Screening for a novel antimicrobial interaction for combination therapy

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1. Summary

Antibiotics could be one of man’s greatest discoveries over the last century, decreasing mortality and greatly improving our life expectancy. Over the last decades a careless overuse of these types of drugs are turning treatable infections unresponsive to treatment. One way of combating resistance in bacteria is to use combinational therapy that targets diverse sites on the bacteria and makes it possible to more efficiently kill the bacteria. These master project experiments were done on a clinically relevant multi drug resistant gram-negative bacterial species, *Klebsiella pneumoniae*. The bacteria had been cultivated from patient isolates. The strains were evaluated in regards to their susceptibility to a given combination of antibiotics with the use of a “Static time-kill” experimental assay. With this *in vitro* method cultures of bacteria were exposed to static concentrations of an antibacterial agent over a predetermined period of time as well as a combination of antibacterial agents. The synergistic combination found was further evaluated and characterized by additional “static time-kill” experiments with other clinical strains of K. pneumonia. The result of the experiments indicates that there is a novel synergistic effect between the antibiotics Colistin and Meropenem on *K. pneumoniae*. 
2. Background

2.1 A brief history of antibiotics

Since their discovery over seventy years ago, antibiotics have been our trusted weapons in the fight against bacterial infections, including life-threatening infections acquired in hospitals. The introduction of antibiotics was one of, perhaps the most, important developments in modern medicine. It all started with Alexander Flemmings accidental discovery in 1928. Even though many before him had observed antimicrobial inhibition of bacteria his discovery and development, with the help of Florey and Chain, of penicillin started the golden age of antibiotics. The actual name “antibiotics” was later given by Selman Abraham Waksman in a publication accepted in 1947. It was so named, he argued, because it was an antagonistic chemical towards bacterial growth. Soon after penicillin had been introduced to the general public another antibiotic was introduced, Streptomycin. Penicillin, which primarily has an effect on gram-positive bacteria, was thus complemented by a chemical that had its effect on gram-negative bacteria. From these two chemicals an arsenal of effective antimicrobials was developed. This had a dramatic effect on the western society with a drastic decrease in mortality from infections. The impact was so great that in 1969 US Surgeon General William H Stewart was quoted saying that - “The time has come to close the book on infectious disease”

2.2 The rise of resistance

This quote from Alexander Fleming paints a clear picture of what was to come after the golden age of antibiotics:

“It is not difficult to make microbes resistant to penicillin in the laboratory by exposing them to concentrations not sufficient to kill them, and the same thing has occasionally happened in the body... ...and by exposing these microbes to non-lethal quantities of the drug makes them resistant.” - Alexander Fleming, Nobel Prize lecture 1945

From the first introduction of antibiotics a resistance in some strains of *Staphylococcus* sp. was observed but as the antibiotic development was still finding new chemicals to keep resistance at bay this was thought of with little concern. Nowadays resistance has developed into a massive issue and is becoming an ever more increasing problem in the medical care of patients all over the world. Numbers from the World Health Organization show that only in Europe approximately 25.000 people die from a serious resistant bacterial infection each year. Most of those infections are acquired in health care settings. The problem with resistance has prompted the world health organization to focus the World Health Day 2011 on antibiotic resistance. Although most would agree that resistance needs to be addressed, there are almost no antimicrobial drugs being developed by pharmaceutical companies. The reason for this is due to several factors, but the key issue is that is too high of a risk and too low profit for the drug companies in developing new antibiotics. A worrying development is that for the last 15 years gram-positive bacteria and their increasing multidrug resistance has been in the loop. Companies directed effort on finding novel antimicrobial agents towards them. A few new pharmaceuticals have seen the light of day and have helped in the fight against resistance. Unfortunately, these few novel antimicrobials were not paralleled with the ongoing
resistance development in gram-negative bacteria. As a result of this a growing number of reports are being presented that demonstrates a vast increase in infections caused by gram-negative microorganisms for which no sufficient therapeutic options exist.\textsuperscript{12}

2.3 Combating resistance with combinations?

Hospitals are settings that spend and prescribe a lot of antibiotics. This in combination with patients having a weakened immune system makes it a perfect setting to select for multi resistance in bacteria. There are many ways of which to combat the spread of resistance but two very essential parts are to improve hygiene in hospitals and limit the over prescription of antibiotics to the general public.\textsuperscript{13} There is also a more direct way of approaching the problem where a combinational antibiotic therapy targets diverse sites on the bacteria. This makes it possible to successfully kill bacteria that otherwise would be resistant to one of the antibiotics used. This approach can never replace the need for development of new antibiotics but it can slow down resistance development in many bacterial species.

2.5 Extended-Spectrum β-Lactamases

Naturally occurring β-lactamases produced by bacteria have probably been around long before humans entered the scene. This is true for many gram-negative bacteria, which possess a chromosomal β-lactamase that gives them an edge in the evolutionary race against β-lactam producing competitors.\textsuperscript{14} The first appearance of the plasmid mediated β-lactamases in gram-negative bacteria was found in \textit{E. coli} and described in 1965, named “TEM-1” (the name originates from the patient it was first isolated from, a man named Temoniera).\textsuperscript{15} A few years later another plasmid-mediated β-lactamase was found in \textit{K. pneumoniae} and \textit{E. coli}, known as “SHV-1” (sulfhydryl variable-1).\textsuperscript{16} Bacteria bearing these plasmids were still sensitive against some β-lactams the cephalosporins and they were widely used in healthcare. In the 1980s the first β-lactamase that was able to hydrolyze all of the cephalosporins was described. It was isolated in Germany from a \textit{K. pneumoniae} and given the name “SHV-2”. This meant that there were enzymes on the plasmids that were active also against the extended spectrum β-lactam antibiotics, hence the name “Extended Spectrum β-Lactamases” (ESBL). ESBLs are most prevalent in the Enterobacteriaceae family and particularly in \textit{K. pneumoniae} and \textit{E. coli} but can also be found in \textit{Pseudomonas aeruginosa} and \textit{Acinetobacter baumannii}. There are now over 130 TEM-types and more then 50 SHV-types of β-lactamases confined to ESBL-plasmids with an ever more increasing number.\textsuperscript{14} There are also members of the ESBL-family that do not have their origin in the SHV or TEM, the most prominent and clinically relevant of those are the CTX-M types of ESBLs. They are distinguished by their ability to hydrolyze the β-lactam variant cefotaxime (The name ‘CTX’ is actually an abbreviation for ‘ceftaximase’ which refers to the hydrolytic activity it has) rather than the ceftazidimes. They can be divided in to five major groups: CTX-M1, CTX-M2, CTX-M8, CTX-M9 and CTX-M25\textsuperscript{17}. According to recent statistics for the period of 2004 to 2007 there has been a marked shift in Europe of the distribution of ESBLs towards a dramatic increase of the CTX-types in comparison to the SHV- and TEM-types.\textsuperscript{18} There are also clinically relevant, still scarce, ESBL-plasmid types that are not confined to the major families which mainly include the TLA, GES, IBC, VEB, SFO-1, BES-1 and BEL-1 types\textsuperscript{17}. Of great worry, but fortunately still uncommon, are the carbapenemases which
are a diverse group of β-lactamases that are not only active against most of the β-lactams but also against the carbapenems. This is of concern as the carbapenems are the only β-lactams that are still active against some ESBL-carrying bacteria and often the antibiotic of choice for treatment. Statistics collected from 2004 to 2007 regarding the prevalence of ESBL-producing strains among Enterobacteriaceae in 22 European countries indicated an increase in isolates with resistance to many antibiotics commonly used against resistant infections.

