDP-N-acetylglucosamine enolpyruvyl transferase (MurA) responsible for initiating the first step in the peptidoglycan synthesis (15,38,39). If first-line treatment is not available, oral fosfomycin can be used to treat *E. coli* cystitis (35,37). The most common resistance mechanism is the presence of the fosfomycin inactivating enzyme FosA which is intrinsic in some Gram-negatives such as *K. pneumoniae* and *P. aeruginosa* (37). One limiting factor for usage of fosfomycin is the resistance development seen during treatment (34,35,38). Importantly, fosfomycin resistance develops at a lower frequency with higher fosfomycin concentrations (38).

There are several advantages to fosfomycin usage: cross-resistance is rare due to its unique mechanism of action, less toxicity in comparison to other alternative treatment options (e.g., aminoglycosides and polymyxins) and

oral administration (40). The use of fosfomycin should be avoided for complicated UTIs or non-UTIs due to inadequate concentrations at the infection site (35,37). Emergence of fosfomycin resistance has been seen both *in vitro* and among clinical isolates and the development of resistance is concentration dependent (38,41). If fosfomycin treatment is considered for severe infections, the Swedish strategic programme against antibiotic resistance (STRAMA) recommends that it should always be used in combination with a second active drug to reduce resistance development during treatment (35).

Fluoroquinolones

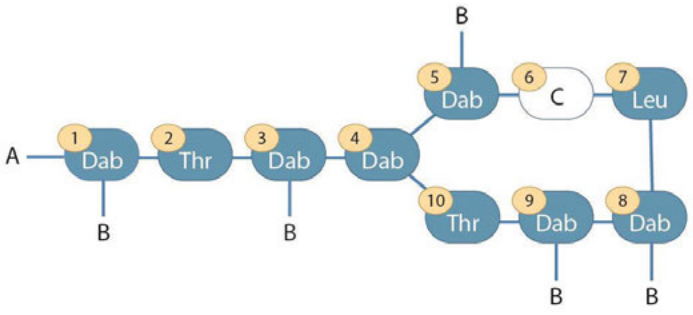
A few decades ago, quinolones were widely used in the treatment of UTIs but it is not very efficient in treating other infections (42). The addition of fluorine to the quinolone molecule increased the lipophilic characteristics, cell penetration and increased affinity for the drug targets: the DNA gyrase and the DNA topoisomerase IV. This was the starting point of a new class of antibiotics: the fluoroquinolones with increased antibacterial activity and improved pharmacological properties. The DNA gyrase is a bacterial enzyme responsible for managing DNA tangles and supercoils formed during DNA replication and transcription (42). The DNA topoisomerase IV shares similar structures with the DNA gyrase but is responsible for relaxation of supercoiled DNA.

Although fluoroquinolones (e.g., ciprofloxacin or levofloxacin) are effective agents against ESBL-*Enterobacterales* in cystitis, limited usage is recommended to preserve the effectiveness of the drugs and use less toxic alternatives (35,37). The Infectious Diseases Society of America (IDSA) recommends ciprofloxacin as one of many treatment alternatives for complicated UTIs (37). In Sweden, only one third of the ESBL-producing *E. coli* isolates are susceptible to the fluoroquinolone ciprofloxacin (35). The use of fluoroquinolones is limited by the high rate of resistance development as few mutations in the DNA gyrase is sufficient to cause a resistant phenotype (7,35). Ciprofloxacin and quinolones can in some cases be clinically useful as they offer generally efficient CNS permeability (35).

Polymyxin antibiotics

Polymyxins, polymyxin B and polymyxin E (colistin), are last resort drugs that are used in the treatment of complicated infections caused by Gram-negative bacteria (29,34,43). The substances are derived from the plant

bacterium *Paenibacillus polymyxa* and the drugs are a composition of several substances: polymyxin B (polymyxin B1 and B2) and colistin (colistin A/E1 and colistin B/E2) (29,43). The chemical structures of polymyxins are very similar consisting of ten amino acids: a tripeptide and a cyclic heptapeptide bound to various amino acid and sidechain alterations (Figure 6).



	Substance	Alteration
A	polymyxin B1/ colistin A (E1) polymyxin B2/ colistin B (E2)	6-methyloctanoic acid 6-methylheptanoic acid
B	polymyxin B and colistin colistimethate (CMS)	amino group ($-NH_2$) $NH-CH_2-SO_3H$
C	polymyxin B colistin	D-Phenylalanine D-Leucine

Figure 6: The chemical structure of polymyxins. The core structure of polymyxins (in blue) consists of a hydrophobic tail (amino acids 1-3) and a heptapeptide (amino acids 4-10). Differences between polymyxin substances are caused by the listed amino acid or sidechain alterations (A-C).
Abbreviations: Dab, diaminobutyric acid; Thr, Threonine; Leu, Leucine

Polymyxins are amphipathic i.e., comprised of hydrophilic and lipophilic parts. The amines of L-α,γ-diaminobutyric acid (Dab) are ionized at physiological pH allowing polymyxins to target the negatively charged phosphate groups of lipid A (29,43). This interaction displaces the cations (Ca^{2+} and Mg^{2+}) responsible for bridging adjacent LPS molecules and disrupts the outer membrane (Figure 7). The hydrophobic tail of the polymyxin molecules is then inserted into the fatty acyl chain layer of the lipid A structure. The phospholipid layer of the inner membrane is later disrupted, as well as the type II NADH-menaquinone oxidoreductase (NDH-2) responsible for bacterial respiration.

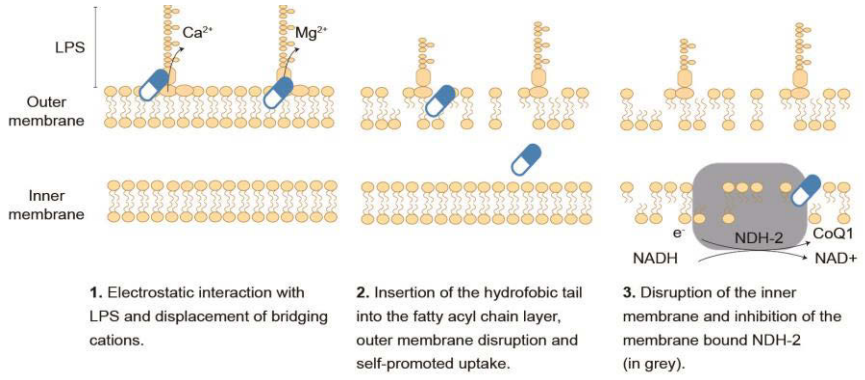


Figure 7: The mechanism of action by polymyxins. The amphiphilic nature of polymyxins facilitates electrostatic interaction with negatively charged phosphate groups of the lipid A moiety in LPS (1). The outer membrane is disrupted by insertion of the hydrophobic tail into the fatty acyl chain layer (2). Finally, the inner membrane is disrupted and NDH-2 responsible for bacterial respiration is inhibited (3).

Despite similar chemical structures and mechanism of action polymyxin B and colistin have different pharmacokinetic properties (29,43). Colistin is administered as a prodrug (colistimethate sodium, CMS) with renal activation and polymyxin B is administered intravenously. The use of polymyxins is associated with nephrotoxicity, which is why it is normally regarded as a last resort drug (44). Studies of toxicity are difficult to interpret as critically ill patients have multiple underlying diseases, the level of toxicity is dose-dependent and various definitions of toxicity are used in the literature. Even so, it is clear that polymyxin therapy is associated with nephrotoxicity. A recent systematic review and meta-analysis reported double risk of nephrotoxicity associated with the use of polymyxins in comparison to other therapies (OR 2.23; 95% CI 1.58-3.15) (44). It was also suggested that there is an increased probability for nephrotoxicity with colistin versus polymyxin B (P = 0.005; OR 1.65; 95% CI 1.16-2.35). Although contrary results have been reported, the difference in pharmacokinetic properties between colistin and polymyxin B is the widely accepted theory behind this (29,34,44). In a recent publication, a new agent was reported to neutralize the cell surface of polymyxin B resistant *E. coli* harboring *mcr-1* thereby improving the incorporation of polymyxin B to *E. coli* (45).

Antimicrobial susceptibility testing

In Europe, the European Committee on Antimicrobial Susceptibility Testing (EUCAST) is responsible for harmonizing antimicrobial susceptibility testing (AST) methodology (46,47). Also, clinical breakpoints for resistance are determined to facilitate interpretation of AST results and guide selection of treatment alternatives in the clinical setting. The measured zone diameters and/or MIC values for a given pathogen and antimicrobial agent is translated into defined susceptibility categories: susceptible (S); susceptible, increased exposure (I) or resistant (R) (Table 2) (48).

Table 2: EUCAST definitions of susceptibility categories.

Susceptibility category	Abbreviation	Definition
Susceptible, standard dosing regimen	S	High likelihood of therapeutic success using a standard dosing regimen of the agent.
Susceptible, increased exposure	I	High likelihood of therapeutic success because exposure to the agent is increased by adjusting the dosing regimen or by its concentration at the site of infection.
Resistant	R	High likelihood of therapeutic failure even when there is increased exposure.

Variability of the MIC measurements is occurring, but assay variation between different AST methods and laboratories explains only a small part of the problem. A previous study reports that over half of the variability seen could be explained by biological variation (49). However, the most commonly used methods for AST are influenced by external factors such as evaporation, binding to lab ware, solubility, pH, temperature and selection of nutrient media, all of which should be standardized (50). The variability of MIC values can have a large impact on treatment success when the "true MIC" is near a clinical breakpoint. In order to come as close as possible to the "true MIC", EUCAST recommends standardized AST methodology and quality control strains for a given pathogen and antimicrobial. Repeated measurements are also recommended and not more than a 2-fold variation between replicates is accepted. Importantly, one has to

remember that the MIC-value is determined for the entire bacterial population as a whole. The phenotype of the selected colonies used for MIC-determination will represent the whole population. Inadequate antimicrobial treatment can select for these individuals and reoccurring infections can occur (51). The methods used for AST are usually determined by available guidelines for standardization, budget, workforce and feasible logistics.

Dilution

The reference method for AST of rapidly growing aerobic bacteria is broth microdilution (BMD) except for mecillinam and fosfomycin, where agar dilution is the reference method (ISO 20776-1:2006) (47,52). When using BMD, a defined amount of bacteria (approximately 5 x 10^5 CFU/mL) is allowed to grow in liquid media with different antimicrobial concentrations in a 2-fold dilution series. In agar dilution, the antimicrobial agents at different concentrations are added into the agar before casting the plates and approximately 5 x 10^5 CFU/mL is plated. In both BMD and agar dilution, the antimicrobial susceptibility is defined by the minimum inhibitory concentration (MIC) which is the lowest concentration of a drug that completely inhibits visible bacterial growth (47). These methods are generally not used in a clinical laboratory as it requires an increased workload and large volumes of reagents.

Disc diffusion

The most commonly used method for AST in clinical laboratories is the disc diffusion method. Direct disc diffusion can be performed by covering the agar evenly with bacterial suspension using a swab before placing a disk containing a defined amount of antibiotic on the surface (50). A defined amount of bacterial suspension can also be standardized to approximately 5 x 10^5 CFU/mL. The diameter (in mm) of the resulting inhibition zone surrounding the antibiotic disc is then correlated with antimicrobial susceptibility breakpoints. Assessing and determining the susceptibility once the patient sample reaches the clinical laboratory takes 16-24 h in general. This method is cost-effective and easy to perform. However, it has many limitations as automation is difficult to incorporate and it is not applicable for slow-growing or fastidious bacteria.

Epsilometer testing (Etest)

Another widely used method in the clinical setting is the Etest. A plastic strip containing a range of concentrations of a certain antibiotic is placed on an agar plate covered with a bacterial suspension of approximately 5 x 10^5 CFU/mL (50). After incubation, an inhibition zone appears and the MIC is easily interpreted using the indicators on the Etest itself. The simplicity and accuracy of the Etest is an advantage over other methods but the antibiotic-specific inaccuracy and inconsistent behavior is a large drawback.

Automated systems

In order to decrease the workload generally required for traditional AST methods, semi or complete automation has been introduced to the clinical laboratories. The use of a pipetting robot can increase the reproducibility and decrease the manual workload. There are also automated systems detecting growing bacteria on the basis of turbidity (e.g., Vitek) or fluorescence (e.g., Sensititre ARIS 2X). Computer integration allows for quick and standardized interpretation of the results. One limitation of these systems is the panel capacity, which usually has a fixed number of samples to analyze at a time. In addition, the panel is typically standardized for a certain bacterial species, antibiotic type or range of concentrations. Furthermore, most systems still offer less reproducibility, sensitivity and reliability compared to traditional AST methods (50).

