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Evaluation of Double and Triple Antibiotic Combinations Including Colistin for NDM-producing *Klebsiella pneumoniae*

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Table of Contents

ABSTRACT

The New Delhi metallo-β-lactamase (NDM)-producing organism *Klebsiella pneumoniae* is becoming a danger around the world because of its antibiotic resistance profile. Combination antibiotic therapy is the best choice in the treatment of such a resistant strain. During this project, we compared 9 strains with 4 antibiotic regimens including multiple combinations consisting of colistin, rifampicin and meropenem. The combination of colistin + rifampicin was the most effective regimen, demonstrating a bactericidal effect in 3 out of 9 strains and a bacteriostatic effect in 6 strains. The combination was most effective against the strain with the lowest MIC value of rifampicin (12 mg/L). The triple combination of colistin + rifampicin + meropenem was also very effective, demonstrating a bactericidal effect in 3 out of 9 strains and a bacteriostatic effect in 5 strains. We also found that the colistin + meropenem combination was effective against a strain that was sensitive to meropenem (MIC= 0.75 mg/L). This combination was, however, not effective against a strain with intermediate sensitivity to meropenem (MIC= 4 mg/L). Surprisingly, one of the meropenem resistant (MIC _ > 32 mg/L) strains was sensitive to the colistin+ meropenem combination. Colistin monotherapy had no bactericidal or bacteriostatic effects. Colistin should thus be combined with another effective drug in antibiotic therapy.

ABBREVIATIONS

Cmax	Maximum concentration of drug in serum
CFU	Colony-forming unit (s)
CST	Colistin
ESBL	Extended-spectrum β-lactamase
EUCAST	European Committee on Antimicrobial Susceptibility Testing
FOF	Fosfomycin
KPC	*Klebsiella pneumoniae* carbapenemase
MBL	Metallo-β-lactamase
MDR	Multidrug resistant
MEM	Meropenem
MIC	Minimum inhibitory concentration
NDM	New Delhi metallo-β-lactamase
OXA	Oxacillinase
PBP	Penicillin binding protein
RIF	Rifampicin
SHV	Sulfhydryl variable
TEM	Temoniera
UTI	Urinary tract infection
VIM	Verona intergron-encoded metallo-β-lactamase

INTRODUCTION

Nowadays, bacterial antibiotic resistance is a threat to global health. Gram-negative bacteria have recently received more attention due to the risk of them developing chromosomal resistance and spreading resistant genes via plasmids or transposons to other strains that they may come in contact with. Extended- spectrum- β- lactamases (ESBLs) which are enzymes carried by non-fermentative Gram-negative bacteria belonging to the Enterobacteriacae family emerged in the 1990s and since then, no new single drugs have been able to treat the bacteria in this family.

Penicillin was first produced by pharmaceutical companies in the 1940s. It contains a β-lactam ring which binds to and inactivates penicillin binding proteins that build up the peptidoglycan of a bacterial cell wall. In the 1960s and 1970s, Temoniera (TEM-) and Sulfhydryl variable (SHV-) broad-spectrum-β-lactamases appeared, typically found in *Escherichia coli* and *Klebsiella pneumoniae*, which hydrolyze the β-lactam ring[1]. In the 1980s, cephalosporin which is active against bacteria producing broad-spectrum-β-lactamases and contains an oxyimino-β-lactam ring, had been launched. Six months later, β-lactamases which had acquired affinity for oxyimino-β-lactam antibiotics were first reported in India and are generally called ESBLs[2].

β-lactamases can be classified into two systems: Ambler molecular and Bush-Jacoby-Medeiros functional classifications (Table 1[3]). According to the Ambler system, classes A, C and D are serine β-lactamases while class B contains the so-called metallo-β-lactamases (MBLs), which are zinc-dependent carbapenemases. In the other system, among more than 200 different β-lactamases, there are three main groups of penicillinases, cephalosporinases and metallo-β-lactamases.