2.5.1 Antibiotics against ESBL-plasmids
ESBLs are able to hydrolyze aztreonam, cephalosporins and penicillins. There is however a great difference in the hydrolytic activity against the mentioned antibiotics between different ESBLs. For example the CTX-M types appear to have less activity against cefotaxime than ceftazidime in contrast to the TEM and the SHV types that typically have the opposite hydrolytic activity. Consequently ESBL-producing pathogens may have different susceptibility towards the above mentioned agents which also makes them hard to correctly diagnose in a laboratory. In the Enterobacteriaceae family, which is one of the most common ESBL-producers, frequent co-expression of resistance has been shown for floxquinolones, aminoglycosides, tetracyclines and trimethoprim/sulfamethoxazole. In many cases, and as long as there is no known resistance towards it, carbapenems are the drug of choice against the Enterobacteriaceae family. Agents that still show good efficacy towards ESBL-producers and may prove invaluable if the spread of resistance continues, include temocillin, fosfomycin, nitrofurantoin and the polymyxins. Particularly polymyxin has in clinical use retained excellent antimicrobial activity. It is however only a matter of time before resistance emerges also against these drugs, but with a prudent use this development can be slowed down.

2.4 Klebsiella pneumoniae – a growing problem
Members of the genus Klebsiella belong to the Enterobacteriaceae family. They have a prominent capsule that gives them an enhanced virulence and a mucoid appearance in isolated colonies. The most commonly isolated member is K. pneumoniae. They inhabit the colon of humans but can also be found in the intestinal and urinogenital tracts. It can cause varying degrees of community acquired lung infections, specifically primary lobar pneumonia. Patients hospitalized with compromised lung function are of increased risk of a pneumonia infection as they often have trouble clearing oral secretions from the lower respiratory tract. K. pneumoniae is most often diagnosed by cultivation on agar plates with selective media. They are also included in the group of pathogens that cause nosocomial infections or hospital-acquired infections, often producing urinary tract infection. K. pneumoniae can be a carrier of the ESBL-plasmid giving it a multitude of resistance depending on which ESBL it acquires. There are also some strains of K. pneumoniae that carry a chromosomally mediated resistance, one example of this and of great concern are the carbapenemases (KPC). According to the Swedish institute for communicable disease control there has been a rapid increase of K. pneumonia with ESBL-plasmids in recent years in Swedish hospitals. Much of this is due to overprescription of antibiotics which causes a selection towards resistance. The same indication can be found all over Europe. Klebsiella infections are a growing problem.
and it will only increase in magnitude and seriousness. There is therefore a pressing matter to try to control the spread of resistance in this bug.

2.6 Polymyxins – our last defense?
This old forgotten class of antibiotics has made its return to the medical scene since some infections have become resistant to almost all known classes of antibiotics. Resistance to the polymyxin class is of great worry as it is our last line of defense against multi-drug resistant gram-negative bacteria. If it should fall there is almost nothing left.

2.6.1 Polymyxins
The polymyxins is a small class of antibiotics, the group consists of five quite similar chemical compounds named polymyxin A-E that were discovered in the late 1940s. Although all five polymyxins show antibacterial activity, only polymyxin B and polymyxin E (which is also named colistin) have been used in clinical practice. Polymyxins are produced by strains of *Bacillus polymyxa* and colistin is produced by the subspecies *Bacillus Colistinus*.

2.6.2 Chemical structure
Colistin has a chemical structure that is built up by a cationic cyclic decapeptide linked to a fatty acid chain through an α-amide linkage (figure 1). It is a large molecule that has a weight of 1750 Da. The amino acid parts of the molecule are D-leucin, L-threonine, and L-α-γ-diaminobutyric acid. If the compound is identified as colistin A the L-α-γ-diaminobutyric acid is linked to a fatty acid residue, 6-methyloctan-oic acid. If the fatty residue is a 6-methyl-heptanoic acid it is named colistin B. These two types of colistin are therapeutically indistinguishable, in fact, the mixture of the two compounds is called colistin E and different batches of pharmaceutical preparation may contain different amounts of these two compounds. There are two forms of colistin available on the market today, colistin sulfate and colistimethate sodium (also called colistin sulfonyl methate, pentasodium colistimethanesulfate and colistin methanesulfate). Colistimethate sodium is less toxic but also less potent. Throughout this text colistimethate sodium is referred as colistin because colistin sulfate is rarely used in current treatment regimes.

![Figure 1. Molecular structure of colistin.](image-url)
2.6.3 Mechanism of action

The polymyxins are bactericidal to most types of gram-negative bacteria. They interact with the lipopolysaccharide layer (LPS) that only bacteria possess and disorganize the structures that is responsible for maintaining the osmotic equilibrium of the cell. Colistin displaces calcium ions (Ca$^{2+}$) and magnesium ions (Mg$^{2+}$), which have important functions in stabilizing the LPS-molecule. This makes the LPS-molecule loose its net charge and become negatively charged, which in turn leads to a local disturbance of the outer membrane. The result of this process is an increase in the permeability of the cell envelope, loss of cell substances and consequently cell death. When a polymyxin is added to a population of non-resistant bacteria a rapid release of internal cell material can be measured. In addition to having an antimicrobial activity it also possesses a potent anti-endotoxin effect by its high affinity to the LPS lipid A. Colistin binds to multiple sites on the LPS molecule and neutralizes its effect on the immune system.

Polymyxins have been shown to have an excellent bactericidal activity against most gram-negative aerobic bacteria like *Acinetobacter*, *Klebsiella*, *Salmonella*, *Shigella*, *Citrobacter* and *Enterobacter* species. The spectrum has also been shown to include *Pseudomonas aeruginosa*, *Escherichia coli*, *Morganella morganii*, *Haemophilus influenzae*, *Yersinia pseudotuberculosis* and several mycobacterium species including *M. tuberculosis*. Some gram-negative bacteria like *Pseudomonas mallei*, *Burkholderia cepacia*, *Brucella* species, *Proteus* species, *Providencia* species, *Edwardsiella* species and *Serratia* species all have a natural resistance against Colistin.