Polymerase chain reaction (PCR)

An effective and direct method to eliminate preparation of bacterial cultures, reduce incubation times and practical labor is the use of PCR. This method is the most efficient and rapid molecular based tool for detection of bacterial resistance genes associated with a certain resistance phenotype. As an example, this method is often used in detecting various β-lactamases conferring a known resistance phenotype. In short, a PCR is based on DNA amplification of a specific gene of interest which is identified using carefully designed primers. Each amplification doubles the amount of DNA and facilitates confirmation of gene presence. There are multiple PCR systems and workflows for interpretation available. One large drawback is the fact that you can only detect what you have knowledge about thus, yet unknown resistance genes cannot be discovered. On the other hand, genotypic methods are generally rapid, direct, sensitive and specific.

Whole genome sequencing

Whole genome sequencing (WGS) is a tool for generating an almost complete DNA sequence of an organism. There are multiple platforms to use for sequencing and the large amount of data is impossible to interpret as a whole. Knowledge on resistance genes and associated phenotypes could in theory predict antimicrobial susceptibility. However, in practice it is difficult to predict the magnitude of expression of these genes and thus the level of resistance. In a report from 2020, WHO and EUCAST recently stated that WGS cannot be used as a substitute for current phenotypical methods as guidance for selecting appropriate treatment alternative (53,54). The lack of understanding of genotype-phenotype translation is a huge limitation and WGS also requires investments in infrastructure, techniques and big-data storage as well as standardization of bioinformatics methods. On the other hand, WGS is not dependent on subjective visual examination of results, specific bacterial growth conditions or species limitations of certain phenotypical methods used for AST. In addition, WGS is a useful tool for surveillance of infectious agents. One can easily determine if a sample is of human or animal origin or whether an already known resistance gene is encoded by a plasmid or on the chromosome. This information is of importance when mapping out transmission routes. There are databases such as CARD or ResFinder which have made an attempt to link acquired genotype to phenotype and are being updated regularly as our knowledge on resistance mechanisms continues to improve (55,56).

Antimicrobial resistance

Antimicrobial resistance in Gram-negatives is often multifactorial and can be a combination of intrinsic, adaptive and acquired mechanisms (Figure 8) (28,57–60). Intrinsic resistance is the innate ability to survive antimicrobial treatments through e.g., low membrane permeability and/or active drug efflux. Adaptive resistance is the change in gene expression that allows the bacteria to upregulate efflux or enzymatic activity and/or downregulate porins in presence of antimicrobials. Susceptible organisms can acquire resistance by acquisition of genetic materials such as plasmids encoding antibiotic-degrading enzymes or alternative drug targets that render a resistant phenotype. There are three mechanisms of how genetic material can be transferred and are commonly referred to as horizontal gene transfer (HGT): conjugation (genetic transfer of plasmids or integrative conjugative elements via pilus), transduction (transfer of bacterial genetic material by bacteriophages) and transformation i.e., uptake of extracellular DNA that is incorporated in the genome of the recipient (61).

Resistance mechanisms are generally grouped as follows: I) prevention of access to the drug target by decreased permeability of the bacterial membrane or active transport of the drug out of the cell, II) antibiotic inactivation by bacterial enzymes, III) reduced affinity for the antibiotic through alterations of the drug target, or IV) bypassing of the biochemical pathway affected by the antibiotic.

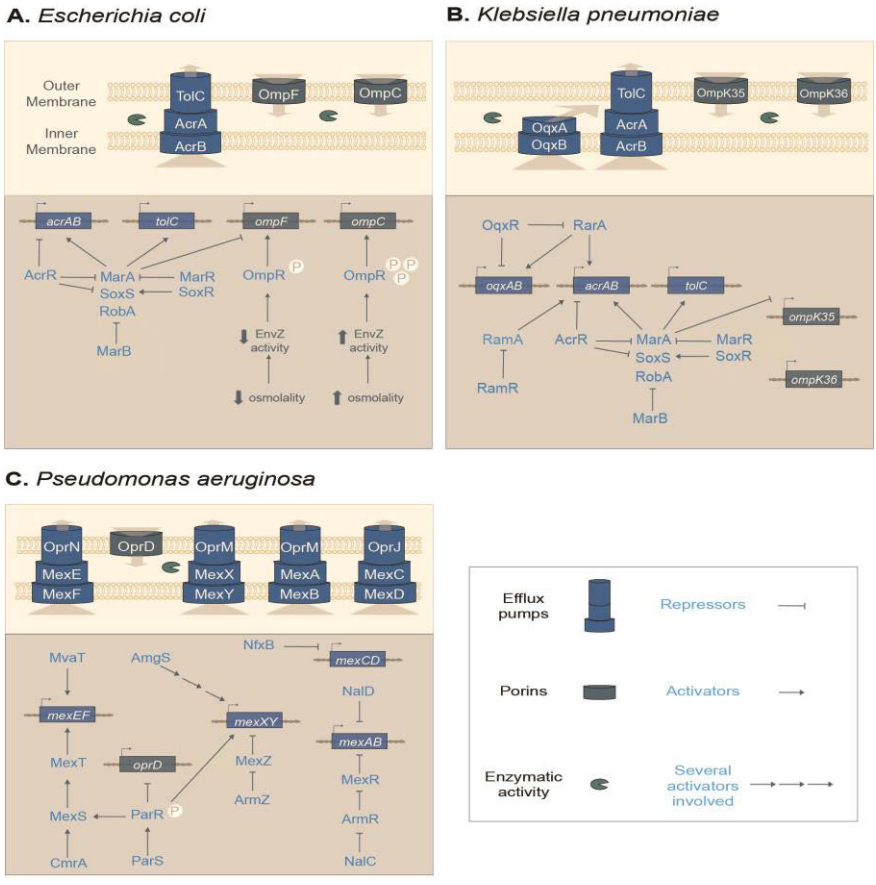


Figure 8: Overview of the genetic regulation of resistance mechanisms in Gram-negative bacteria.

The bacterial membrane barrier

The complex structure of the Gram-negative cell wall results in intrinsic resistance to many antibiotics as a result of low permeability (9). Small and hydrophobic molecules can normally cross the phospholipid bilayer and Gram-negative bacteria utilize channels such as porins to transport e.g. nutrients (14). This is a mechanism hijacked by some antibiotics to enter the cell. Efflux pumps present in the bacterial membrane are responsible for removal of harmful substances such as toxins and antimicrobials that made its way into the cell (57,58). In addition, bacterial enzymes e.g., β-lactamases inactivating β-lactam antibiotics, are normally located between the outer and inner membrane. Also, *K. pneumoniae* has the ability to induce

polymyxin resistance by shedding capsular polysaccharides binding polymyxins (62).

Porin deficiency

The size and hydrophobicity of a drug influences the way it may enter the bacterial cell. Large and hydrophobic drugs such as rifampicin cannot cross the outer membrane which results in intrinsic resistance of Gram-negative bacteria. Smaller and hydrophilic drugs can cross the membrane through porins which are transmembrane proteins forming a channel. Various types of porins exist and they can be classified as specific or non-specific based on their activity (63). In addition, the size of the channel is also an important factor for permeability of drugs and its role in antimicrobial resistance. Porin deficiencies caused by genetic variations is a common resistance mechanism in Gram-negatives and often associated with carbapenem resistance (57,63–65).

Non-specific porins OmpC and OmpF are found in *E. coli* while their homologues OmpK35 and OmpK36 are found in *K. pneumoniae*. The porins of *E. coli* are much more studied than those in *K. pneumoniae* but genetic comparisons have revealed many similarities (66). The porin channels of OmpF and OmpK35 are larger and allow entry of several antibiotics. As a result, OmpF or OmpK35 deficiency impacts resistance much more than alterations in OmpC and OmpK36 (63,66). OmpF is the major porin involved in β-lactam resistance in *E. coli* with great impact on aztreonam resistance among others (63). Tetracyclines can utilize the OmpF porin in *E. coli* but the more lipophilic minocycline crosses the membrane as well (67,68). The bacterial membrane barrier and intrinsic resistance is especially reinforced in *P. aeruginosa* due to the lack of large non-specific porins such as OmpF and OmpK35 (60). *P. aeruginosa* expresses several smaller highly specific porins such as OprD which facilitates entry of basic amino acids and porin loss has been associated with carbapenem resistance (69).

Efflux activity

The major efflux pumps of Gram-negative bacteria belongs to the Resistance-Nodulation-Division (RND) efflux pump family which function as a tripartite complex composed of the RND protein (the inner membrane component), the membrane fusion protein (MFP, the periplasmic component), and the outer membrane protein (OMP) (13). Together, these proteins form a continuous channel and the substrate molecules can be transported across the bacterial membrane using the proton-gradient force.

Adaptive resistance can occur as a result of induced efflux activity in the presence of antibiotics. However, resistance has also been associated with mutations in transcription regulators altering the efflux activity.

Enterobacterales such as *E. coli* and *K. pneumoniae* share the major efflux pump AcrAB-TolC, although it is much more studied in *E. coli* (58). AcrAB-TolC has a wide substrate specificity due to the large binding pocket of AcrB and efflux of the following substrates are seen in both *E. coli* and *K. pneumoniae*: β-lactams, chloramphenicol, fluoroquinolones, minocycline and rifampicin (58,70). Different substrates bind diverse parts of the binding pocket. As an example, the small sized minocycline molecule travels to the upper sub-pocket while rifampicin (a large substrate) initially binds to the proximal binding site followed by conformational change and later binding to the distal binding site. Increased efflux activity has also been associated with certain genetic variations in transcription regulators. The AcrAB-TolC efflux is activated by the *rob-sox-mar* regulon and inhibited by the global transcription factor AcrR (71). In *K. pneumoniae*, another regulator, RamA, has also been found to activate AcrAB-TolC efflux (72). Efflux of tetracyclines is mediated by Tet-proteins located in the cytoplasmic membrane and are found in Gram-negative as well as Gram-positive bacteria (73). Most Tet-proteins cause resistance to tetracycline but not minocycline or tigecycline, but have some variation in substrate specificity, e.g., TetA confers resistance mainly to tetracycline, while TetB confers resistance to tetracycline and minocycline but not tigecycline (73). The recently described efflux pump OqxAB has been found to be structurally similar to other efflux pumps of the RND family. As it is located in the inner membrane it is sometimes dependent on the outer membrane channel TolC (74). OqxAB mainly exports fluoroquinolones but has also been associated with increased resistance against chloramphenicol and cefoxitin (59,74,75). OqxAB is negatively regulated by OqxR and upregulated by RarA which also induces AcrA transcription (58,59). The *rarA-oqxABR* locus is chromosomally encoded in *K. pneumoniae* and in *E. coli* most often located on plasmids (58,76).

Resistance in *P. aeruginosa* is to a great extent caused by multiple efflux pumps; MexAB-OprM, MexEF-OprN, MexCD-OprJ and MexXY-OprM being the most prominent ones (57). MexEF-OprN and MexCD-OprJ are associated with high-level fluoroquinolone resistance while MexAB-OprM, MexEF-OprN and MexXY-OprM play a larger role in β-lactam resistance (57,58). MexXY-OprM has been the reason for aminoglycoside resistance when enzymatic activity could not be identified (57,58). Even if

a specific pump is the major one, many efflux pumps share substrate specificity and can compensate for lost efflux activity (57,58). The *mexXY* operon is negatively regulated by the MexZ repressor but presence of antibiotics alleviate the repression through ArmZ inhibiting MexZ repression (77). Modifications of the AmgRS and ParRS two-component systems are involved in regulation of efflux in *P. aeruginosa*. Mutations associated with efflux of aminoglycosides has been identified in AmgS that indirectly induces MexXY-OprM transcription (57,78). Efflux of aminoglycosides, fluoroquinolones and cefepime are caused by MexXY-OprM activation by phosphorylated ParR (79).

Enzymatic resistance

The presence of β-lactamases is the primary β-lactam resistance mechanism (28). These bacterial enzymes inactivate β-lactam antibiotics by cleavage of the β-lactam ring. *Enterobacterales* are very much associated with β-lactam resistance due to high prevalence of β-lactamase genes encoding ESBLs and carbapenems such as New Delhi metallo-beta-lactamase 1 (NDM-1), oxacillinase (OXA-48) and *K. pneumoniae* carbapenemase (KPC) (28). Carriage of several enzymes on plasmids or gene cassettes is a common phenomenon and contributes to increased β-lactam resistance. Genetic material encoding β-lactamases is very rapidly spread among bacteria, even among different species (61). β-lactam resistance in *P. aeruginosa* is often associated with overproduction of the chromosomal cephalosporinase AmpC but over 200 Pseudomonas Derived Cephalosporinase (PDC) variants have been described so far (57). Intrinsic resistance in *P. aeruginosa* against fosfomycin is mediated by chromosomally encoded FosA that inactivates the drug; an enzyme that can be found in other species due to HGT (80). Horizontally-acquired aminoglycoside modifying enzymes are frequently found among Gram-negatives and often in large numbers (57).

Drug target variations

Gram-negative bacteria utilize many ways of varying the drug target and obstruct antimicrobial effects. The gene encoding the target can be inactivated or mutated but a reason for intrinsic resistance can simply be lack of drug target. Another way of creating resistance is to increase the abundance of the target through gene amplification (81). Producing a unique version of the drug target can also result in resistance as seen in *Vibrio fischeri* which is intrinsically resistant to fosfomycin due to its sole structure of

MurA (82). Also, a single mutation in *murA* (C115D) has been found to confer resistance to fosfomycin in *E. coli* (83).