The most common class A β-lactamases are SHV-1 and TEM-1 ESBLs, which are penicillinases found in common Gram-negative rods (*E. coli, K. pneumoniae* etc.) with weak activity against cephalosporins[1] (not shown in Table 1). ESBLs develop from simple point mutations in the β-lactamase encoding gene that can also be transferred from one organism to another. They are active against all penicillins and cephalosporins. The CTX-M-15-producing ESBL gene jumps from chromosome to a plasmid whose enzymes were detected first in India and since then, continued to be the dominant ESBL, especially in *E. coli* and *K. pneumoniae*[4,5].

Bush-Jacoby-Medeiros system	Ambler system	Enzyme: carbapenemases	Function	Known organisms
Group 2 penicillinases	A	KPC	Hydrolyzes all β-lactam antibiotics; weakly inhibited by clavulanate	*K. pneumoniae,* Enterobacteriaceae
Group 3 metallo-β-lactamases	B	MBLs (NDM, IMP, VIM, GIM, SPM)	Hydrolyzes all β-lactams except aztreonam; not inhibited by clavulanate; zinc-dependent; inhibited by EDTA	*Pseudomonas aeruginosa*, Acinetobacter spp, Enterobacteriaceae
Group 2 penicillinases	D	OXA	Oxacillin hydrolyzing; weakly activated by carbapenems	*P. aeruginosa, A. baumannii*, Enterobacteriaceae
Group 1 cephalosporinases	C	-	ESBLs; resistant to all β-lactams except carbapenems; not inhibited by clavulanate	Enterobacteriaceae except *Salmonella, Klebsiella*

Table 1. Classification of β-lactamase enzymes showing mainly carbapenemases[3]

Modified from Rice LB and Bonomo RA (2007) Mechanisms of Resistance to Antibacterial Agents. In: Murry PR, Jorgensen JH, Pfaller MA, et al (eds). Manual of Clinical Microbiology, 9th ed. Vol.1. 1114-1130. American Society for Microbiology, Washington DC.

Class C β-lactamases are primarily cephalosporinases, encoded on the bacterial chromosome, e.g. Amp C-type ESBLs, which are strongly active against extended-spectrum cephalosporins except carbapenems and are not inhibited by clavulanic acid[1] (not shown in table 1). Class D β-lactamases are OXA-type penicillinases found mainly in Gram-negative rods. They can hydrolyze oxacillin, cloxacillin and related penicillins and are poorly inhibited by clavulanic acid. They are categorized as both ESBL and carbapenemase because some of them are less active against carbapenems[6].

β-lactamases in class B are zinc-dependent metallo-enzymes and are broadly active against all β-lactam antibiotics including cephamycins and carbapenems but not monobactams (aztreonam)[1,5]. The clinically important carbapenemases in *E. coli* and *K. pneumoniae* are OXA-type, serine *K. pneumoniae* type carbapenemases (KPC) and MBLs (VIM-, IMP- and NDM-types) (Table 1). The most common carbapenemase: class A KPCs is active against all β-lactams including penicillins and cephalosporins, weakly active against monobactams, carbapenems etcetra., and are mildly inhibited by clavulanic acid[7]. They were first detected in North Carolina, U.S.A., in 1996[8] and since then have spread around the world.

The Verona Integron-encoded metallo-β-lactamases (VIM) in the so-called MBL gene cassette was inserted into the class 1 integron variable region. They are active against all β-

lactams except monobactams and cannot be inhibited by clavulanic acid. VIM was originally detected in Greek hospitals and later spread to Italy[9,10].

Other MBLs, called New Delhi Metallo-β-lactamases-1 (NDM-1), carry their characteristic gene: *bla* $_{\text{NDM-1}}$ on a large plasmid in *K. pneumoniae* and *E. coli*, and are easily transferred to other species. NDM-1 isolates were first extracted from a Swedish patient who had travelled to New Delhi, India. NDM-1 can also be described as a transmissible genetic element with the potential to encode multiple resistance genes. The initial sources of the samples taken from the regions in India were community-acquired infections but now, NDM-1 is a potential threat in hospitals as well as the public[2,11]. Isolates have been reported from India, Pakistan, Bangladesh, the UK and so forth; some are only susceptible to colistin which is the last resort among antibiotics and are therefore classified as multi drug resistant (MDR).