2.6.4 Resistance

Even though colistin shows great effect on many gram-negative bacteria, resistance occurs and has been seen in patients. There are at least two ways in which polymyxin susceptible bacteria can become resistant. One way is the stepwise adaptation to polymyxin present in the growth medium, the phenotype is altered in favor of resistance but looses this in the absence of polymyxin (adaptive resistance). This resistance is characterized by a range of different outer membrane alterations. The second type is mutational resistance, which can result in increase in the outer membrane protein H1 levels and a decrease of Mg$^{2+}$ content of the cell envelope. The proposed mechanism of this is that H1 replaces Mg$^{2+}$, which then effectively stops colistin from inserting itself in to the LPS-molecule as H1 lack an electrostatic charge that is needed. There has recently been demonstrated that, although seldom, *Acinetobacter baumanii* can become totally resistant to polymyxins and this is mediated by a complete loss of the lipopolysaccharide layer. One would then assume that this essential part of the bacteria leads to a major loss in viability but the bacterial doubling time was essentially unchanged. As mentioned previously the chemical structures of the polymyxin class are very similar and resistance to one polymyxin mediates resistance to the entire class. Resistance does seem, for some reason, to be less frequent than with other antibiotic classes, e.g. there have been extensive use of colistin administered to cystic fibrosis patient against *P. aeruginosa* for over 10 years but no inherited resistance to colistin has yet to be confirmed.
2.6.5 Clinical use
Colistin is considered a last line antibiotic by clinicians because of its poor reputation regarding adverse effects. It is however very effective and it is sometimes the only remaining treatment option for patients suffering from gram-negative multidrug resistant bacteria (MDR). There are three common routes of administration of colistin: intravenous, where colistin is administrated through a vein, aerosolized, in which colistin is inhaled as a fine powder, often used in patients with cystic fibrosis against common pneumonia and finally, colistin administered in to a cavity within the body.37

2.6.6 Toxicity and adverse effects
Early studies done with colistin indicate adverse effects. In 1977 288 patients hospitalized with infection and cystic fibrosis were treated with colistin in a 317 day therapy in order to establish what the manifestations were and the incidence rate of potential adverse effects. The patients were monitored continuously over the course of the treatment. Adverse renal reactions occurred during 20.2% of colistin utilizations and were generally reversible. Acute tubular necrosis occurred during 1.9% of utilizations, manifestations of neurological tissue damage (neurotoxicity) from colistin developed during 7.3%. 13 deaths could be linked to adverse effect from the colistin treatment.38 There are studies that suggest that there is a higher sensitivity to colistin in patients with cystic fibrosis. In a group of patients with cystic fibrosis of 31 that were treated with colistin 29% showed some type of neurotoxic event after recommended colistin dose administration.39 Both neurotoxicity and renal failure are generally considered as dose dependent and the discontinuation of therapy usually leads to full recovery of the patients.38 More recent data indicate that the adverse effects of colistin might be less than early studies demonstrated. In one study from 2005, 19 patients with a four week colistin treatment were observed in relation to toxicity from colistin and none of the patients developed any type of adverse effects.40 2003 a study on critically ill patients given colistin as a last resort and out of a patient count of 28 no adverse effects could with certainty be linked to the colistin treatment.41 Although the observed patient groups were smaller than early studies they are still of relevance, and did find lower frequency of toxic events. These seemingly conflicting observations may have their explanation in the supportive treatment being offered to critically ill patients in the recent studies in comparison with the older ones. There is also the possibility that the colistin being used in the older studies contain an unknown fraction of colistin sulfate which could give a much higher toxic effect than the newer colistin formulation containing exclusively colistimethate sodium.24

2.7 Project aim
The main goal of this project was to evaluate colistin in 2-combination experiments using time-kill methods in search for a novel combination at therapeutically relevant concentrations. A second goal was be to characterize possible interactions further.
3. Study design

3.1 Antibiotics selected as combination partners

Colistin was chosen as the main antibiotic to evaluate combinations for this project. The reason for this choice is the increasing resistance to other antibiotics commonly used against gram-negative bacteria and the consequent renewed interest and usage in this “forgotten” class of antimicrobials in clinics all over the world.\textsuperscript{42, 43, 44} Since the time-kill method used is time consuming it limits the number of combinations that can be tested. The antibiotics used in combination with colistin were therefore chosen with the aim to cover as many different mechanisms as possible. There were seven different antibiotics chosen including colistin, description and motive for this is stated below.

![Figure 2. Targets of antibiotics used in the project.](image)

### 3.1.1 Meropenem

Meropenem is one of the later antibiotics to be added to the β-lactams, first synthesized in the late eighties. Meropenem is a part of the carbapenem subclass which differs from the β-lactams in that they have a carbon atom bound to position 1 instead of sulfur.\textsuperscript{45} Imipenem, a predecessor to meropenem with a similar spectrum was one of the first antibiotics to be fully synthesized in the carbapenem subclass.\textsuperscript{46} When imipenem was administered for the first time it was soon discovered that it was quickly degraded by the enzyme dehydropeptidase (DHP-1) found in the kidneys.\textsuperscript{47} This in turn led up to new
types of carbapenems which had structural modifications that made them stable against DHP-1. Among these new drugs was meropenem with its broad antibacterial spectrum and unique chemical structure (figure 3).48

Figure 3. The hydroxymethyl side-chain protects the β-lactam ring and gives stability against β-lactamases; 2, the β-lactam ring confers a high affinity for penicillin binding proteins, target structure of all β-lactam antibiotics; 3, the carbapenem nucleus is responsible for an ultra-broad spectrum of antibacterial activity; 4, the methyl group at C1 provides more resistance to DHP-1 than imipenem; 5, the C2 substituent is responsible for its high activity against gram-negative bacteria.49

Meropenem, like all β-lactams inhibits the last step in the cell wall synthesis. The cell wall structure can be found in almost all bacteria and is mainly responsible for the structural integrity of the bacteria. The structure is largely built up by peptidoglycans in a similar manner in most bacterial species.50 The synthesis of the cell wall is governed by a number of enzymatic steps and the last enzymes involved in the process are anchored in the plasma membrane, the anchored enzymes are also called penicillin binding proteins (PBP) because they are covalently bound and inactivated by β-lactam antibiotics. When meropenem binds the PBP the process of synthesis of the cell wall is halted which in turn leads to death of the microorganism. Meropenem is able to have such extremely broad antibacterial spectrum because of the variable affinity to specific PBPs in a range of different bacterial species. Although it is well documented how meropenem and PBPs interacts the final mechanism leading to bacterial cell death is not completely understood49

Meropenem is rapidly becoming one of the few β-lactams that still has a good effect on clinically relevant strains such as ESBL-producing Klebsiella pneumoniae. An effective combination with colistin could possibly limit the risk of resistance development.