There are also known alterations in different genes encoding PBPs targeted by β-lactams. As an example, one mutation in *dacB* encoding PBP4, inactivated the gene and resulted in high level β-lactam resistance in a *P. aeruginosa* isolate (84). The inactivation also triggered overproduction of chromosomal AmpC. Further, an insertion sequence in *ftsI* encoding PBP3, resulted in elevation in MIC for several β-lactams (85).

Quinolone-resistance determining regions are found in both *gyrA* and *parC*, encoding the DNA gyrase and the DNA topoisomerase IV, respectively (86,87). Mutations in these regions change the protein conformation and alters the quinolone affinity. Several mutations give rise to resistance and for *E. coli* the most common sites for mutations are Ser83 and Asp87 in GyrA which are key residues in binding to quinolones (87,88).

Aminoglycoside resistance can arise due to methylation of the 16S ribosomal RNA (89). The methylation sterically hinders the drug from binding to the ribosomal RNA. A study from Japan reported high-level resistance against almost all clinically useful aminoglycosides (MIC >1024 mg/L) among *P. aeruginosa* and *Serratia marcescens* isolates (90). The reason was alterations in the *rmtA* and *rmtB* genes encoding 16S ribosomal RNA methyltransferases. A plasmid-borne methylase encoded by the *armA* gene was initially found in *K. pneumoniae* and has since then been found worldwide in other bacterial species (91).

The most common reason for polymyxin resistance in Gram-negative bacteria is modification of LPS which reduces the negatively charged phosphate groups. This occurs mainly through addition of 4-amino-L-arabinose (L-Ara4N), phosphoethanolamine (PEA) and/or galactosamine to the negatively charged phosphate groups of lipid A (29). The most dreaded mechanism is the emergence of the plasmid-encoded *mcr-1* initially identified in *E. coli*. Today MCR-1 to 8 are known to catalyze the transfer of phosphoethanolamine (PEA) onto lipid A (29,92). Resistance can also arise within the bacteria as multiple regulators and two-component systems are involved in managing the LPS modifications in Gram-negative bacteria (29,62). PhoP/PhoQ and PmrA/PmrB are triggered by environmental changes such as low Mg^{2+} and pH-levels and modify transcription of a number of genes (29). The same systems are also upregulated during pol-

ymyxin therapy, inducing adaptive resistance. For *K. pneumoniae*, disruption of the *mgrB* gene encoding a 47-amino acid transmembrane protein that inhibits PhoP is a key player in polymyxin resistance (62). Also, multiple mutations in *pmrA* and *pmrB* have been linked to polymyxin resistance in *K. pneumoniae* and *P. aeruginosa* (62). In *E. coli*, PhoP can induce transcription of a small sRNA from *mgrR* which inhibits *eptB* thereby causing PEA addition to Kdo.

Antimicrobial combination therapy

When monotherapy fails, antimicrobial combination therapy has been used as a treatment option against MDR Gram-negatives (34,93–95). The increase of carbapenemase–producing *Enterobacterales* escalated the use of antimicrobial combination therapy in early 2000s. Polymyxin combinations have been commonly used for severe infections caused by carbapenemase-resistant Gram-negative bacteria (34). Antibiotics in combination can yield increased activity or complement each other by targeting different sites. However, clinical evidence on combination therapy is still limited. Although several methods are used in research to screen for efficient antimicrobial combinations, no diagnostic tool is available for use in a clinical microbiology lab.

Methods used in screening for efficient antimicrobial combinations *in vitro*

Static time-kill experiments

Static time-kill experiments are considered the standard method in screening of synergistic effects of antimicrobial combinations (96,97). Separate test tubes are dedicated to growth control, monotherapies and the combination separately. A standardized bacterial inoculum of ~ 6 $\log_{10}$ CFU/mL is added to the tube and after a 0 h sample, antibiotics can be added to the test tube. Multiple testing and viable counts are possible before the experiment is ended, normally after 24 h. The killing effect can be classified as synergistic (defined as ≥2 $\log_{10}$ decrease in CFU/mL by the combination in comparison to the most effective monotherapy at the same concentration), indifference (<1 $\log_{10}$ CFU/mL decrease in bacterial density) or additive (>1 and <2 $\log_{10}$ CFU/mL decrease in bacterial density). A bactericidal effect is defined as ≥3 $\log_{10}$ CFU/mL decrease in bacterial density in comparison to the start inoculum.

The method is relatively time-consuming, labor intensive and offers evaluation of only a small range of concentrations and the killing effect is influenced by the larger inoculum size (in comparison to AST). Also, the method has received criticism as the bacterial killing effect is usually determined at 24 h only which can mask the effect by antibiotics dosed more regularly. However, time-kill experiments offer a low limit of detection (1 $\log_{10}$ CFU/mL), the possibility to sample at multiple time-points and the results have had good correlation with clinical outcome (96).

Checkerboard

Checkerboards are also frequently applied and utilizes MICs to define synergistic effects (97). Multiple antibiotic concentrations, commonly ranging from 1/32 to 4 x MIC, are pipetted onto a 96-well plate as monotherapy and in combination before a standardized bacterial inoculum is added to the plate. The effect of the combination (FIC_{index}) is determined using the fractional inhibitory concentration (FIC) calculated for each drug separately:

$$FIC_{drug\ NN} = \frac{MIC_{drug\ NN\ in\ combination}}{MIC_{drug\ NN\ monotherapy}}$$

$$FIC_{index} = FIC_{drug\ A} + FIC_{drug\ B}$$

The inhibitory effect is defined as follows: synergy ($FIC_{index} \leq 0.5$), indifference (FIC_{index} of >0.5 but ≤ 4) or antagonism ($FIC_{index} >4$).

The checkerboard is a relatively simple method which is easy to perform in comparison to time-kill experiments despite evaluation of multiple concentrations. However, it allows for interpretation at only one time point and has a high limit of detection (~7 $\log_{10}$ CFU/mL).

Time-lapse microscopy (the oCelloScope)

Another way of measuring real-time bacterial growth is to utilize digital time-lapse microscopy (98). Our group previously evaluated the use of the oCelloScope (Philips BioCell A/S, Allerød, Denmark) as a screening tool for identifying promising combinations (99,100). In this assay, a bacterial inoculum of ~6 $\log_{10}$ CFU/mL and antibiotics are placed in a flat-bottomed 96-well plate. The plate is covered with a transparent adhesive film before being placed in the microscope which is located within an incubator of 37°C. The software utilizes a bottom search function to determine the best focus for each well at the start of the experiment. In our experiments, five images of each well were taken every fifteen minutes for 24 h. A strength of the microscope for this field of application is the leaning camera which facilitates imaging of a solution. All the images from the same horizontal plane are combined into an image stack before analysis.

The UniExplorer software version 6.0 (Philips BioCell A/S, Allerød, Denmark) analyzes the images generated during the screening. A combination of the Background Corrected Absorption (BCA) and Segmentation and Extraction of Surface Area (SESA) algorithms has been found to best describe

the bacterial density. BCA measures differences in intensity in comparison to the first images generated while SESA measures the area of detected objects (pixels). The use of both algorithms minimizes the rate of false positives. BCA cannot separate condensation, filamentous bacteria or colored media/antibiotics from bacterial growth. SESA on the other hand, is unreliable at high bacterial concentrations as the value decreases as cells start to overlap. For this reason, we utilize $SESA_{max}$ i.e., the highest measured SESA-value during the 24 h period. Cut-off values indicating a bacterial density of $>10^6$ CFU/mL after 24 h were set to >8.0 for BCA and >5.8 for maximum SESA value ($SESA_{max}$). Antibiotic combinations inhibiting bacterial growth (BCA and/or $SESA_{max}$ below cut-off), while monotherapy failed to do so, were considered promising.

Dynamic time-kill experiments

Dynamic *in vitro* models can be used to mimic patient pharmacokinetics and such a model was previously developed by Löwdin et al. (101) and later modified by Christer Malmberg (personal communication). In short, diluent media is supplemented to a vessel containing the bacterial culture using a constant rate peristaltic pump (P-500, Pharmacia, Sweden). The bacteria grow in solution in the vessel and a filter prevents the bacteria from escaping. The flowrate settings are set to obtain a certain half-life calculated as follows:

$$F = V * \frac{\ln 2}{t_{½}}$$

Antimicrobial dosing and sampling for viable counts are possible at multiple time points. In a more recent publication, Hickman et al. evaluated the pharmacodynamics of double and triple combinations were the use of computer-controlled syringes dynamically added antibiotics to compensate for the difference in half-lives (102). As for static time-kill experiments, the bacterial killing effect was based on viable counts and differences in $\log_{10}$ CFU/mL between monotherapy and the combinations.

One limitation of such models is the increased workload in setting up and managing the systems. The material costs are also relatively high in comparison to a static time-kill experiment. The experiments also have a relatively high failure rate due to leakage from the vessel and environmental contamination. The limit of detection is very low (1 $\log_{10}$ CFU/mL) and the possibility to mimic pharmacokinetics *in vitro* is a huge advantage.

In recent years, the usage of the hollow fiber infection model (HFIM) (FiberCell® Systems Inc, New Market, USA) has increased for mimicking pharmacokinetics to evaluate pharmacodynamics (103). The principal is similar to the set up described above as medium is supplemented to a bacterial compartment using a pump. A big difference is the closed bacterial compartment in which semi-permeable fibers facilitate circulation of media while retaining the bacteria in the cartridge. The compartments containing the antibiotics and the bacteria are separated which facilitates sampling of bacterial loads and antibiotic concentrations throughout the experiment. The cost for infrastructure, cartridges and running remains high and no performance standards exist even if suggestions have been published (103).

Aim of the current thesis

The main objective of this thesis was to find novel antibiotic combinations effective against MDR Gram-negative bacteria in an attempt to meet the clinical need of new treatment alternatives. As a part of a Joint Programming Initiative on Antimicrobial Resistance (JPIAMR) called CO-ACTION, previously neglected or disused antibiotics were included in the polymyxin B combinations. In addition, antibiotics from several antibiotic classes were included. Also, the project aimed at providing a better understanding of the mechanisms behind synergy which can be important information in diagnostics and selection of suitable combinational therapy. The study design was based on the following questions:

1. Which combinations are most effective?
2. Which resistance mechanisms exist and can resistance be suppressed by using combinations?
3. Which are the mechanisms behind the observed interactions with specific combinations?

A great obstacle in treating Gram-negative pathogens is their complex membrane surrounding the bacteria. The foundation of this work is the membrane disrupting mechanism of action of polymyxin B. The prevailing theory regarding the synergistic effect obtained with polymyxin B combinations is that membrane disruption could facilitate entry of a second drug or reduce efflux activity (29,104). Further, polymyxin combinations will not be as efficient in circumventing other resistance mechanisms such as enzymatic activity or drug target alterations. A lot of emphasis was put on genetic analysis to elucidate underlying resistance mechanisms in order to find potential associations with synergistic effects. The long term goal with this thesis is to make a contribution in improving treatment options and clinical outcome in patients infected by MDR Gram-negative bacteria.

Paper I

Efficacy of antibiotic combinations against multidrug-resistant *Pseudomonas aeruginosa* in automated time-lapse microscopy and static time-kill experiments.

Background

Difficult-to-treat *P. aeruginosa* is emerging and clinical evidence and *in vitro* data for antimicrobial combination therapy is lacking (105). There are usually several mechanisms for resistance in *P. aeruginosa* strains such as decreased permeability of the outer membrane, increased efflux and enzymatic activity (65,80,106,107). It is therefore important to identify promising regimens and to better understand the mechanisms behind synergistic interactions. This paper is a part of an initiative where previously neglected or disused antibiotics were included in polymyxin B combinations to hopefully revitalize their clinical use.

Material and Methods

In this study, four clinical strains of MDR *P. aeruginosa* provided by the Public Health Agency of Sweden were included. The antibacterial effect of polymyxin B in combination with 13 different antibiotics (amikacin, aztreonam, cefepime, chloramphenicol, ciprofloxacin, fosfomycin, linezolid, meropenem, minocycline, rifampicin, temocillin, thiamphenicol and trimethoprim) at clinically relevant concentrations was assessed. Antibiotics were selected to represent several classes and antibiotics not normally effective against *P. aeruginosa*. Automated time-lapse microscopy (the oCelloScope) was used as a screening tool to find promising regimens. Combinations with enhanced effect in comparison to monotherapy were then evaluated using time-kill experiments. The strains were extensively

characterized in terms of antimicrobial susceptibility and genetic background. Genes encoding enzymatic activity, porins, efflux pumps or regulators were considered.