Many surveillance studies on β-lactamases have been conducted which has alarmed the scientific community around the world. In a study of the first outbreak in Scandinavia, the occurrence of ESBL-producing *K. pneumoniae* strains increased compared with the previous year's data[12]. Similarly, one of the studies in the Asia-Pacific area showed that an increasing rate of ESBLs was found in countries like India, China, Thailand and Vietnam[13]. Moreover, a high prevalence of carbapenem-resistant *K. pneumoniae* (CRKP) was reported in the USA in a 12 years cohort surveillance study[14]. The spread of multi-resistant CRKP also seems to be happening in Norway and Sweden[15]. The European Antimicrobial Resistance Surveillance Network (EARS-Net) confirmed that there was a significant rise of CRKP resistance in six out of eight European countries during a period of six years[16]. A three years report from an Israeli hospital showed that the increasing trend of CRKP might be caused by rapid dissemination of the strain[17].

All in all, MDR bacteria have created difficult to treat and dangerous diseases because of their production of aforementioned enzymes, and their mobile genetic elements, leading to rapid and easy dissemination of infection. Poor hygienic conditions, increased use of antibiotics and misuse, easily available over the counter antimicrobial agents, poor microbiological facilities, poor drug quality, lack of new antibiotics production and many other factors favor the selection of resistant microbes. The emergence of acquired microbial resistance dictates the need for continuous surveillance to guide empirical therapy. Therefore, it is important to study the emergence and the determinants of antimicrobial resistance and there is an obvious need to devise appropriate strategies for antimicrobial control.

Carbapenems (e.g., ertapenem, meropenem, imipenem) are β-lactams that are widely prescribed broad-spectrum antibiotics. They are active against all groups of organisms and are especially used for treatment of severe infections with ESBL-producing Enterobacteriaceae but they are not active against all oxacillin resistant staphylococci and some selected Enterobacteriaceae. However, some carbapenem resistance has been reported in KPC-producing and NDM-1-producing *K. pneumoniae*. Meropenem is the first line of therapy in treatment of infections of the central nervous system. The most important side effects are drug hypersensitivity, neuromascular disorders like seizures and it is very dangerous to use meropenem in patients with penicillin allergy[1,5,18,19,20].

Monobactams (Aztreonam) are narrow-spectrum antibiotics that are active only against aerobic, Gram-negative bacteria. They are usually administered by intravenous, intramascular or inhalation methods but more importantly, it is safe to use in patients with a penicillin allergy. Both carbapenems and monobactams are bactericidal. Their mechanisms are similar to that of penicillin which blocks peptidoglycan cross linking by binding penicillin binding proteins in the bacterial cytoplasm, causing bacterial cell wall disruption[20].

Fosfomycin is a small molecule that inhibits the first step in cell wall synthesis by acting as an analog of phosphoenolpyruvate[21]. It exhibits a broad spectrum of antimicrobial activity against both Gram-positive and –negative bacteria, especially in treatment of urinary tract pathogens such as vancomycin-resistant Enterococci (VRE), *E. coli*, Klebsiella and Enterobacter. Fosfomycin resistance genes are chromosomally mediated and are quite common. Parenteral fosfomycin should be combined with other antibiotics in systemic infections[22].

Rifampicin is a potent inhibitor of prokaryotic DNA-dependent RNA polymerases at the transcription level. It is one of the first lines of drugs in the treatment of *Mycobacterium Tuberculosis* infection and is active against chronic staphylococcal infections. Rifampicin resistance develops quickly during treatment, so it should be used in combination with other antibiotics instead of monotherapy[23,24].