3.1.2 Daptomycin
Daptomycin was discovered in the late eighties. It is one of the newer antibiotic used to combat infections. It is a large molecule with a molecular weight of 1620.67 kDa. Daptomycin has a unique mechanism of interaction; a hydrophobic extension is inserted and irreversibly bound to the cell membrane of gram-positive bacteria.51 The process is K⁺ dependent and it is postulated that oligomers are formed in the membrane, which leads to the formation of K⁺ channels in response to the daptomycin binding.52 This consequently leads to a depolarization of the membrane and a rapid cell death. The process might be more complicated since it has been suggested that Mg²⁺-ions and ATP
play an unknown part in the process. Daptomycin is active against most gram-positive bacteria and is often used when a Vancomycin resistant strain is found. It has no documented effect on gram-negative bacteria. Daptomycin has been reported to have some toxicity involving gastrointestinal disturbance, headache and injection site reactions. Especially in early trials toxicity towards the skeletal muscles were shown to be frequent. In trials done in recent years much less adverse reactions are demonstrated and daptomycin is considered to be well tolerated by a very large number of patients. Because of daptomycins prolonged effect after administration one dose every 12 h is often enough, in non complicated infections. It is therefore an attractive drug to be used on outpatients and has been done so with great success. Resistance to daptomycin can occur through a series of different steps and has been studied in mostly S. aureus where daptomycin has been shown to have reduced binding efficiency against some isolates. There are also reports of changes in the membrane towards a more positively charged surface which then repels the daptomycin molecule. There are difficulties selecting for daptomycin resistant isolates in single-step experiments which suggest that there are more than one mutation leading to resistance.

Daptomycin is one of the newer antibiotics and thus resistance towards it is still uncommon. It has an effect on most gram-positive bacteria. A combination with colistin against K. pneumoniae would be surprising and novel, but could be theorized from the fact that colistin “strips” the cell from the outer membrane, exposing the target peptidoglycan layer otherwise hidden.

3.1.3 Vancomycin

More than 50 years ago the compound vancomycin was isolated from bacteria found in soil collected in the jungle of Borneo. The initial name was at first simply 05865. Soon after its isolation it was found that 05865 had a high degree of bactericidal activity against staphylococci and furthermore, the staphylococci did not seem able to develop significant resistance to the compound. This was welcome news to medical society as drug resistant staphylococci were on the verge of developing resistance to almost all known antibiotics. Soon after the compound 05865 was introduced to the market and labeled “Vancomycin”. There was however a problem with this new drug, in early studies done in the 1950s it was shown to give a high degree of Ototoxicity (ear damage) and nephrotoxicity. This was likely because the early preparations contained a high degree of impurities. After the introduction of methicillin and other antistaphylococcal penicillins, which had a just as strong effect, and none of the adverse effect of vancomycin, its popularity dropped. But, in the 1970s as the purification process was refined and animal studies done showed no Ototoxicity and little Nephrotoxicity, Vancomycin was once again regarded as a good treatment option. This uncontaminated vancomycin was soon in high demand as Methicillin-resistant staphylococci were increasing around the world. Vancomycin like all glycopeptides is a large molecule that has it mechanism of action at a late stage in the bacterial cell wall peptidoglycan synthesis. It inhibits incorporation of the crucial parts N-acetylmuramic acid (NAM) and N-acetylglucosamine (NAG) to the backbone glycan chains by forming stable complexes with hydrogen bonding. The vancomycin molecule is large and cannot pass through the LPS-layer of gram-negatives and thus they are not affected.
In a recent article a surprising synergistic effect with colistin and vancomycin had been found on strains from the bacteria *Acinetobacter baumannii*. There is therefore a good reason to evaluate the combination with different species of gram-negative bacteria.

### 3.1.4 Ciprofloxacin

Ciprofloxacin is a broad spectrum antibiotic that most often has excellent *in vitro* effect on gram-negative bacteria. The drug also exhibits effect on gram-positive but it is not as strong as on gram-negatives. Ciprofloxacin belongs to the fluoroquinolones and has the characteristic mechanism of action of interfering with replication. Fluoroquinolones inactivate DNA-gyrase which is responsible for rewinding DNA after being copied. As this is essential for the replication it kills the bacteria if it is stopped. As ciprofloxacin has a very broad spectrum it is used to treat a multitude of infections. Like all fluoroquinolones it is however associated with more adverse effects when compared with other antibiotics. This manifests itself mainly as inflammation in tendons and/or nerve damage. It can in very rare cases be severe reactions but it is usually reversible when treatment is stopped.

Fluoroquinolones have their own unique mechanism of action with a well established effect on both gram-negative and gram-positive bacteria. A combination with colistin and ciprofloxacin could present itself as a very effective additive effect.

### 3.1.5 Telavancin

In the 1980s resistance against vancomycin started to emerge in enterococci. The concern then arose that the gene responsible for resistance would jump over to Methicillin resistant *Staphylococcus aureus* (MRSA) strains. This prompted research to develop new antibiotics that could take the place of vancomycin in treating MRSA. Telavancin was one of the lipoglycopeptid derivatives developed from the vancomycin molecule for treatment of resistant gram-positive infections. It is in many ways similar to vancomycin but a hydrophobic side chain and a hydrophilic group has been attached to one of the amino acids. Telavancin acts in a dual fashion, one mechanism inhibiting the cell wall synthesis by covalently binding and forming a stable complex with one of the precursors responsible for producing the cell wall. When bound to the complex the precursor is unable to fulfill its task, which in turn leads to cell death. Telavancin has been shown to have a 10-fold greater activity than vancomycin in forming this complex in MRSA. The second mode of action is what appears to give telavancin its rapid bactericidal effect compared to vancomycin a concentration dependent depolarization of the cell membrane, which also mediates leakage of cytoplasmic ATP and K⁺. Telavancin has a similar spectrum as vancomycin in most aspects but has been shown to still have a stronger effect on vancomycin intermediate *S. aurues* than vancomycin. It is however not active against of fully vancomycin resistant bacterial strains. Telavancin is mostly used in complicated skin and skin structure infections. It is unfortunately associated with more adverse effects than vancomycin but although relevant they are seldom very serious. As MRSA isolates are becoming less and less susceptible to vancomycin the need for additional antibiotics with activity against these potentially life-
threatening infections are increasing. Telavancin is an important treatment option and its usefulness is most likely to increase.\textsuperscript{70}

There have been previous reports on vancomycin and colistin synergy as stated above. Telavancin has an even stronger antimicrobial effect than vancomycin and enhanced effect against MRSA. Telavancin has a molecular composition similar to vancomycin, a novel combination with colistin would in theory be plausible, and this is why it was chosen for the project.

\subsection*{3.1.7 Rifampicin}

First fully synthesized in 1959 makes it one of the older antibiotics. It was first for sale in 1967. When administered the tolerance is most often excellent but about 4% of the patients develop liver damage of some sort\textsuperscript{73}, very few get serious symptoms. One of the key elements in treating \textit{Mycobacterium} infections with rifampicin is as part of a combination cocktail used with good success rate. The cocktail contains a varying mix of other active antibiotics against \textit{Mycobacterium}, but as rifampicin is the key element in treatment it is always present. In fact, many of the other antibiotics are given to prevent resistance development to rifampicin.\textsuperscript{74} In recent years it has also been associated with treatment of MRSA and \textit{in vitro} studies done have found rifampicin treatment in combination with other active antibiotics a likely viable option.\textsuperscript{75,76} Rifampicin inhibits transcription by interfering with a DNA-bound and actively transcribing RNA-polymerase. It does this by binding to the β-subunit with high affinity. This effectively puts a halt to transcription and kills the bacteria.\textsuperscript{77} Gram-negative bacteria are either not affected by rifampicin or less sensitive than gram-positives.