Results and discussion

Promising combinations effective against *P. aeruginosa*

Enhanced activity with the combination compared to the most active single antibiotic was found in 39 of the 52 (75%) experimental setups assessed using time-lapse microscopy. The most promising combinations with enhanced effect against all four strains were polymyxin B in combination with aztreonam, cefepime, fosfomycin, linezolid, minocycline, thiamphenicol or trimethoprim. The time-kill experiments could confirm additive or synergistic activity at one or several time points in 27 of the 39 (69%) promising combinations resulting from the initial screening. Polymyxin B in combination with aztreonam, cefepime or fosfomycin was effective against all four strains also according to this method. Combinations including meropenem, minocycline or thiamphenicol also remained promising as they were superior to monotherapy in 3 of 4 strains.

Strong efflux activity circumvented

Efflux systems are very abundant in *P. aeruginosa* in comparison to other Gram-negative species (65,106). Hyperactivation of the MexAB-OprM efflux system was suspected in all strains due to several mutations in previously known regulators. Further, intact sequences of *mexT* encoding a MexEF-OprN efflux regulator were also found in all strains suggesting basal efflux activity. Both MexAB-OprM and MexEF-OprN transport β-lactam antibiotics out of the cell which is a resistance mechanism circumvented by the membrane disrupting effect of polymyxin B (29,57,58). MexXY-OprM activity could potentially be lowered in two strains as the repressor encoding gene *mexZ* was intact (106). Genes encoding proteins involved in MexCD-OprJ efflux were also found but no speculation on efflux activity could be made due to varying genotypes.

Polymyxin B combinations and enzymatic degradation of antibiotics

Several aminoglycoside modifying enzymes were found in all strains which could explain the limited effect seen for polymyxin B and amikacin combinations (33,108). Enzymatic activity is hypothetically a resistance mechanism not circumvented by polymyxin B combinations (29). The level of enzymatic activity against a specific antibiotic is of importance and a number of β-lactamases were found to exhibit relatively low hydrolytic

effect on two of the β-lactam antibiotics used in combination with polymyxin B in Paper I. Despite the presence of β-lactamases, additive and synergistic effects with polymyxin B in combination with aztreonam or cefepime were frequently found. In contrast, the high hydrolytic effect by oxacillinases such as $bla_{\text{OXA-50}}$ and $bla_{\text{OXA-10}}$ against temocillin could be the reason for the lack of effect by polymyxin B in combination with temocillin (109,110). The polymyxin B and fosfomycin combination showed synergistic or additive effect in all four strains despite high fosfomycin MICs (≥64 mg/L) and presence of *fosA* encoding an enzyme inactivating fosfomycin. FosA is highly conserved in *P. aeruginosa* and a known reason for intrinsic resistance (80). Both polymyxin B and fosfomycin target components of the bacterial membrane and their simultaneous actions have been found successful in combination therapy previously (39).

Multifactorial meropenem resistance

In all three carbapenem resistant isolates several possible reasons for resistance were found: decreased permeability (non-functioning porin due to disrupted *oprD* gene), potentially increased efflux activity (MexAB-OprM hyperactivation) and genes encoding antibiotic-degrading enzymes, e.g. $bla_{\text{OXA-50}}$ and bla_{PAO} (65,106). Polymyxin B in combination with meropenem had bactericidal and synergistic or additive effect in all strains with meropenem resistant phenotype which is in line with previous results demonstrating *in vitro* synergy against MDR *P. aeruginosa* (95,111). The involvement of membrane associated resistance mechanisms, such as efflux and porin loss, in the meropenem-resistance phenotype probably increased the chance of synergy by the combination. Polymyxin B and meropenem in combination did not perform better than meropenem monotherapy in the meropenem susceptible strain.

Polymyxin B combinations dodging intrinsic resistance

Increased activity of polymyxin B combinations including minocycline, thiamphenicol or rifampicin, antibiotics normally not effective against *P. aeruginosa* due to decreased permeability or efflux activity, was also found (112,113). Interestingly, combinations including thiamphenicol performed better than those including chloramphenicol although both antibiotics have similar mode of action (114). The presence of chromosomal *catB7* in *P. aeruginosa* could have an effect but usually results in low-level chloramphenicol resistance (115). Even if synergistic or additive effect was found for the combinations, no genetic explanation could be found on this set of

data. Emerging resistant subpopulations was suggested as a potential reason (51). Unfortunately, no data on drug target alterations were included in the screening.

Conclusion

Several indications on β-lactam specific efflux and presence of β-lactamases were found in all strains. The membrane disrupting effect of polymyxin B would theoretically affect efflux activity but not enzymatic activity. The success of polymyxin B and aztreonam and cefepime combinations could be explained by the relatively low enzymatic activity against the β-lactams included. Further, meropenem resistance was also associated with decreased permeability through non-functioning *oprD* gene and the polymyxin B combination was superior to monotherapy in all resistant strains. Continued evaluation of the promising combinations found in this study is needed to assess potential clinical implementation.

Paper II

Evaluation of polymyxin B in combination with 13 other antibiotics against carbapenemase-producing *Klebsiella pneumoniae* in time-lapse microscopy and time-kill experiments.

Background

Carbapenem-producing *K. pneumoniae* is a high priority pathogen as treatment options are lacking and high mortality is seen in critically ill patients (94,116,117). As resistance is usually multifactorial and co-resistance is common, polymyxins have already become a cornerstone in the treatment of these bacteria (34). In order to find additional treatment alternatives, this study aimed to find promising polymyxin B combinations effective against *K. pneumoniae*. Previously neglected or disused antibiotics were included in the combinations to hopefully revitalize their clinical use. As lack of knowledge regarding combination therapy is prevalent, an extensive genetic characterization was included to study the genotype-phenotype relationship between resistance mechanisms present and treatment success by the combinations.

Material and Methods

Five clinical *K. pneumoniae* strains with MDR phenotype and carbapenemases-production were provided by the Public Health Agency of Sweden. An initial screening of promising polymyxin B combinations was performed using time-lapse microscopy. Polymyxin B was combined with 13 different antibiotics (amikacin, aztreonam, cefepime, chloramphenicol, ciprofloxacin, fosfomycin, linezolid, meropenem, minocycline, rifampicin, temocillin, thiamphenicol and trimethoprim) at clinically relevant concentrations. The selection of antibiotics was made to represent several antibiotic classes which were previously neglected or disused. As an example, minocycline and rifampicin are also intrinsically resistance to *K.*

pneumoniae. Promising combinations with enhanced effect in comparison to monotherapy in the initial screening were further evaluated using time-kill experiments. Antimicrobial susceptibility and genetic background were extensively studied and genes involved in polymyxin B resistance, enzymatic activity, porins, efflux pumps or regulators were considered.

Results and discussion

Promising combinations effective against *K. pneumoniae*

Of the 65 antibiotic combination/strains setups included in the initial screening 23 showed enhanced effect in comparison to monotherapy. Polymyxin B in combination with minocycline, rifampicin and fosfomycin performed exceptionally well with superior activity versus monotherapy against five, five and four strains, respectively. Of the 23 combinations evaluated in time-kill experiments 21 showed synergistic or additive effect at one or more time points. Combinations including polymyxin B and minocycline, fosfomycin or rifampicin showed promising results also in time-kill experiments. Polymyxin B in combination with minocycline had synergistic effect at 24 h in four of five strains with a bacteriostatic and a bactericidal effect in two of the strains. The combinations including rifampicin was synergistic at 24 h against all four strains evaluated in time-kill experiments and a bactericidal or bacteriostatic effect was seen in three and one strain respectively. For the polymyxin B and fosfomycin combination, synergistic effect was seen at one or more time points against four strains but regrowth was common. Frequent regrowth *in vitro* has been reported previously for polymyxin B and fosfomycin against KPC-producing *K. pneumoniae* which could potentially limit the clinical use of the combination (118–120).

Genotype, a better predictor than phenotype

Minocycline resistance is commonly caused by the presence of *tetB,* which encode an efflux pump or through porin loss and decreased permeability (68). The polymyxin B and minocycline combination was successful in four of five strains included in the study. None of the strains encoded *tetB* but the strain that was indifferent to the combination was associated with a disrupted *ompK35* sequence. The combination was also successful in strains with polymyxin B resistance associated with a mutated *ccrB* gene or IS-insertion in the *mgrB* promotor. A previous study on carbapenemase-producing *K. pneumoniae* has also reported synergistic effect by the polymyxin B and minocycline combination in polymyxin B resistant strains (121). Huang et al. reported that the effect of the combination decreased

with increased polymyxin B MIC, but here we find synergistic effect in strains with MICs as high as 16 and 32 mg/L. Taken together, this illustrates that the resistance phenotype is a poor predictor for treatment success for combinations. Instead, knowledge on genotype and resistance mechanisms affecting the antibiotics used in combinations is of importance.

Increased enzymatic activity resists polymyxin B combinations

All strains included in the study had a rifampicin MIC of >32 mg/L but only one strain carried the *arr-2* gene encoding a rifampicin inactivating enzyme (122). The polymyxin B and rifampicin combination was superior to monotherapy in the strains not encoding *arr-2*. This strengthens the hypothesis regarding the decreased effect of polymyxin B combinations in strains encoding enzymatic activity. This was also seen for the polymyxin B and aztreonam combination showing synergistic effect in one aztreonam susceptible strain at 1 and 3 h. The susceptible strain encoded one carbapenemase only while the other strains harbored CTX-M-15 or KPC for example, enzymes hydrolyzing aztreonam (123). In other words, polymyxin B and aztreonam combinations were superior to monotherapy in strains lacking enzymes hydrolyzing aztreonam. In contrast, polymyxin B in combination with fosfomycin showed synergistic effect in four strains despite the presence of *fosA* encoding a fosfomycin inactivating enzyme.

Polymyxin B and meropenem showed synergistic or additive effect against all three strains evaluated in time-kill experiments. In line with previous reports sequence variations in *ompK35* and *ompK36* seem to have a small influence on resistance and the success of combination therapy (124,125). Synergistic effect was seen after 24 h for one strain encoding bla_{VIM-1} only and additive effect was seen at 3 and/or 6 h for the remaining two strains harboring bla_{KPC}, bla_{TEM-1a} and/or bla_{SHV}. In contrast, the two strains with intermediate effect by the polymyxin B and meropenem combination encoded bla_{NDM-1} or bla_{OXA-48}, which can degrade meropenem, and six additional β-lactamases. Taken together, the fewer enzymes harbored by a strain, despite presence of carbapenemases, the bigger success for the polymyxin B and meropenem combination. This trend is in line with previous studies indicating that the accumulative effect of several enzymes is of importance (124). Observational clinical data advocate that meropenem should always be used in combination, and only if the meropenem MIC value is ≤ 8 mg/L, against carbapenem resistant *Enterobacterales* (35,117). However, this data is almost exclusively based on KPC-producing *K. pneu-*

moniae and our results indicates that the type and number of carbapenemases determines the success of the polymyxin B and meropenem combination.

Conclusion

Polymyxin B in combination with fosfomycin, minocycline or rifampicin was found to be most successful in treatment of carbapenemase-producing *K. pneumoniae*. However, the regrowth seen for combination therapy including fosfomycin might limit the clinical usage. Even so, further investigation on promising combinations in a larger group of strains would be of interest to draw more solid conclusions on genotype-phenotype associations found in this study.

Paper III

Interactions of polymyxin B in combination with aztreonam, minocycline, meropenem and rifampicin against *Escherichia coli* producing NDM and OXA-48-like carbapenemases.

Background

Polymyxin B in combination with other drugs such as β-lactams, minocycline and rifampicin have shown *in vitro* synergy against carbapenemase-producing *Enterobacterales* (Paper I-II) (126–128)). However, the effect against *E. coli* in particular, has been poorly studied in comparison to *K. pneumoniae*. This study aimed to further evaluate the effect by polymyxin B in combination with aztreonam, minocycline, meropenem and rifampicin against carbapenemase-producing *E. coli*. To increase the knowledge behind synergistic interactions, an extensive genetic analysis was performed. In addition, statistical calculations were used to find associations between genetic background and the combination effect.

Material and methods

Twenty carbapenemase-producing *E. coli* isolates originally collected from hospitalized patients in Oman during 2015 were included in the study. A 24 h time-lapse microscopy assay was used to screen for promising effects by polymyxin B in combination with aztreonam, minocycline, meropenem and rifampicin at multiple clinically achievable concentrations. After the 24 h time-lapse microscopy, a spot assay was added to generate CFU/mL data as samples were spotted on MH-II plates. In addition, the same samples were also plated on MH-II plates with polymyxin B at a concentration of 4 x MIC. To further investigate the potential emergence of resistance, a selection of isolates growing on the polymyxin B plates were subject to polymyxin B susceptibility testing. Association between

the genetic background and the effect of the combination was calculated using Fisher's exact test.

Results and discussion

Combinations effective against carbapenemase-producing *E. coli*

The time-lapse microscope assay and the spot test showed good conformity. Synergistic and bactericidal effects were identified by the spot test for 22/23 of the promising combinations resulting from the screening. Polymyxin B in combination with minocycline or rifampicin were the most successful regimens with synergistic and bactericidal effects against 11/20 and 9/20 strains, respectively. Polymyxin B in combination with a β-lactam was less successful. The screening identified positive interactions for combinations including meropenem only in 3/20 strains and the combinations including aztreonam were not superior to monotherapy. Although growth on plates containing polymyxin B was seen bacterial concentrations were typically ≤ 2 CFU/mL and no increase in MIC was recorded.