Colistin is one of the sulphomethyl derivatives of the polymyxins called polymyxin E. It is a peptide antibiotic with five positive charges in physiological solutions. Free colistin base is dissolved in the colistin sulphate salt. Polymyxins disrupt the Gram-negative bacterial membrane by acting like a cationic detergent[25]. This polypeptide antibiotic is active against most of Gram-negative bacilli, and especially useful against carbapenem resistant *E. coli*, *K.*

pneumoniae, Pseudomonas and Acinetobacter. Colistin had previously been abandoned due to nephrotoxicity in patients. Nowadays, increasing amount of antibiotic resistance make colistin a last resort antibiotic and the current purification techniques are lowering the toxicity of the drug[26]. Although the optimal dose for colistin is still under investigation, a current recommended target steady state concentrations are at 2-2.5 mg/L[27]. The maximum concentration of colistin achieved at steady state during 8 hour administrations was at 2.3 mg/L and it remained at the same level for a significant amount of time because of its half-life of 14.4 hours[26]. Although reported resistance to colistin is rare, it has increased in *K. pneumoniae* during treatment[28,29]. In order to prevent the selection of resistance, colistin should always be administered in combination with other effective antibiotics[30,31].

Combination antibiotic therapy has long been used to improve clinical outcomes, particularly associated with a high rate of morbidity and mortality in patients with chronic bacteremia, necrotizing penumonia and other severe infections. Clinical data have revealed that combination therapy gave a better outcome than monotherapy for severe infections with carbapenemase-producing *K. pneumoniae* when the combination therapy included colistin, a carbapenem, tigecycline, fosfomycin or an aminoglycoside[32,33]. A synergistic effect seems to be achieved in antibiotic combinations. When colistin acts on the cell wall of Gram-negative bacteria, a rapid change in the permeability of the cytoplasmic membrane arises allowing the entry of a second drug. In *in vitro* studies, colistin plus rifampicin or carbapenems showed a synergistic effect for *P. aeruginosa* and *Acinetobacter baumannii*[34,35]. Greek hospitals have been guided to use meropenem combination with gentamycin or colistin for the carbapenemase-producing *K. pneumoniae* infections if the MIC value is less than 4 mg/L[36]. Although many studies have proved that combination therapy has a better outcome, potential adverse effects like increased drug toxicity, interactions, and antagonism should be considered during treatment.

Before treating patients, *in vitro* time-kill experiments are useful for finding appropriate combinations of antibiotics. The time-kill (killing curve or killing rate) method measures the amount of surviving micro-organisms along a time interval during a therapeutic regimen. Although it is time consuming to perform, interesting drugs can be tested against clinically important bacteria before animal experimentation and clinical evaluations are performed. Moreover, it gives information about the rate and degree of killing of an individual drug or combination, compared to others[37]. In addition, the information given by the kinetic time-kill method can be correlated with clinical situations and laboratory experiments, because human

pharmacokinetics can be simulated in a kinetic time-kill model. The data from kinetic time-kill methods can therefore potentially be applied in clinical treatment.

The spiral gradient method is useful for microbiological studies and is done via a spiral plater. It is a stylus-like instrument that deposits a set amount of sample containing antibiotics with bacteria in a spiral pattern on a rotating agar plate. This allows for easy determination of the number of colonies in the sample. The advantages of this method in microbiological routine procedure of the bacterial enumeration are in saving time and due to the lower amount of labor required[38].

While dealing with antimicrobial agents in microbiological analyses, drug carryover effects might be encountered. This phenomenon can occur when the drug inhibits the growth of the colonies present in that given dilution. The drug carryover effected zone may be seen clearly in lower dilutions of the sample on the plates[39,40].

AIMS

The aims of the current report are

- To evaluate double and triple antibiotic combinations including colistin against NDM-producing *K. pneumoniae* isolates *in vitro*, at clinically relevant antibiotic concentrations, using time-kill experiments.
- To validate if spiral plating can be used instead of manual plating in this project.