In this project rifampicin was the only which inhibited transcription. No publications were found on a colistin-rifampicin combination with static time-kill utilizing serum concentrations. That is why it was chosen for the project.

\subsection*{3.1.8 Fosfomycin}

In 1969 phosphonomycin, as it was first called, was isolated from various strains of \textit{Streptomyces}. To this day it belongs to its own class and no other member has been introduced to the marked. Fosfomycin has a unique mechanism of action with a broad spectrum of bactericidal activity including both gram-positive and gram-negative species of bacteria.\textsuperscript{78} It inhibits the cell wall biosynthesis as it inactivates the enzyme MurA. The enzyme catalyzes an irreversible last step of the peptidoglycan biosynthesis. When MurA is bound by fosfomycin the forming of the peptidoglycan layer is canceled resulting in death of the bacteria. Fosfomycin uses a protein transport system as its sole method of entry present in all fosfomycin sensitive bacteria. The loss of this system is the primary cause of resistance.\textsuperscript{79} This resistance also seems to develop faster than with other antibiotic which, unfortunately, makes it unsuitable for sustained monotherapy.\textsuperscript{80} One alternative that possible could prevent the fast resistance development is to combine fosfomycin with another active antibiotic.\textsuperscript{78} The early studies on fosfomycin showed a low efficacy which in turn lead to skepticism about it usage. In the mid 1970s proper studies in combination with deeper understanding of the mechanism involved gave a very different result, demonstrating a good effect on many different bacteria.\textsuperscript{78} It has
traditionally been used to treat urinary tract infections but a recent article describes a
good success rate also in other infection with multiresistant Enterobacteriaceae.\textsuperscript{81}

Fosfomycin is an effective antibiotic on many types of bacteria but as resistance to it
develops fast it have had a restricted usage. This has limited the spread of resistance and
it now represents a possible alternative to other antibacterial drugs that rapidly are
becoming ineffective. Very few publications describing a colistin and fosfomycin
combination exist and no one with the method used in this project. A novel combination
with colistin would in theory be plausible which makes it an excellent candidate for the
project.

4. Methods to evaluate additivity

4.3 Common definitions of synergy
The following two definitions of synergy are only briefly explained and no further
explanations or discussions are made. To fully investigate the best way to model synergy
is not in the scope of this project and the only point made is that there is lack of
consensus among scientist in this field of study.

There are two widely used methods to define synergy. Bliss independence, assumes that
the relative effect of one drug is independent the presence of other drug. Bliss
independence then states that effects are additive rather than doses.\textsuperscript{82} The second is
Loewe additivity, which assumes that two drugs act on a target through a similar
mechanism and the joint effect of these two will have an increased effect on the target.
This is a dose dependent model and it is often referred to as dose additivity.

Both of these methods have their own drawbacks. Loewe additivity presumes that the
maximum effect (E_{\text{max}}) of each drug is the same, this is however seldom the case. It also
presumes that this will be true in a combination, this is also seldom the case. If both of
the drugs have the same maximum effect then, Loewe additivity states, the only thing that
can differ is the effectiveness of the halfway between the baseline and maximum effect
i.e. the EC\textsuperscript{50} pictured in figure 7.

Bliss independence does have some advantages in comparison with Loewe additivity. It
does not presume that Emax is the same for each drug and it also allows for the EC\textsuperscript{50} and
Emax of the drugs to change. As the Emax is presumed to differ in a combination of
drugs anything less than a presumed increase of the expected effect is antagonism. That
means that even a slight additive effect is said to be antagonistic if it falls under the scope
of the expected additive effect of the two drugs.
At a recent symposium Professor Alan Forrest advocated scrapping these ideas and only look at total killing activity. If the total killing activity is increased by the combination it is worthwhile to study further, even if it is not strictly synergistic based on Bliss Independence or Loewe Additivity.  

4.2.1 Checkerboard and other end-point based assays

This technique is one of the most frequently used to assess antimicrobial combinations in vitro. This probably has its explanation in that it is easy to understand, the necessary mathematics needed to interpret the results are simple, it can readily be performed in clinical laboratories and it is the most used method in published studies done on synergistic combinations. As figure 4 demonstrates it is done in a microtiter plate with two separate antibiotic dilution series in relation to a previously tested MIC of the bacterium. The antibiotics are mixed with each other in the wells resulting in a unique concentration in each well. Most checkerboards are read after 24 hours incubation. The red circle represents the combined MIC of the bacteria detected by the naked eye. The green wells represent bacterial growth. When the results are interpreted a simple mathematic formula is used to evaluate the data named fractional inhibitory concentration index (FICI). Each drug is calculated on its expected FIC by dividing the combined MIC of those drugs with the MIC of the drug alone as seen in the example below. The FICI is then calculated for each of the drugs present in that well. A FICI of ≤0.5 is regarded as synergistic, additivity as 1.0 and antagonism is often defined as ≥2.0. This method is based on Loewe additivity, as the dose influence on end-point cell concentration is measured, and synergy is set as measured effects greater than the calculated additive dose effect. Theoretically, <1 should be a synergistic combination, but to allow for random fluctuations always present in MIC-tests, an arbitrary limit of 0.5 is the standard. 

---

**Figure 7. Bliss independence and Loewe additivity**

![Bliss Independence and Loewe additivity graphs](image-url)
Figure 4. An example of a checkerboard producing a synergistic combination.

There are also a few types of diffusion methods performed to evaluate synergy. They all utilize the fact that antimicrobials diffuse in agar. Most of them have a major advantage in that the equipment necessary are commercially available and they are very easy to perform. Paper strips or disks soaked in antimicrobial solutions are used in some fashion and modification depending on specific method. As bacteria grow on an agar plate with disks or strips they will be inhibited by the antimicrobials as they diffuse on the plate. Figure 5 demonstrates a type of method were two E-tests are placed in a 90° angle from each other and the joint effect of the two will give an indication of a potential synergistic effect.85 84

4.2.3 Limitations of point based techniques

This technique only gives inhibitory data at a single time-point, often 24 hours, as no regard is taken on the state of the microbes over the time up until that point, i.e. only the sum of all events is presented, but not “each” event by itself. Furthermore, you often have no information about the end-state except that the CFU/ml is below the limit visible to
the naked eye. It is quite possible that an empty well or empty patch of agar contains a high number of slower growing bacteria or other adaptive resistance phenotypes