Antimicrobial non-susceptibility does not preclude synergy

Despite the fact that all strains had intermediate resistance to polymyxin B and an intermediate or resistant minocycline phenotype, synergy was seen in approximately half of the strains in both cases. The presence of *tetB* was associated with synergistic effect by polymyxin B and minocycline (P = 0.0281). In addition, statistically significant associations between polymyxin B and minocycline synergy were also found for *marB* and *marR* encoding regulators of AcrAB-TolC efflux (129). This finding supports the hypothesis that the membrane disrupting effect by polymyxin B can circumvent resistance mechanisms such as efflux activity (29).

Enzymatic activity remains an obstacle for β-lactams

Contrary to the current recommendations for difficult-to-treat carbapenemase-producing *Enterobacterales* infections, our results indicate that polymyxin B in combination with meropenem is not a successful treatment alternative for NDM and OXA-48-producing *E. coli* (34,130). The observational clinical data supporting the use of such combinations are mainly based on KPC-producing *K. pneumoniae* and its effect on *E. coli* and non-KPC isolates is less studied (34,131). In the current study, polymyxin B and β-lactams in combination were not sufficient to circumvent the enzymatic activity of the strains included. Although polymyxin B in combination with meropenem showed synergistic and bactericidal effect in 2/20 strains, no association between genetic background and the success

of the combination was found. However, β-lactamases with hydrolytic effect against the β-lactams included in the study was frequently found among the isolates. Metallo-β-lactamases (MBLs) such as NDM were found in 15/20 strains (NDM-1, n = 6; NDM-5, n = 8; NDM-7, n = 1) and hydrolyze meropenem (132). Aztreonam offers interesting features in treatment of carbapenemase-producing isolates due to its stability to MBLs. The presence of $bla_{\text{CTX-M-15}}$, encoding the β-lactamase CTX-M-15, with high affinity for aztreonam was seen in 14/20 strains (28,133). When using antimicrobial combinations or β-lactam-β-lactamase inhibitor combinations, it is important to consider strain specific genetic determinants in addition to the susceptibility profiles of the drugs in the combination. Knowledge on specific enzymes present causing the carbapenem-resistant phenotype is an important key in finding the optimal treatment strategy.

Evaluating the contribution of different genetic traits to combination effects

Gram-negative bacteria are intrinsically resistant to rifampicin which was also seen in the current study with MIC-values ranging from 8-32 mg/L. As mentioned, all strains showed intermediate resistance to polymyxin B and synergistic and bactericidal effect by the polymyxin B and rifampicin combination was identified in 9/20 strains. Interestingly, the genetic analysis revealed positive associations between synergistic effect by the combination and several mutations in the *arnT* gene (P = 0.005-0.022). The *arnT* gene encodes the ArnT enzyme which adds positively charged moieties to the negatively charged phosphate groups of lipid A which could decrease the effect of polymyxin B (9). On the other hand, alterations of lipid A could also influence the rigidity of the membrane itself and increase the permeability for rifampicin. The cation-linkages between the negatively charged phosphate groups is an important factor for membrane integrity (29). In contrast, the mutation C27Y in *eptA* (also encoding an enzyme altering the negatively charged feature of lipid A) was negatively associated with polymyxin B and minocycline synergy (P = 0.0499). Mutant *lpxB* and *lpxK* were however associated with polymyxin B and minocycline synergy (P = 0.0499 and P = 0.0499 respectively) (9). The enzymes encoded by *lpxB* and *lpxK* are important for lipid A biosynthesis. Minocycline itself has the ability to directly chelate Ca^{2+}, which is present in the cation-linkages between lipid A molecules which could also contribute to the synergistic effect of the combination by increasing permeability (29,134).

Conclusion

Positive interaction with polymyxin B combinations against NDM and OXA-48-group carbapenemases was more common for combinations including minocycline or rifampicin in comparison to β-lactams. The β-lactamase activity among the isolates included in the study was not circumvented by the combination. For combinations including minocycline presence of resistance mechanisms encoding efflux activity was frequently associated with the success of the combination. Alterations of lipid A was associated with synergy by polymyxin B combinations including minocycline or rifampicin. Taken together, there is still a lot to learn about the interactions of the antibiotics in combinations and how the genetic background influences the resistance mechanisms involved.

Paper IV

In vitro pharmacodynamics of polymyxin B in combination with minocycline against carbapenemase-producing *Klebsiella pneumoniae*.

Background

Carbapenem-resistant *Enterobacterales* are increasing in prevalence and polymyxin combinations are often used because other treatment alternatives are lacking. In previous screenings, a synergistic effect by polymyxin B in combination with minocycline was seen against *E. coli*, *K. pneumoniae* and *P. aeruginosa* in time-lapse microscopy and static time-kill experiment (Paper I-III). This study aimed to assess the effect of polymyxin B in combination with minocycline and its effect against carbapenemase-producing *K. pneumoniae.* The effect was assessed using a dynamic *in vitro* model mimicking patient concentrations of antibiotics, which vary over time.

Materials and methods

The clinical pharmacokinetic profile of polymyxin B and minocycline, alone and in combination, was simulated in a modified version of a previously published dynamic model (101). An *in silico* model based on static time-kill experiments (135) was utilized to predict the best fitted dosing used *in vitro* to generate the patients-pharmacokinetics of the antibiotic in combination (136,137). To confirm that the desired antibiotic concentrations were maintained in the *in vitro* model a biological concentration determination method was used. Monotherapy and the combination were tested against two clinical isolates of *K. pneumoniae* harboring $bla_{\text{OXA-48}}$ or $bla_{\text{KPC-3}}$ with varying phenotypic resistance to polymyxin B and minocycline. The pharmacodynamics were evaluated during 72 h and viable counts were performed on MH-II plates with and without polymyxin B or

minocycline at concentrations of 4 x and 8 x MIC to assess antibiotic susceptibility post treatment. Viable counts were performed after 24 h and again after 48 h to detect slow growing subpopulations. Isolates growing on antibiotic plates from the first replicate growing on antibiotic plates post treatment were subjected to MIC determination using BMD.

Results and discussion

Treatment success of the polymyxin B and minocycline combination

Synergistic and bactericidal effects were seen from 12 h and onwards against strain ARU705 which was susceptible to minocycline and showed intermediate resistance to polymyxin B. No obvious genetic reason for decreased polymyxin B susceptibility was found. ARU984 had a more resistant phenotype for both polymyxin B and minocycline. A synergistic effect against this strain was seen only between 4 and 8 h. While no obvious reason for decreased minocycline susceptibility was identified, the strain carried several mutations in genes associated with polymyxin B resistance: *pmrB* (T246A) and *ccrB* (C68S) (138).

Resistance development during treatment

Minocycline and polymyxin B monotherapy against ARU705 resulted in regrowth of isolates post treatment. All isolates growing on polymyxin B plates had a MIC increase of more than 2 steps. Isolates collected post treatment was not associated with an increase in minocycline MIC and growth on antibiotic plates was more likely a result of an inoculum effect. Few isolates growing on plates containing polymyxin B or minocycline was seen for the combination and no increase in polymyxin B or minocycline MIC was identified. In contrast, emergence of ARU984 subpopulations with decreased susceptibility was frequently seen with both the single and combination regimens. Of note, growth on PAP plates was also seen for the growth control and a selective pressure by the plate itself was noted. An increase in minocycline MIC was again rarely seen but a polymyxin B MIC increase was seen for 89% of the isolates. This indicates that a polymyxin B heteroresistant subpopulation was present in ARU984.

Clinically relevant concentrations for treatment success

Polymyxins are associated with nephrotoxicity and should be used with caution. In the current experiments, the polymyxin B concentration varies between 2 and 1 mg/L after 4 h while an average plasma concentration above 1 mg/L is considered too high and contributes to increased nephrotoxicity (34). The concentration levels of the simulated PK-profile

were effective against the most susceptible strain ARU705. For a heteroresistant strain such as ARU984, where few treatment alternatives exist, a continuous infusion of a relatively high concentration would be necessary.

Conclusion

The polymyxin B and minocycline combination could be a good alternative for treatment of carbapenemase-producing *K. pneumoniae* with moderate resistance profiles. The combination was not as successful against a seemingly heteroresistant strain.

Concluding remarks and future perspectives

Promising polymyxin B combinations

This thesis can report several promising polymyxin B combinations. Paper I identified polymyxin B in combination with aztreonam, cefepime or fosfomycin as effective alternatives in treating MDR *P. aeruginosa*. Papers II – IV focused on carbapenemase-producing *E. coli* and *K. pneumoniae* and polymyxin B combinations including minocycline or rifampicin were considered most promising after the screening in papers II and III. The assessment of polymyxin B and minocycline in paper IV showed that the combination may be a promising treatment alternative. Paper I-IV also highlights the importance of knowledge on resistance levels and resistance mechanism present as the combination was not as successful against a strain with elevated resistance phenotype and a polymyxin B heteroresistant subpopulation.

Treatment success and genetic background

Several associations between genetic background and treatment success by polymyxin B combinations were found. This work highlights the importance of knowledge on resistance mechanisms and the interactions of the drugs combined. Polymyxin B combinations had a large impact on the MDR-phenotype in *P. aeruginosa* which to a great extent is associated with elevated efflux activity. Elevated efflux is a resistance mechanism circumvented by the membrane disruptive effect of polymyxin B. The absence of β-lactamases with hydrolytic effect against the antibiotics included increased the chance of treatment success by polymyxin B and β-lactam combinations. The isolates included in paper II and III harbored enzymatic activity against the specific β-lactams used in combination with polymyxin B. As a result, the combination was not as successful as the mode of action of polymyxin B did not affect the enzymatic activity. Instead, polymyxin B combinations including minocycline or rifampicin were more successful. Both minocycline and rifampicin monotherapy have permeability issues in

Gram-negative bacteria which is a mechanism influenced by the mode of action of polymyxin B.

Predicting the success of combination therapy

Even the most promising combinations have limited usage in the presence of certain resistance levels or mechanisms. The possibility to include such combinations in the clinical routine analysis setting would require thoroughly developed diagnostics tools. Also, today's lack of consensus on how to prepare your samples prior to sequencing, selection of sequence platform, cut offs for quality and assembly could influence the end results. As an example, minor differences between various β-lactamases could be lost by small deviations in WGS-data handling. Having to map out the exact resistance mechanism in order to select treatment, might be too much work practically in a clinical microbiology lab.

Associating genotype with phenotype requires a lot of knowledge and complex interpretation of results. Furthermore, multifactorial antimicrobial resistance and genotype based diagnostics where you only detect what you have knowledge about and will not notice unknown events. Databases linking genotype and phenotype exist and are being updated but they are still dependent on human input and new knowledge. Optimistically, future solutions using e.g., artificial intelligence could substitute the flat human learning curve. Phenotypic based diagnostics could have an advantage but again, then we will not know what we are missing. However, development of phenotypical assays for identifying efficient antimicrobial combinations in the clinical setting offers an interesting alternative (139). Antimicrobial combination is a promising future alternative for when no other options are available. Usage in the clinical setting would require further studies and development of a feasible workflow for diagnostics.

Sammanfattning på svenska

Innan Alexander Flemming upptäckte penicillinet år 1928 var det inte möjligt att behandla infektioner orsakade av antibiotika. Storskalig produktion av antibiotika blev inte möjligt förrän år 1942, men gjorde då stor skillnad för tillfrisknandet av amerikanska soldater under andra världskriget. Sedan dess har tillgången på antibiotika varit en grundförutsättning för modern sjukvård och möjliggör idag operationer, vård av förtidigt födda barn och behandling av cancer.

Resistenta bakterier har ökad motståndskraft mot vanligt förekommande antibiotika vilket gör att de kan överleva en antibiotikakur. Faktum är att opassande eller avbruten behandling kan gynna resistens. Diagnostik som fastställer vad som orsakat infektionen och hur den bör behandlas är grundläggande för att motverka resistensutveckling. I bästa fall utplånas infektionen av antibiotikakuren, patienten tillfrisknar snabbt och efterföljande biverkningar uppkommer inte. Idag saknar många länder fortfarande lämplig diagnostik och tillgången på antibiotika kan även vara begränsad. Utvecklingen av antibiotikaresistens begränsar behandlingsalternativen ytterligare och i vissa fall finns inga alternativ alls, en utveckling som Alexander Flemming varnade för redan på 40-talet.

Denna doktorsavhandling har studerat antibiotikakombinationer för att bemöta den ökande bristen på alternativ vid svårbehandlade bakterieinfektioner. Idag är kunskapen om antibiotikakombinationer väldigt begränsad och användningen är ofta baserad på kliniska observationer. Avhandlingens främsta syfte har därför varit att hitta nya kombinationer med effekt mot svårbehandlade infektioner. Dessutom har stor vikt även lagts vid att försöka utreda och förstå varför kombinationen fungerar eller inte. En viktig beståndsdel i kombinationerna har varit antibiotikan polymyxin B vilket bryter ner den svårgenomträngliga yta som omger och skyddar vissa bakterier. Denna yta förhindrar effekt av många antibiotika då de inte kan ta sig in i bakteriecellen. Den allmänt accepterade mekanismen för kombinationsbehandling innehållande polymyxin B är att ett andra antibiotika skulle kunna verka när den svårgenomträngliga ytan bryts ner.