MATERIALS

1. Bacterial Strains

Clinical isolates of New Delhi metallo-beta-lactamase (NDM)-1-producing *Klebsiella pneumoniae* strains and Verona integron-encoded metallo-beta-lactamase (VIM)-1-producing *K. pneumoniae* were obtained from the Department of Clinical Microbiology, Karolinska University Hospital, Stockholm, Sweden. NDM-1- producing *K.pneumoniae* strains NDM-KP K1, NDM-KP K6, NDM-KP K9, NDM-KP IR15, NDM-KP IR18K, NDM-KP IR19K and NDM-KP IR20K were for antibiotic susceptibility tests. For validation between spiral plating and manual plating with beads VIM-1-producing *K. pneumoniae* strains VIM-KP ÖN 2211 and VIM-KP T14789, NDM-1-producing *K. pneumoniae* strains NDM-KP IR8, NDM-KP IR20K and NDM-KP IR62E were used. Main experiments; static time-kill experiments were performed with NDM-1- producing *K. pneumoniae* strains NDM-KP K1, NDM-KP K6, NDM-KP K9, NDM-KP IR8, NDM-KP IR15, NDM-KP IR18K, NDM-KP IR19K, NDM-KP IR20K and NDM-KP IR62E, but for kinetic time-kill experiments only NDM-1-producing *K. pneumoniae* strains NDM-KP IR8 was used. Population analysis profiles were tested with NDM-1-producing *K. pneumoniae* strains, NDM-KP IR20K and NDM-KP IR62E.

2. Culture Media (Appendix 1) and solutions

Mueller Hinton II Broth [41] and Mueller Hinton II agar [42] plates (Becton, Dickinson & Co., Sparks, USA) were used for all the experiments in this report. The agar powder had adjusted cations (especially calcium and magnesium) because they can alter colistin activity. Phosphate Buffered Saline (PBS) pH 7.4 ($NaCl+Na_2HPO_4+KCL+KH_2PO_4$) (Merck, Darmstadt, Germany), NaCl (Merck, Darmstadt, Germany)[43], 99.99% methanol (Merck, Darmstadt, Germany), 70% ethanol, Dimethylsulfoxide (Sigma-Aldrich, St.Louis, USA)[44], 0.5% McFarland turbidity standard and Sterile water.

3. Antibiotic susceptibility testing

MIC determination of the NDM-1-producing *K. pneumoniae* strains in this report was performed using E-test strips of aztreonam (ATM), ciprofloxacin (CIP), colistin (CST), fosfomycin (FOF), meropenem (MEM), rifampicin (RIF) and tigecycline (TGC) (bioMérieux SA, Marcy-l´Etoile, France) according to the instructions of the manufacturer.

4. Antibiotics

Aztreonam powder (A6848, Sigma-Aldrich, St.Louis)[45], colistin sulphate salt powder (C4461, Sigma-Aldrich, St.Louis)[46], phosphomycin disodium salt powder (P5396, Sigma-Aldrich, St.Louis)[47] and Rifampicin powder (R3501, Sigma-Aldrich, St.Louis)[48] and Meropenem trihydrate powder[49] (BX 080121A, AstraZeneca, Södertälje, Sweden) were used for respective experiments.

5. Glass wares and plastics

5L, 500ml, 200ml, 80ml empty glass bottles, sterile glass beads in tubes, glass tubes for weighing antibiotic powder, Dilution glass tubes, Sterile empty 90 mm Petri dishes, 15ml polypropylene falcon tubes[50] in order to reduce the colistin binding to the wall of the tube, 13 ml polystyrene tubes for inoculum, 0.6 ml eppendorf tubes, 1.5 ml eppendorf tubes, inoculation loops, swabs, 5000 µl, 1000 µl, 200 µl, 40 µl pipettes and 5ml, 1000 µl, 200 µl pipette tips (Sarstedt), Etest applicator kit.

6. Easy Spiral Pro Automatic Spiral Plater (Interscience, Bois Arpents, France) [51]

A complete description of this part of the materials section is given in appendix 3 and 4.