4.2.4 Time-kill curves

The killing curve technique measures the effect of the antimicrobial agent being tested over time. The method is one of the most reliable in accurately giving a picture of the dynamic antimicrobial action. However, the technique is tedious due to large amount of sampling and seriously limits the number of isolates and antimicrobial concentrations and combinations that can be tested. It is therefore essential that the antimicrobial concentrations being tested are chosen carefully in order to reduce the number of experiments. Usually when performing time-kill curves, the basis for the concentrations chosen are the MIC values and several multiples of MIC concentrations are tested in one experiment.\(^{84}\) When using time-kill for evaluating an antibiotic combination you generate killing-curves for each combination and compare those with single treatment and a control. It is generally accepted as one of the highest standards for synergy testing. The classical definition of synergy in time-kill is defined as a reduction of ≥2 \(\log_{10}\) cfu/mL with the combination compared with the cfu/mL of the most potent single drug at a set end point. Antagonism is defined as >2 \(\log_{10}\) cfu/mL increase with the combination compared with the cfu/mL of the most active single drug. Indifference is defined as <2 \(\log_{10}\) cfu/mL difference between the cfu/mL of the combination compared with the cfu/mL of the most potent single drug.\(^{84}\) It is possible to use several other definitions of synergy, such as Bliss Independence with regards to final CFU/mL, rate of cell death or growth or area under the bacteriological curve.

A weakness of the combination static time-kill method is how the design is generally developed to find combinations with relation to MIC. It does not take to account the toxic effect of the antibiotic, protein binding by human plasma or the actual dose given to patients i.e. even though a synergistic combination is found according to the classic definition, the concentrations might not be practically attainable in the patient. A different approach is to use the documented plasma concentration at steady state as a measure of
antibiotic concentration in the experiments. This approach allows for a more direct link to a clinical setting and a more realistic combination effect. It also enables more data to be produced as dose evaluation of each antibiotic is already done by previous studies. However, when choosing this design there is a chance that dose ratios that could still work are missed.

4.4 Synergy definition in the project

Since great concern has been expressed lately regarding the above stated definitions of synergy the classical definition of synergy was used. The data was also evaluated by assuming first order exponential death and regrowth, which allows the measurement of specific growth or death rate. This is the rate of change ($\mu$) which we then can statistically compare between single drug and combination. Analysis of variance (ANOVA) was used to compare the rate of change for colistin ($\mu_c$) versus rate of change with meropenem ($\mu_m$) versus rate of change with meropenem + colistin ($\mu_{c+m}$), which gives: ($\mu_c$ vs. $\mu_m$ vs. $\mu_{c+m}$). If $\mu_{c+m}$ is found to be significantly larger than for each drug alone we conclude that the effect of the combination is better than each drug alone. Rate of change also gives a more dynamic picture of the interaction in the experiments as it gives more information about what happens between the start and end points of the experiment.
5. Results

5.1 Results of drug screening

Summary of data analysis on two-combinational static time-kill experiments using Excel and Graphpad (fig 8, table 1). By the standard definition of synergy in static time-kill, only one combination was synergistic on the *K. pneumoniae* strain with ESBL that was used for the screening: colistin with meropenem. No other combination was found to be synergistic with the classical definition and no combination was found to be antagonistic and is thus defined as indifferent (table 1). All antibiotics combinations were done in two replicate experiments. It was not possible to do any statistical analysis with the initial screening as the number of replicates of each experiment were too few.

Figure 8. Data summary of two-combination static time-kill experiments with 95% confidence bars. All experiments done in three replicates. The synergistic effect is present only in the Meropenem Colistin diagram.
<table>
<thead>
<tr>
<th>Combination partners with Colistin</th>
<th>Synergy</th>
<th>Indifference</th>
<th>Antagonism</th>
</tr>
</thead>
<tbody>
<tr>
<td>Meropenem</td>
<td>x</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Daptomycin</td>
<td></td>
<td>x</td>
<td></td>
</tr>
<tr>
<td>Vancomycin</td>
<td></td>
<td>x</td>
<td></td>
</tr>
<tr>
<td>Ciprofloxacin</td>
<td></td>
<td>x</td>
<td></td>
</tr>
<tr>
<td>Telavancin</td>
<td></td>
<td>x</td>
<td></td>
</tr>
<tr>
<td>Fosfomycin</td>
<td></td>
<td>x</td>
<td></td>
</tr>
<tr>
<td>Rifampicin</td>
<td></td>
<td>x</td>
<td></td>
</tr>
</tbody>
</table>

Table 1: summary of screening results
5.2 Results of Colistin Meropenem study

Ten strains of clinical isolates of *K. pneumoniae* with ESBL were included in this follow-up study and further evaluated with static time-kill.

Figure 9. Data summary of all strains in two-combination static time-kill experiments with 95% confidence bars. All experiments done in two replicates.
In figure 9 all strains are evaluated alone with two replicates per experiments. Synergistic effects with the classical definition (compared to both of the single antibiotics) at 2h with the meropenem and colistin combination are seen in strains 510, 511 and 514. When instead looking at the end time-point (24h) strains 509, 515, 516 and 517 show a synergistic effect with the classical definition.

Figure 10. Data analysis of the colistin and meropenem combination including all strains

Figure 10 demonstrates a data summary of all strains regarded as a group. A synergistic effect can be seen at the second time point (2h) and at the end time point (24h).

Figure 11. Showing a summary of SDR/SGR from 0 to 2 hours and 6 to 24 hours
As previously mentioned, another possibility to evaluate synergy is to look at a quantification of the effects, such as growth rate or death rate. We assume 1:st order exponential kill between start time and hour 1 (SDR), and growth from time 6 to 24, as is generally seen with colistin and these strains. If there is a significant change in death or growth rate, we can say that the combination is better than single treatment. This is summarized in figure 10. Statistics were done with a ANOVA using Bonferronis multiple comparison post-test.

### Meropenem and Colistin combination on 10 strains of *Klebsiella pneumoniae*.

<table>
<thead>
<tr>
<th>Strains</th>
<th>SDR</th>
<th>SGR</th>
<th>(\geq 2) log_{10} cfu/mL 2h</th>
<th>(\geq 2) log_{10} cfu/mL 24h</th>
</tr>
</thead>
<tbody>
<tr>
<td>509</td>
<td>(+)</td>
<td>NS</td>
<td>(+)</td>
<td>+</td>
</tr>
<tr>
<td>510</td>
<td>NS</td>
<td>NS</td>
<td>+</td>
<td>(+)</td>
</tr>
<tr>
<td>511</td>
<td>(+)</td>
<td>NS</td>
<td>+</td>
<td>(+)</td>
</tr>
<tr>
<td>512</td>
<td>(+)</td>
<td>NS</td>
<td>(+)</td>
<td>(+)</td>
</tr>
<tr>
<td>513</td>
<td>NS</td>
<td>NS</td>
<td>(+)</td>
<td>(+)</td>
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<tr>
<td>514</td>
<td>NS</td>
<td>NS</td>
<td>+</td>
<td>(+)</td>
</tr>
<tr>
<td>515</td>
<td>(+)</td>
<td>NS</td>
<td>(+)</td>
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<td>516</td>
<td>(+)</td>
<td>NS</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>517</td>
<td>(+)</td>
<td>NS</td>
<td>(+)</td>
<td>+</td>
</tr>
<tr>
<td>518</td>
<td>NS</td>
<td>NS</td>
<td>(+)</td>
<td>(+)</td>
</tr>
<tr>
<td>All strains*</td>
<td>+</td>
<td>(+)</td>
<td>+</td>
<td>(+)</td>
</tr>
</tbody>
</table>