Studierna genererade flera lovande polymyxin B kombinationer som var effektiva mot svårbehandlade bakterier. Kombinationerna som studerades lyckades penetrera den skyddande ytan men hade ingen effekt vid förekomst av ämnen som inaktiverar antibiotika. Metoderna och tillvägagångssättet som använts i denna avhandling erbjuder ett effektivt och bra tillvägagångssätt för att studera effekten av antibiotikakombinationer.

Acknowledgements

There are many people I would like to thank for contributing to the finalization of my PhD thesis. I would especially like to express my gratitude to the following people:

My supervisors, **Thomas Tängdén**, **Linus Sandegren** and **Pernilla Lagerbäck**. Thank you for giving me the opportunity and for providing me with a wide foundation of understanding of the field of antibiotic resistance.

Present and previous colleagues: **Lisa Allander**, **Karin Vickberg**, **Elin Fermér**, **Herin Oh**, **Ayda Shams**, **Pikkei Wistrand-Yuen**, **Christer Malmberg**, **Cecilia Johansson**, **Annabel Peyravi Latif**, **Hanna Montelin**, **Marcus Hong**, **Björn Herrmann**, **Eva Tano**, **Carl Påhlson**, **Lena Kask**, and **Anna Nilsson**. Without your knowledge and support, there would not be a thesis today!

Co-authors: **Christian Giske**, **Hissa Al-Farsi**, **Marcus Hong**, **Chenyan Zhao**, **Elisabet Nielsen**, **Lena Friberg** and **Kari-Pekka Skarp**. Your contribution to our shared publications truly widened the perspective.

My extended lab family: **Linnea Flinkfeldt**, **Jessie Torpner**, **Emma Davies** and **Jenny Fernberg**. Thank you for all the positive energy and laughter throughout the years.

Thank you CrossFit Vanheim for keeping me sane during the past four and a half years. Thank you **Niklas Björk** and **Robert Åkerström** for providing such a platform. Also, a big thank you to all instructors and my workout buddies who all contribute to the atmosphere in this place. Some burpees a day keep the doctor away!

And finally, to my family and friends, thank you for your continuous love and support.

References

1. Jim O'Neill. AMR Review Paper - Tackling a crisis for the health and wealth of nations. The Review on Antimicrobial Resistance (AMR); 2014.
2. Wright P. Norman George Heatley. The Lancet. 2004 Feb 7;363(9407):495.
3. PENICILLIN'S FINDER ASSAYS ITS FUTURE; Sir Alexander Fleming Says Improved Dosage Method Is Needed to Extend Use Other Scientists Praised Self-Medication Decried. N Y Times. 1945 Jun 26;
4. The World Health Organisation. Global Framework for Development & Stewardship to Combat Antimicrobial Resistance. (Switzerland, 2018).
5. Tacconelli E, Carrara E, Savoldi A, Harbarth S, Mendelson M, Monnet DL, et al. Discovery, research, and development of new antibiotics: the WHO priority list of antibiotic-resistant bacteria and tuberculosis. Lancet Infect Dis. 2018 Mar;18(3):318–27.
6. Murray, R. Patrick, Rosenthal, S. Ken, Pfaller, A. Michael. Medical Microbiology. 7th ed. Philadelpia, PA: Elsevier Saunders; 2013.
7. Wilson, A. Brenda, Salyers, A. Abigail, Whitt, D. Dixie, Winkler, E. Malcolm. Bacterial Pathogenesis. 3rd ed. Washington, DC: ASM Press; 2011.
8. Moyes RB, Reynolds J, Breakwell DP. Differential staining of bacteria: gram stain. Curr Protoc Microbiol. 2009 Nov;Appendix 3:Appendix 3C.
9. Bertani B, Ruiz N. Function and Biogenesis of Lipopolysaccharides. EcoSal Plus. 2018 Aug;8(1).
10. Silhavy TJ, Kahne D, Walker S. The bacterial cell envelope. Cold Spring Harb Perspect Biol. 2010 May;2(5):a000414.
11. Moffatt JH, Harper M, Boyce JD. Mechanisms of Polymyxin Resistance. Adv Exp Med Biol. 2019;1145:55–71.
12. Costa TRD, Felisberto-Rodrigues C, Meir A, Prevost MS, Redzej A, Trokter M, et al. Secretion systems in Gram-negative bacteria: structural and mechanistic insights. Nat Rev Microbiol. 2015 Jun;13(6):343–59.
13. Fernando DM, Kumar A. Resistance-Nodulation-Division Multidrug Efflux Pumps in Gram-Negative Bacteria: Role in Virulence. Antibiotics. 2013 Mar 18;2(1):163–81.
14. Nikaido H. Molecular basis of bacterial outer membrane permeability revisited. Microbiol Mol Biol Rev MMBR. 2003 Dec;67(4):593–656.
15. Typas A, Banzhaf M, Gross CA, Vollmer W. From the regulation of peptidoglycan synthesis to bacterial growth and morphology. Nat Rev Microbiol. 2011 Dec 28;10(2):123–36.
16. Macheboeuf P, Contreras-Martel C, Job V, Dideberg O, Dessen A. Penicillin binding proteins: key players in bacterial cell cycle and drug resistance processes. FEMS Microbiol Rev. 2006 Sep;30(5):673–91.

17. Huszczynski SM, Lam JS, Khursigara CM. The Role of Pseudomonas aeruginosa Lipopolysaccharide in Bacterial Pathogenesis and Physiology. Pathog Basel Switz. 2019 Dec 19;9(1):E6.
18. Amor K, Heinrichs DE, Frirdich E, Ziebell K, Johnson RP, Whitfield C. Distribution of core oligosaccharide types in lipopolysaccharides from Escherichia coli. Infect Immun. 2000 Mar;68(3):1116–24.
19. Follador R, Heinz E, Wyres KL, Ellington MJ, Kowarik M, Holt KE, et al. The diversity of Klebsiella pneumoniae surface polysaccharides. Microb Genomics. 2016 Aug;2(8):e000073.
20. Opoku-Temeng C, Kobayashi SD, DeLeo FR. Klebsiella pneumoniae capsule polysaccharide as a target for therapeutics and vaccines. Comput Struct Biotechnol J. 2019 Oct 25;17:1360–6.
21. Goh KGK, Phan M-D, Forde BM, Chong TM, Yin W-F, Chan K-G, et al. Genome-Wide Discovery of Genes Required for Capsule Production by Uropathogenic Escherichia coli. mBio. 2017 Oct 24;8(5):e01558-17.
22. Walker KA, Miller VL. The intersection of capsule gene expression, hypermucoviscosity and hypervirulence in Klebsiella pneumoniae. Curr Opin Microbiol. 2020 Apr;54:95–102.
23. Chen L, Zou Y, She P, Wu Y. Composition, function, and regulation of T6SS in Pseudomonas aeruginosa. Microbiol Res. 2015 Mar;172:19–25.
24. Zhao F, Yang H, Bi D, Khaledi A, Qiao M. A systematic review and meta-analysis of antibiotic resistance patterns, and the correlation between biofilm formation with virulence factors in uropathogenic E. coli isolated from urinary tract infections. Microb Pathog. 2020 Jul;144:104196.
25. Ernst CM, Braxton JR, Rodriguez-Osorio CA, Zagieboylo AP, Li L, Pironti A, et al. Adaptive evolution of virulence and persistence in carbapenem-resistant Klebsiella pneumoniae. Nat Med. 2020 May;26(5):705–11.
26. European Centre for Disease Prevention and Control. Antimicrobial resistance in the EU/EEA (EARS-Net) - Annual Epidemiological Report 2019. Stockholm: ECDC; 2020.
27. Paczosa MK, Mecsas J. Klebsiella pneumoniae: Going on the Offense with a Strong Defense. Microbiol Mol Biol Rev MMBR. 2016 Sep;80(3):629–61.
28. Tooke CL, Hinchliffe P, Bragginton EC, Colenso CK, Hirvonen VHA, Takebayashi Y, et al. β-Lactamases and β-Lactamase Inhibitors in the 21st Century. J Mol Biol. 2019 23;431(18):3472–500.
29. Jian Li, Roger L. Nation, Keith S. Kaye. Polymyxin Antibiotics: From Laboratory Bench to Bedside, Advances in Experimental Medicine and Biology 1145. Springer Nature Switzerland AG; 2019.
30. the World Health Organization. 2020 Antibacterial agents in clinical and preclinical development: an overview and analysis. Geneva, 2021.
31. Jalde SS, Choi HK. Recent advances in the development of β-lactamase inhibitors. J Microbiol Seoul Korea. 2020 Aug;58(8):633–47.
32. Krause KM, Serio AW, Kane TR, Connolly LE. Aminoglycosides: An Overview. Cold Spring Harb Perspect Med. 2016 Jun 1;6(6).
33. Ramirez MS, Tolmasky ME. Aminoglycoside modifying enzymes. Drug Resist Updat Rev Comment Antimicrob Anticancer Chemother. 2010 Dec;13(6):151–71.

34. Tsuji BT, Pogue JM, Zavascki AP, Paul M, Daikos GL, Forrest A, et al. International Consensus Guidelines for the Optimal Use of the Polymyxins: Endorsed by the American College of Clinical Pharmacy (ACCP), European Society of Clinical Microbiology and Infectious Diseases (ESCMID), Infectious Diseases Society of America (IDSA), International Society for Anti-infective Pharmacology (ISAP), Society of Critical Care Medicine (SCCM), and Society of Infectious Diseases Pharmacists (SIDP). Pharmacotherapy. 2019;39(1):10–39.
35. Behandling av infektioner orsakade av ESBL-producerande Enterobacteriaceae ochkarbapenemresistenta gramnegativa bakterier. Strategigruppen för rationell antibiotikaanvändning och minskad antibiotikaresistens (STRAMA); 2018.
36. Prescott WA, Nagel JL. Extended-interval once-daily dosing of aminoglycosides in adult and pediatric patients with cystic fibrosis. Pharmacotherapy. 2010 Jan;30(1):95–108.
37. Tamma PD, Aitken SL, Bonomo RA, Mathers AJ, van Duin D, Clancy CJ. Infectious Diseases Society of America Guidance on the Treatment of Extended-Spectrum β-lactamase Producing Enterobacterales (ESBL-E), Carbapenem-Resistant Enterobacterales (CRE), and Pseudomonas aeruginosa with Difficult-to-Treat Resistance (DTR-P. aeruginosa). Clin Infect Dis Off Publ Infect Dis Soc Am. 2020 Oct 27;
38. Falagas ME, Athanasaki F, Voulgaris GL, Triarides NA, Vardakas KZ. Resistance to fosfomycin: Mechanisms, Frequency and Clinical Consequences. Int J Antimicrob Agents. 2019 Jan;53(1):22–8.
39. Skarzynski T, Mistry A, Wonacott A, Hutchinson SE, Kelly VA, Duncan K. Structure of UDP-N-acetylglucosamine enolpyruvyl transferase, an enzyme essential for the synthesis of bacterial peptidoglycan, complexed with substrate UDP-N-acetylglucosamine and the drug fosfomycin. Struct Lond Engl 1993. 1996 Dec 15;4(12):1465–74.
40. Múñez Rubio E, Ramos Martínez A, Fernández Cruz A. Fosfomycin in antimicrobial stewardship programs. Rev Espanola Quimioter Publicacion Of Soc Espanola Quimioter. 2019 May;32 Suppl 1:62–6.
41. Falagas ME, Kastoris AC, Kapaskelis AM, Karageorgopoulos DE. Fosfomycin for the treatment of multidrug-resistant, including extended-spectrum beta-lactamase producing, Enterobacteriaceae infections: a systematic review. Lancet Infect Dis. 2010 Jan;10(1):43–50.
42. Ezelarab HAA, Abbas SH, Hassan HA, Abuo-Rahma GE-DA. Recent updates of fluoroquinolones as antibacterial agents. Arch Pharm (Weinheim). 2018 Sep;351(9):e1800141.
43. Ayoub Moubareck C. Polymyxins and Bacterial Membranes: A Review of Antibacterial Activity and Mechanisms of Resistance. Membranes. 2020 Aug;10(8):181.
44. Wagenlehner F, Lucenteforte E, Pea F, Soriano A, Tavoschi L, Steele VR, et al. Systematic review on estimated rates of nephrotoxicity and neurotoxicity in patients treated with polymyxins. Clin Microbiol Infect Off Publ Eur Soc Clin Microbiol Infect Dis. 2021 Jan 6;S1198-743X(20)30764-3.