Table 2: Evaluation of the statistical significance of the effect using ANOVA analysis on SDR (0-2h) and SGR (0-6h), and synergistic effect measured as \(\geq 2\) log_{10} cfu/mL at 2 h and 24 h

+ - indicates that the combination was better (statistically significant change of \(\mu\) or above 2 log cfu/ml change) compared to both antibiotics alone

(+)- indicates that the combination was better compared with one drug, but not the other

NS – not significantly better according to the one- and two way ANOVA test

* All strains analyzed as a single group

When all the strains were compared as a total group in the hours 0 - 2 there was a significant difference in growth rate/death rate between the combination and the single treatments. The combination had a statistically significant higher death rate compared to both of the single dose antibiotics. When this was done on hours 6 to 24 the results indicate that the combination had a lower growth rate compared to colistin single dose treatment, but not meropenem treatment. Note that this does not exclude the possibility that there is a better effect, only that there is not enough data to see a statistically significant difference, as only two replicates were made with each strain.

### 6. Discussion

#### 6.1 Drug screening

The screening found a synergistic effect of meropenem and colistin combination as shown in the table 2. The effect is present after 2 hours and at 24 hours. The increased effect is not strong nevertheless it is still present. The other antibiotics did not have any effect, neither single nor in combination with colistin. Some of them, like rifampicin or ciprofloxacin were expected to have some effect on the bacteria but with the two replicates done this was not seen. No MIC was established to see degree of resistance.
towards these two antibiotics, as the time frame of the project did not allow for that. It can only be speculated that the screening strain used was insensitive to rifampicin and ciprofloxacin at these concentrations, further experiments should be done to establish this.

There are some weaknesses with the combination screening. First of all it is done with only one strain and, second, every combination is only done in two replicates. Both of these problems are the consequence of the method used, which is very time consuming and requires a large set of laboratory material. It might be the case that a less obvious combination would be seen if more experiments were done and with a larger number of strains included. This would also allow for a deeper statistical analysis. Both figure 8 and figure 9 demonstrates the rapid effect of colistin in the initial hour. This very strong bactericidal effect in many cases hides the full extent of an additive effect. The strong effect in the initial hours can with the combination be under limit of detection thus hiding any additional effect beyond that the effect of the combination could thus be even greater than what we have seen here.

6.2 Colistin Meropenem study

If the classical definition of synergy at 24 hours is used to interpret the results, a positive combinational effect is present. A problem with only using this method to interpret the data is that it only takes into account a comparison between endpoints. This inherently gives it the same problem as FICI-tests. This was one of the reasons a second statistical method was used.

When a two way ANOVA is done with the SDR and SGR of the ten samples and each different strain is compared on its own against the combination and the single dose antibiotics it gives a non significant result as the sample size is too low. No certain assumption can then be made on the individual strains other than that that more experiments need to be done to be able to draw any certain conclusions. However, what the statistic show is that if the 10 strains are treated as group a superior effect by the combination against single dose treatment can be said to be present. These ten strains are randomly picked and can therefore speculatively be treated as a representative population of Klebsiella pneumoniae with ESBL susceptible to meropenem and colistin. If this assumption is done then this combination of meropenem and molistin is a novel interaction that potentially can be used on patients bearing in mind that no speculation is made on the toxic effect of the combination.

When each strain are evaluated alone it is interesting to see that the effect was more obvious in the initial screening than in the later follow up. This was even though the ten strains show susceptibility to both colistin, which has a clinical breakpoint at ≤ 2, and meropenem, which also has a clinical breakpoint at ≤ 2. It can only be speculated what the reason for this is. It might be that the ESBL plasmid was different from the initial strain used, the bacteria were more prone to develop a small resistance during the experiments or that a subpopulation of the strains tolerated a higher degree of colistin or meropenem.
6.3 The problem with synergy methods

A number of reviews and articles have pointed out the lack of coherence between different methods to test combinations of antibiotics. An important point to this discussion is made with this master thesis. In almost all of the experiments done during the project and especially, with the exception of the meropenem + colistin combination, when colistin are evaluated a regrowth of the bacteria occurs. If a method was used that only evaluated the difference between time point one and an endpoint, if the endpoint was set to hour 24, it would give a result that showed that colistin has no effect on the bacteria. However, since in vivo you also have the immune system to take into account, it is sometimes enough to quickly lower the bacterial burden with antibiotics, so that the immune system then can finish the job. As the clinical evidence for colistin antimicrobial effect is great this leads to the conclusion that point based methods have some obvious drawbacks.

6.4. Conclusions

The synergistic combination that was found during this project is novel. There have been other studies done on meropenem and colistin but they are few and none with the use of static time-kill and steady state serum concentrations. The combination was effective in that it had colistin’s rapid bactericidal effect with meropenem’s ability to stop regrowth. It acts within the hour, reducing the bacterial count to a very low level. Meropenem then keeps the bacterial count under the limit of detection. As antibiotic resistance in clinically relevant pathogens is increasing this combination could possibly be used in hospitals as a way to reduce the risk of resistance development for both colistin and meropenem in K. pneumoniae with ESBL plasmids sensitive to meropenem. There is already an abundance of different resistances, provided by the ESBL-plasmids or chromosomally expressed by K. pneumoniae, it is of grave importance to limit the spread of this resistance development to keep our last resort antibiotics still effective.

Looking at the graphs picturing the individual strains used, seven out of ten show a synergistic effect. Three in the initial hour and four at the end of the experiment, it could then be speculated that in a clinical setting 7 out of 10 in a patient group could benefit from using the combination meropenem and colistin instead of the antibiotics on its own.

If more work is to be done with this combination to truly search for its potential additional time-kill experiments need to be done with more strains of K. pneumoniae. In a further perspective there is possible a positive clinical potential for this combination which then needs to be evaluated clinically.

7. Material and methods

7.1 Static time-kill

Strains were brought up from the minus 70 freezer and grown in Mueller-Hinton II broth at 37°C ~150 rpm shaker in growth room. The next day the bacteria were plated on Mueller-Hinton II agar-plates and incubated overnight at 37°C. A single colony was picked and added to Mueller-Hinton II broth and incubated overnight in 37°C ~150 rpm shaker in growth room. 20 µl of the o.n. culture was diluted into 2 ml of fresh broth and
allowed to grow in 37°C ~150 rpm rocking water bath for 1.5h to achieve a bacterial cell concentration of ~10^8 cfu/ml in an exponential growth phase. The antibiotics were dissolved in standard phosphate buffer saline (PBS) to a concentration of 10.000µg/ml. The antibiotics were further diluted to the desired concentration (see table 2). After 1.5 hour, 20 µl of the bacterial cultures were added to the growth tubes to a total volume of 2 ml to achieve a concentration of ~10^6 cfu/ml. Samples were taken from every culture tube, serial diluted in PBS and plated on Mueller-Hinton II agar-plates. During the day the growth tubes were sampled on agar plates on specific time-points previously set. Zero hour samples were taken on plates and sample time-points set to 1, 2, 4, 6, 9, 12 and 24 hours for the antibiotic screening. The time-points for the colistin meropenem screening were set to 0, 2, 6, and 24. Counting of the number of colonies on plates were done after approximately 24 h of incubation.