45. Cho H, Naskar A, Lee S, Kim S, Kim K-S. A New Surface Charge Neutralizing Nano-Adjuvant to Potentiate Polymyxins in Killing Mcr-1 Mediated Drug-Resistant Escherichia coli. Pharmaceutics. 2021 Feb 11;13(2):250.
46. EUCAST Clinical Breakpoint Tables v. 11.0, valid from 2021-01-01. 2021.
47. The European Committee on Antimicrobial Susceptibility Testing. Reading guide for broth microdilution. v 3.0. Valid from 2021-01-01.
48. Gunnar Kahlmeter and the EUCAST Steering Committee. Redefining susceptibility testing categories S, I and R. Valid from 2019-01-01.
49. Mouton JW, Meletiadis J, Voss A, Turnidge J. Variation of MIC measurements: the contribution of strain and laboratory variability to measurement precision. J Antimicrob Chemother. 2018 Sep 1;73(9):2374–9.
50. Khan ZA, Siddiqui MF, Park S. Current and Emerging Methods of Antibiotic Susceptibility Testing. Diagnostics [Internet]. 2019 May 3 [cited 2021 Mar 5];9(2). Available from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC6627445/
51. Nicoloff H, Hjort K, Levin BR, Andersson DI. The high prevalence of antibiotic heteroresistance in pathogenic bacteria is mainly caused by gene amplification. Nat Microbiol. 2019;4(3):504–14.
52. Clinical Laboratory Testing and In Vitro Diagnostic TestSystems. Susceptibility Testing of Infectious Agents and Evaluation of Performance of Antimicrobial Susceptibility Test Devices. Part 1: Reference Method for Testing the In Vitro Activity of Antimicrobial Agents Against Rapidly Growing Aerobic Bacteria Involved in Infectious Diseases. International Standard 20776-1. Geneva, Switzerland: ISO; 2006.
53. The World Health Organisation. GLASS whole-genome sequencing for surveillance of antimicrobial resistance. Geneva, 2020.
54. Ellington MJ, Ekelund O, Aarestrup FM, Canton R, Doumith M, Giske C, et al. The role of whole genome sequencing in antimicrobial susceptibility testing of bacteria: report from the EUCAST Subcommittee. Clin Microbiol Infect Off Publ Eur Soc Clin Microbiol Infect Dis. 2017 Jan;23(1):2–22.
55. Alcock BP, Raphenya AR, Lau TTY, Tsang KK, Bouchard M, Edalatmand A, et al. CARD 2020: antibiotic resistome surveillance with the comprehensive antibiotic resistance database. Nucleic Acids Res. 2020 Jan 8;48(D1):D517–25.
56. Bortolaia V, Kaas RS, Ruppe E, Roberts MC, Schwarz S, Cattoir V, et al. ResFinder 4.0 for predictions of phenotypes from genotypes. J Antimicrob Chemother. 2020 Dec 1;75(12):3491–500.
57. López-Causapé C, Cabot G, Del Barrio-Tofiño E, Oliver A. The Versatile Mutational Resistome of Pseudomonas aeruginosa. Front Microbiol. 2018;9:685.
58. Li X-Z, Plésiat P, Nikaido H. The challenge of efflux-mediated antibiotic resistance in Gram-negative bacteria. Clin Microbiol Rev. 2015 Apr;28(2):337–418.
59. Bialek-Davenet S, Lavigne J-P, Guyot K, Mayer N, Tournebize R, Brisse S, et al. Differential contribution of AcrAB and OqxAB efflux pumps to multidrug resistance and virulence in Klebsiella pneumoniae. J Antimicrob Chemother. 2015 Jan;70(1):81–8.

60. Arzanlou M, Chai WC, Venter H. Intrinsic, adaptive and acquired antimicrobial resistance in Gram-negative bacteria. Essays Biochem. 2017 28;61(1):49–59.
61. Partridge SR, Kwong SM, Firth N, Jensen SO. Mobile Genetic Elements Associated with Antimicrobial Resistance. Clin Microbiol Rev [Internet]. 2018 Oct 1 [cited 2020 Oct 8];31(4). Available from: https://cmr.asm.org/content/31/4/e00088-17
62. Olaitan AO, Morand S, Rolain J-M. Mechanisms of polymyxin resistance: acquired and intrinsic resistance in bacteria. Front Microbiol. 2014;5:643.
63. Choi U, Lee C-R. Distinct Roles of Outer Membrane Porins in Antibiotic Resistance and Membrane Integrity in Escherichia coli. Front Microbiol. 2019;10:953.
64. Chetri S, Singha M, Bhowmik D, Nath K, Chanda DD, Chakravarty A, et al. Transcriptional response of OmpC and OmpF in Escherichia coli against differential gradient of carbapenem stress. BMC Res Notes. 2019 Mar 14;12(1):138.
65. Quale J, Bratu S, Gupta J, Landman D. Interplay of Efflux System, ampC, and oprD Expression in Carbapenem Resistance of Pseudomonas aeruginosa Clinical Isolates. Antimicrob Agents Chemother. 2006 May 1;50(5):1633–41.
66. Sugawara E, Kojima S, Nikaido H. Klebsiella pneumoniae Major Porins OmpK35 and OmpK36 Allow More Efficient Diffusion of β-Lactams than Their Escherichia coli Homologs OmpF and OmpC. J Bacteriol. 2016 01;198(23):3200–8.
67. Schnappinger D, Hillen W. Tetracyclines: antibiotic action, uptake, and resistance mechanisms. Arch Microbiol. 1996 Jun;165(6):359–69.
68. Chopra I, Roberts M. Tetracycline Antibiotics: Mode of Action, Applications, Molecular Biology, and Epidemiology of Bacterial Resistance. Microbiol Mol Biol Rev. 2001 Jun 1;65(2):232–60.
69. Chevalier S, Bouffartigues E, Bodilis J, Maillot O, Lesouhaitier O, Feuilloley MGJ, et al. Structure, function and regulation of Pseudomonas aeruginosa porins. FEMS Microbiol Rev. 2017 01;41(5):698–722.
70. Nakashima R, Sakurai K, Yamasaki S, Nishino K, Yamaguchi A. Structures of the multidrug exporter AcrB reveal a proximal multisite drug-binding pocket. Nature. 2011 Nov 27;480(7378):565–9.
71. Lee JO, Cho K-S, Kim OB. Overproduction of AcrR increases organic solvent tolerance mediated by modulation of SoxS regulon in Escherichia coli. Appl Microbiol Biotechnol. 2014 Oct 1;98(20):8763–73.
72. Schneiders T, Amyes SGB, Levy SB. Role of AcrR and ramA in fluoroquinolone resistance in clinical Klebsiella pneumoniae isolates from Singapore. Antimicrob Agents Chemother. 2003 Sep;47(9):2831–7.
73. Shankar C, Nabarro LEB, Anandan S, Veeraraghavan B. Minocycline and Tigecycline: What Is Their Role in the Treatment of Carbapenem-Resistant Gram–Negative Organisms? Microb Drug Resist. 2016 Aug 26;23(4):437–46.
74. Hansen LH, Johannesen E, Burmølle M, Sørensen AH, Sørensen SJ. Plasmid-encoded multidrug efflux pump conferring resistance to olaquindox in Escherichia coli. Antimicrob Agents Chemother. 2004 Sep;48(9):3332–7.

75. Szabo O, Kocsis B, Szabo N, Kristof K, Szabo D. Contribution of OqxAB Efflux Pump in Selection of Fluoroquinolone-Resistant Klebsiella pneumoniae. Can J Infect Dis Med Microbiol J Can Mal Infect Microbiol Medicale. 2018;2018:4271638.
76. Li J, Zhang H, Ning J, Sajid A, Cheng G, Yuan Z, et al. The nature and epidemiology of OqxAB, a multidrug efflux pump. Antimicrob Resist Infect Control [Internet]. 2019 Feb 22 [cited 2020 Oct 2];8. Available from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC6387526/
77. Kawalek A, Modrzejewska M, Zieniuk B, Bartosik AA, Jagura-Burdzy G. Interaction of ArmZ with the DNA-binding domain of MexZ induces expression of mexXY multidrug efflux pump genes and antimicrobial resistance in Pseudomonas aeruginosa. Antimicrob Agents Chemother. 2019 Sep 16;
78. Lau CH-F, Krahn T, Gilmour C, Mullen E, Poole K. AmgRS-mediated envelope stress-inducible expression of the mexXY multidrug efflux operon of Pseudomonas aeruginosa. MicrobiologyOpen. 2015 Feb;4(1):121–35.
79. Muller C, Plésiat P, Jeannot K. A two-component regulatory system interconnects resistance to polymyxins, aminoglycosides, fluoroquinolones, and β-lactams in Pseudomonas aeruginosa. Antimicrob Agents Chemother. 2011 Mar;55(3):1211–21.
80. Ito R, Mustapha MM, Tomich AD, Callaghan JD, McElheny CL, Mettus RT, et al. Widespread Fosfomycin Resistance in Gram-Negative Bacteria Attributable to the Chromosomal fosA Gene. mBio. 2017 Sep 6;8(4):e00749-17.
81. Sandegren L, Andersson DI. Bacterial gene amplification: implications for the evolution of antibiotic resistance. Nat Rev Microbiol. 2009 Aug;7(8):578–88.
82. Kumar S, Parvathi A, Hernandez RL, Cadle KM, Varela MF. Identification of a novel UDP-N-acetylglucosamine enolpyruvyl transferase (MurA) from Vibrio fischeri that confers high fosfomycin resistance in Escherichia coli. Arch Microbiol. 2009 May;191(5):425–9.
83. Kim DH, Lees WJ, Kempsell KE, Lane WS, Duncan K, Walsh CT. Characterization of a Cys115 to Asp substitution in the Escherichia coli cell wall biosynthetic enzyme UDP-GlcNAc enolpyruvyl transferase (MurA) that confers resistance to inactivation by the antibiotic fosfomycin. Biochemistry. 1996 Apr 16;35(15):4923–8.
84. Moya B, Dötsch A, Juan C, Blázquez J, Zamorano L, Haussler S, et al. Beta-lactam resistance response triggered by inactivation of a nonessential penicillin-binding protein. PLoS Pathog. 2009 Mar;5(3):e1000353.
85. Zhang Y, Kashikar A, Brown CA, Denys G, Bush K. Unusual Escherichia coli PBP 3 Insertion Sequence Identified from a Collection of Carbapenem-Resistant Enterobacteriaceae Tested In Vitro with a Combination of Ceftazidime-, Ceftaroline-, or Aztreonam-Avibactam. Antimicrob Agents Chemother. 2017 Aug;61(8):e00389-17.
86. Garoff L, Huseby DL, Praski Alzrigat L, Hughes D. Effect of aminoacyl-tRNA synthetase mutations on susceptibility to ciprofloxacin in Escherichia coli. J Antimicrob Chemother. 2018 Dec 1;73(12):3285–92.

87. Mehla K, Ramana J. Structural signature of Ser83Leu and Asp87Asn mutations in DNA gyrase from enterotoxigenic Escherichia coli and impact on quinolone resistance. Gene. 2016 Jan 15;576(1 Pt 1):28–35.
88. Hooper DC, Jacoby GA. Topoisomerase Inhibitors: Fluoroquinolone Mechanisms of Action and Resistance. Cold Spring Harb Perspect Med. 2016 Sep 1;6(9):a025320.
89. Jana S, Deb JK. Molecular understanding of aminoglycoside action and resistance. Appl Microbiol Biotechnol. 2006 Mar;70(2):140–50.
90. Galimand M, Courvalin P, Lambert T. Plasmid-mediated high-level resistance to aminoglycosides in Enterobacteriaceae due to 16S rRNA methylation. Antimicrob Agents Chemother. 2003 Aug;47(8):2565–71.
91. Galimand M, Sabtcheva S, Courvalin P, Lambert T. Worldwide disseminated armA aminoglycoside resistance methylase gene is borne by composite transposon Tn1548. Antimicrob Agents Chemother. 2005 Jul;49(7):2949–53.
92. Hinchliffe P, Yang QE, Portal E, Young T, Li H, Tooke CL, et al. Insights into the Mechanistic Basis of Plasmid-Mediated Colistin Resistance from Crystal Structures of the Catalytic Domain of MCR-1. Sci Rep. 2017 Jan 6;7:39392.
93. Martis N, Leroy S, Blanc V. Colistin in multi-drug resistant Pseudomonas aeruginosa blood-stream infections: a narrative review for the clinician. J Infect. 2014 Jul;69(1):1–12.
94. Paul M, Carmeli Y, Durante-Mangoni E, Mouton JW, Tacconelli E, Theuretzbacher U, et al. Combination therapy for carbapenem-resistant Gram-negative bacteria. J Antimicrob Chemother. 2014 Sep;69(9):2305–9.
95. Lenhard JR, Nation RL, Tsuji BT. Synergistic combinations of polymyxins. Int J Antimicrob Agents. 2016 Dec 1;48(6):607–13.
96. Clinical and Laboratory Standards Institute, 1999. M26-ED01:1999. Methods for Determining Bactericidal Activity of Antimicrobial Agents, 1st Edition.
97. White RL, Burgess DS, Manduru M, Bosso JA. Comparison of three different in vitro methods of detecting synergy: time-kill, checkerboard, and E test. Antimicrob Agents Chemother. 1996 Aug;40(8):1914–8.
98. Fredborg M, Andersen KR, Jørgensen E, Droce A, Olesen T, Jensen BB, et al. Real-time optical antimicrobial susceptibility testing. J Clin Microbiol. 2013 Jul;51(7):2047–53.
99. Ungphakorn W, Malmberg C, Lagerbäck P, Cars O, Nielsen EI, Tängdén T. Evaluation of automated time-lapse microscopy for assessment of in vitro activity of antibiotics. J Microbiol Methods. 2017;132:69–75.
100. Ungphakorn W, Lagerbäck P, Nielsen EI, Tängdén T. Automated time-lapse microscopy a novel method for screening of antibiotic combination effects against multidrug-resistant Gram-negative bacteria. Clin Microbiol Infect Off Publ Eur Soc Clin Microbiol Infect Dis. 2018 Jul;24(7):778.e7-778.e14.
101. Löwdin E, Odenholt I, Bengtsson S, Cars O. Pharmacodynamic effects of sub-MICs of benzylpenicillin against Streptococcus pyogenes in a newly developed in vitro kinetic model. Antimicrob Agents Chemother. 1996 Nov;40(11):2478–82.