7.2 E-tests
Strains were brought up from the minus 70 freezer and grown on Mueller-Hinton II agar plates in 37ºC growth room for approximately 24 hours. One colony was taken from the plate using a standard loop and grown in 2 ml Muller-Hinton II broth for 5 hours to reach a density of 0,5 McFarland. A cotton swab was used to spread the bacteria on a Mueller-Hinton II agar plate. E-tests strips were carefully placed on the surface of the agar plate with the use of pinchers. The plate was incubated for 18 hours in 37ºC growth room. The next day the plate was read for its minimum inhibitory concentration (MIC) according to the manufacturer of the E-tests.

7.3 Concentrations of the antibiotics used
In order to look at clinically relevant concentrations of antibiotics, these were chosen to represent steady state serum concentrations, all of which were taken from the literature.

<table>
<thead>
<tr>
<th>Antibiotics</th>
<th>Free serum (SS) Concentrations (µg/ml)</th>
<th>References</th>
<th>Manufacturer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Colistin</td>
<td>2,1</td>
<td>91</td>
<td>Sigma-Aldrich</td>
</tr>
<tr>
<td>Vancomycin</td>
<td>9,09</td>
<td>92</td>
<td>Alpharma</td>
</tr>
<tr>
<td>Ciprofloxacin</td>
<td>0,98</td>
<td>93</td>
<td>Sigma-Aldrich</td>
</tr>
<tr>
<td>Telavancin</td>
<td>3,25</td>
<td>69</td>
<td>Sigma-Aldrich</td>
</tr>
<tr>
<td>Fosfomycin</td>
<td>9,50</td>
<td>80</td>
<td>Sigma-Aldrich</td>
</tr>
<tr>
<td>Rifampicin</td>
<td>1,74</td>
<td>94</td>
<td>Sigma-Aldrich</td>
</tr>
<tr>
<td>Meropenem</td>
<td>6,76</td>
<td>95</td>
<td>Astrazeneca</td>
</tr>
<tr>
<td>Daptomycin</td>
<td>1,65</td>
<td>96</td>
<td>Chiron</td>
</tr>
</tbody>
</table>

Table 3: concentrations of antibiotics used

7.4 Strains used in the experiments
The initial screening strain 449 was the outbreak strain from Uppsala Academic hospital from 2007 obtained from Dan Andersson. Strains used in the follow-up study 509 – 518 were obtained from patients in Kronobergs landstingssjukhus 2011 and sent by mail to the laboratory. MIC was established using E-tests.
<table>
<thead>
<tr>
<th>Specie</th>
<th>Coded Number</th>
<th>Comment</th>
<th>MIC Colistin</th>
<th>MIC Meropenem</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>K. pneumonia</em></td>
<td>ARU 449</td>
<td>Clinical isolate ESBL</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td><em>K. pneumonia</em></td>
<td>ARU 509</td>
<td>Clinical isolate ESBL</td>
<td>0.104</td>
<td>0.023</td>
</tr>
<tr>
<td><em>K. pneumonia</em></td>
<td>ARU 510</td>
<td>Clinical isolate ESBL</td>
<td>0.147</td>
<td>0.034</td>
</tr>
<tr>
<td><em>K. pneumonia</em></td>
<td>ARU 511</td>
<td>Clinical isolate ESBL</td>
<td>0.210</td>
<td>0.034</td>
</tr>
<tr>
<td><em>K. pneumonia</em></td>
<td>ARU 512</td>
<td>Clinical isolate ESBL</td>
<td>0.210</td>
<td>0.023</td>
</tr>
<tr>
<td><em>K. pneumonia</em></td>
<td>ARU 513</td>
<td>Clinical isolate ESBL</td>
<td>0.170</td>
<td>0.104</td>
</tr>
<tr>
<td><em>K. pneumonia</em></td>
<td>ARU 514</td>
<td>Clinical isolate ESBL</td>
<td>0.417</td>
<td>0.023</td>
</tr>
<tr>
<td><em>K. pneumonia</em></td>
<td>ARU 515</td>
<td>Clinical isolate ESBL</td>
<td>0.397</td>
<td>0.085</td>
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<tr>
<td><em>K. pneumonia</em></td>
<td>ARU 516</td>
<td>Clinical isolate ESBL</td>
<td>0.255</td>
<td>0.023</td>
</tr>
<tr>
<td><em>K. pneumonia</em></td>
<td>ARU 517</td>
<td>Clinical isolate ESBL</td>
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<td>0.047</td>
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<tr>
<td><em>K. pneumonia</em></td>
<td>ARU 518</td>
<td>Clinical isolate ESBL</td>
<td>0.417</td>
<td>0.032</td>
</tr>
</tbody>
</table>

Table 4: Strains used in the experiments
ND – not done

7.5 Data analysis and Equations

All statistics were done in Excel and Graphpad. The statistic tests used were one- and two way analysis of variance (ANOVA) with Bonferroni’s multiple comparison. Replicate data was averaged and 95% confidence intervals were calculated in Excel.

Specific death rate/specific growth rate were defined as: $\mu = \ln\left(\frac{N_2}{N_1}\right)/\Delta t$.

Where $N_1$ is the mean log cfu/ml of the replicates at the first time point. $N_2$ is the mean log cfu/ml of the replicates at the second time point. $\Delta t$ is the change in time between the two time points. The first time interval (0-2h) was chosen since most strains decreased in cfu/ml up until 2h and the second time interval (6-12h) was chosen since most strains show regrowth from this point forward. The specific death rate was calculated for each strain between 0-2h, while the specific growth rate was calculated for each strain between 6-24h, as it was generally observed for all strains that there was still a decline in the number of viable cells after 2h, and an increase between 6 and 24h.

Acknowledgements

To Pernilla and especially Christer for the greatest of patience and help during the project. Your guidance and assistance throughout the project was essential for its completion. I would also like to thank all the rest of the staff at the antibiotic research unit: Juliana, Matti and Patricia for your help and support.
8. References


73. Tripathi Rifampicin Adverse Effects. (2011). vid <http://www.lungindia.com/article.asp?issn=0970-2113;year=1991;volume=9;issue=3;spage=111;epage=115;aulast=Tripathi;type=0>


84. Lorian, V., M.D. *Antibiotics in laboratory medicine*. (Williams & Wilkins: Baltimore, Maryland, 1996).