102. Hickman RA, Hughes D, Cars T, Malmberg C, Cars O. Cell-wall-inhibiting antibiotic combinations with activity against multidrug-resistant Klebsiella pneumoniae and Escherichia coli. Clin Microbiol Infect Off Publ Eur Soc Clin Microbiol Infect Dis. 2014 Apr;20(4):O267-273.
103. Sadouki Z, McHugh TD, Aarnoutse R, Ortiz Canseco J, Darlow C, Hope W, et al. Application of the hollow fibre infection model (HFIM) in antimicrobial development: a systematic review and recommendations of reporting. J Antimicrob Chemother. 2021 Aug 12;76(9):2252–9.
104. Bowers DR, Cao H, Zhou J, Ledesma KR, Sun D, Lomovskaya O, et al. Assessment of minocycline and polymyxin B combination against Acinetobacter baumannii. Antimicrob Agents Chemother. 2015 May;59(5):2720–5.
105. Rigatto MH, Vieira FJ, Antochevis LC, Behle TF, Lopes NT, Zavascki AP. Polymyxin B in Combination with Antimicrobials Lacking In Vitro Activity versus Polymyxin B in Monotherapy in Critically Ill Patients with Acinetobacter baumannii or Pseudomonas aeruginosa Infections. Antimicrob Agents Chemother. 2015 Oct;59(10):6575–80.
106. Masuda N, Sakagawa E, Ohya S, Gotoh N, Tsujimoto H, Nishino T. Substrate specificities of MexAB-OprM, MexCD-OprJ, and MexXY-oprM efflux pumps in Pseudomonas aeruginosa. Antimicrob Agents Chemother. 2000 Dec;44(12):3322–7.
107. Poirel L, Naas T, Nordmann P. Diversity, epidemiology, and genetics of class D beta-lactamases. Antimicrob Agents Chemother. 2010 Jan;54(1):24–38.
108. Ramirez MS, Tolmasky ME. Amikacin: Uses, Resistance, and Prospects for Inhibition. Mol Basel Switz. 2017 Dec 19;22(12).
109. Alexandre K, Fantin B. Pharmacokinetics and Pharmacodynamics of Temocillin. Clin Pharmacokinet. 2018 Mar;57(3):287–96.
110. Jules K, Neu HC. Antibacterial activity and beta-lactamase stability of temocillin. Antimicrob Agents Chemother. 1982 Sep;22(3):453–60.
111. Guelfi KC, Tognim MCB, Cardoso CL, Gales AC, Carrara-Marrone FE, Garcia LB. In vitro evaluation of the antimicrobial activity of meropenem in combination with polymyxin B and gatifloxacin against Pseudomonas aeruginosa and Acinetobacter baumannii. J Chemother Florence Italy. 2008 Apr;20(2):180–5.
112. Li XZ, Livermore DM, Nikaido H. Role of efflux pump(s) in intrinsic resistance of Pseudomonas aeruginosa: resistance to tetracycline, chloramphenicol, and norfloxacin. Antimicrob Agents Chemother. 1994 Aug 1;38(8):1732–41.
113. Krishnamoorthy G, Wolloscheck D, Weeks JW, Croft C, Rybenkov VV, Zgurskaya HI. Breaking the Permeability Barrier of Escherichia coli by Controlled Hyperporination of the Outer Membrane. Antimicrob Agents Chemother. 2016 Dec;60(12):7372–81.
114. Neu HC, Fu KP. In vitro activity of chloramphenicol and thiamphenicol analogs. Antimicrob Agents Chemother. 1980 Aug;18(2):311–6.
115. White PA, Stokes HW, Bunny KL, Hall RM. Characterisation of a chloramphenicol acetyltransferase determinant found in the chromosome of Pseudomonas aeruginosa. FEMS Microbiol Lett. 1999 Jun 1;175(1):27–35.

116. Tumbarello M, Trecarichi EM, De Rosa FG, Giannella M, Giacobbe DR, Bassetti M, et al. Infections caused by KPC-producing Klebsiella pneumoniae: differences in therapy and mortality in a multicentre study. J Antimicrob Chemother. 2015 Jul;70(7):2133–43.
117. Daikos GL, Tsaousi S, Tzouvelekis LS, Anyfantis I, Psichogiou M, Argyropoulou A, et al. Carbapenemase-producing Klebsiella pneumoniae bloodstream infections: lowering mortality by antibiotic combination schemes and the role of carbapenems. Antimicrob Agents Chemother. 2014;58(4):2322–8.
118. Bulman ZP, Zhao M, Satlin MJ, Chen L, Kreiswirth BN, Walsh TJ, et al. Polymyxin B and fosfomycin thwart KPC-producing Klebsiella pneumoniae in the hollow-fibre infection model. Int J Antimicrob Agents. 2018 Jul;52(1):114–8.
119. Diep JK, Sharma R, Ellis-Grosse EJ, Abboud CS, Rao GG. Evaluation of Activity and Emergence of Resistance of Polymyxin B and ZTI-01 (Fosfomycin for Injection) against KPC-Producing Klebsiella pneumoniae. Antimicrob Agents Chemother. 2018 Feb;62(2).
120. Fransen F, Hermans K, Melchers MJB, Lagarde CCM, Meletiadis J, Mouton JW. Pharmacodynamics of fosfomycin against ESBL- and/or carbapenemase-producing Enterobacteriaceae. J Antimicrob Chemother. 2017 Dec 1;72(12):3374–81.
121. Huang D, Yu B, Diep JK, Sharma R, Dudley M, Monteiro J, et al. In Vitro Assessment of Combined Polymyxin B and Minocycline Therapy against Klebsiella pneumoniae Carbapenemase (KPC)-Producing K. pneumoniae. Antimicrob Agents Chemother. 2017;61(7).
122. Baysarowich J, Koteva K, Hughes DW, Ejim L, Griffiths E, Zhang K, et al. Rifamycin antibiotic resistance by ADP-ribosylation: Structure and diversity of Arr. Proc Natl Acad Sci U S A. 2008 Mar 25;105(12):4886–91.
123. Doi Y, Paterson DL. Carbapenemase-producing Enterobacteriaceae. Semin Respir Crit Care Med. 2015 Feb;36(1):74–84.
124. Zhang Y, Jiang X, Wang Y, Li G, Tian Y, Liu H, et al. Contribution of β-lactamases and porin proteins OmpK35 and OmpK36 to carbapenem resistance in clinical isolates of KPC-2-producing Klebsiella pneumoniae. Antimicrob Agents Chemother. 2014;58(2):1214–7.
125. Clancy CJ, Chen L, Hong JH, Cheng S, Hao B, Shields RK, et al. Mutations of the ompK36 porin gene and promoter impact responses of sequence type 258, KPC-2-producing Klebsiella pneumoniae strains to doripenem and doripenem-colistin. Antimicrob Agents Chemother. 2013 Nov;57(11):5258–65.
126. Yu Y, Walsh TR, Yang R-S, Zheng M, Wei M-C, Tyrrell JM, et al. Novel partners with colistin to increase its in vivo therapeutic effectiveness and prevent the occurrence of colistin resistance in NDM- and MCR-co-producing Escherichia coli in a murine infection model. J Antimicrob Chemother. 2019 Jan 1;74(1):87–95.
127. MacNair CR, Stokes JM, Carfrae LA, Fiebig-Comyn AA, Coombes BK, Mulvey MR, et al. Overcoming mcr-1 mediated colistin resistance with colistin in combination with other antibiotics. Nat Commun. 2018 Jan 31;9(1):1–8.

128. Urban C, Mariano N, Rahal JJ. In Vitro Double and Triple Bactericidal Activities of Doripenem, Polymyxin B, and Rifampin against Multidrug-Resistant Acinetobacter baumannii, Pseudomonas aeruginosa, Klebsiella pneumoniae, and Escherichia coli. Antimicrob Agents Chemother. 2010 Jun 1;54(6):2732–4.
129. Vinué L, Hooper DC, Jacoby GA. Chromosomal mutations that accompany qnr in clinical isolates of Escherichia coli. Int J Antimicrob Agents. 2018 Mar;51(3):479–83.
130. Infectious Diseases Society of America. Infectious Diseases Society of America Antimicrobial Resistant Treatment Guidance: Gram-Negative Bacterial Infections. 2020.
131. Paul M, Daikos GL, Durante-Mangoni E, Yahav D, Carmeli Y, Benattar YD, et al. Colistin alone versus colistin plus meropenem for treatment of severe infections caused by carbapenem-resistant Gram-negative bacteria: an open-label, randomised controlled trial. Lancet Infect Dis. 2018;18(4):391–400.
132. Khan AU, Maryam L, Zarrilli R. Structure, Genetics and Worldwide Spread of New Delhi Metallo-β-lactamase (NDM): a threat to public health. BMC Microbiol. 2017 Apr 27;17(1):101.
133. Lohans CT, Brem J, Schofield CJ. New Delhi Metallo-β-Lactamase 1 Catalyzes Avibactam and Aztreonam Hydrolysis. Antimicrob Agents Chemother [Internet]. 2017 Dec 1 [cited 2020 Jul 13];61(12). Available from: https://aac.asm.org/content/61/12/e01224-17
134. Dai C, Ciccotosto GD, Cappai R, Wang Y, Tang S, Xiao X, et al. Minocycline attenuates colistin-induced neurotoxicity via suppression of apoptosis, mitochondrial dysfunction and oxidative stress. J Antimicrob Chemother. 2017 Jun 1;72(6):1635–45.
135. Zhao C, Wistrand-Yuen P, Lagerbäck P, Tängdén T, Nielsen EI, Friberg LE. Combination of polymyxin B and minocycline against multidrug-resistant Klebsiella pneumoniae: interaction quantified by pharmacokinetic/pharmacodynamic modelling from in vitro data. Int J Antimicrob Agents. 2020 Jun;55(6):105941.
136. Sandri AM, Landersdorfer CB, Jacob J, Boniatti MM, Dalarosa MG, Falci DR, et al. Population pharmacokinetics of intravenous polymyxin B in critically ill patients: implications for selection of dosage regimens. Clin Infect Dis Off Publ Infect Dis Soc Am. 2013 Aug;57(4):524–31.
137. Welling PG, Shaw WR, Uman SJ, Tse FL, Craig WA. Pharmacokinetics of minocycline in renal failure. Antimicrob Agents Chemother. 1975 Nov;8(5):532–7.
138. De La Cadena E, Mojica MF, García-Betancur JC, Appel TM, Porras J, Pallares CJ, et al. Molecular Analysis of Polymyxin Resistance among Carbapenemase-Producing Klebsiella pneumoniae in Colombia. Antibiot Basel Switz. 2021 Mar 10;10(3):284.
139. Fatsis-Kavalopoulos N, Roemhild R, Tang P-C, Kreuger J, Andersson DI. CombiANT: Antibiotic interaction testing made easy. PLoS Biol. 2020 Sep;18(9):e3000856.

Acta Universitatis Upsaliensis

Digital Comprehensive Summaries of Uppsala Dissertations from the Faculty of Medicine 1788

Editor: The Dean of the Faculty of Medicine

A doctoral dissertation from the Faculty of Medicine, Uppsala University, is usually a summary of a number of papers. A few copies of the complete dissertation are kept at major Swedish research libraries, while the summary alone is distributed internationally through the series Digital Comprehensive Summaries of Uppsala Dissertations from the Faculty of Medicine. (Prior to January, 2005, the series was published under the title "Comprehensive Summaries of Uppsala Dissertations from the Faculty of Medicine".)

Distribution: publications.uu.se
urn:nbn:se:uu:diva-457615

ACTA
UNIVERSITATIS
UPSALIENSIS
UPPSALA
2021