7. Kinetic *in vitro* model (Appendix 5)

Sterilized two-armed spinner flasks (~100 ml), Dilution pump (type P-500; Pharmacia Biotech, Uppsala, Sweden), Dosing pump (model 22, Harvard Apparatus, Hollistion, MA, USA), Computer with in-house developed software ARUDose 2.0 (Antibiotic Research Unit, Department of Medical Sciences, Uppsala University, Sweden), Magnetic stirrers, caps, caps with a silicon membrane, detachable base of the culture flask with tubing , filter support and transparent rubber gaskets, main filter (0.45 µm pore size, Millipore Corporation), prefilter (Sigma-Aldrich), red and gray plastic clamps, butterfly screws, hoses, Luer lock syringe, Converter port, USB cable, power supply

METHODS

1. Determination of Antibiotic Susceptibility

The minimum inhibitory concentration (MIC) was determined by E-test (Epsilometer) strips for seven NDM-1-producing strains. Approximately five well-isolated colonies were picked up with a loop and diluted in approximately 5 ml of Mueller Hinton II broth until the turbidity was similar to 0.5 McFarland turbidity standard. The sterile cotton swab was dipped into the inoculum and streaked on MH II plate evenly. The selected antibiotic E-test strips were placed carefully on each plate using E-test applicator and incubated at 37°C for 18-24 hours. The MIC corresponded to the point of intersection between the zone of inhibition and the strip, on which the value was read[52]. Susceptibility categories were interpreted according to European Committee on Antimicrobial Susceptibility Testing (EUCAST) (Appendix 2). All MIC values were confirmed at least twice.

2. Preparation of selected antibiotics

After calculating the counter ions in the antibiotic powder, the actual antibiotic base was obtained. The concentration of stock solutions were as follow: colistin sulphate salt[46]- 12060 mg/L, meropenem trihydrate[49]- 11400 mg/L, fosfomycin disodium salt[47]- 13400 mg/L, rifampicin[48]- 10000 mg/L to give a solution of 10000 mg/L of antibiotic base. In which, rifampicin (purity ≥ 97% by HPLC) and aztreonam (purity: analytical ≥ 98%) have no counter ions. Although colistin, meropenem and fosfomycin are dissolved in PBS, rifampicin is soluble in methanol. Aztreonam [45] was dissolved as 50000 mg/L in dimethylsulfoxide and further diluted as 1000 mg/L in PBS.

3. Validation between spiral plating and manual plating with beads

In these experiments, all the antibiotic concentrations were targeted as maximum concentrations (Cmax) of unbound protein fraction of drug in human already calculated from literature searches. The target concentrations (Cmax x fraction unbound drug) of antibiotics used were as follow: Aztreonam[53,54]= 91.8 mg/L (204 mg/L x 0.45), colistin[55,56]= 1.465 mg/L (2.93 mg/L x 0.5), meropenem[57]= 50 mg/L (50 mg/L x 1), fosfomycin[58,59]= 240 mg/L (240 mg/L x 1) and rifampicin[60]= 4.35 mg/L (17.4 mg/L x 0.25). The above concentrations of the antibiotics were obtained by diluting them in PBS from already prepared antibiotics (from method 2). Drug combinations included in this study were colistin+ rifampicin, colistin +

meropenem, colistin + rifampicin + meropenem, colistin + fosfomycin + meropenem, colistin + aztreonam + meropenem.

First, one colony was inoculated in MH II broth and bacteria were grown at 37°C shaking water bath for overnight. In order to achieve starting inocula in logarithmic growth phase, approximately 5 x 10^9 cfu/ml bacteria were further inoculated in pre-warmed MH II broth and incubated for 1.5 hours. After that, the start culture was further inoculated into MH II broth to obtain a starting inoculum of approximately 5 x 10^7 cfu/ml and serial dilution of bacteria was made. Targeted amount of antibiotics were added to each prepared set of diluted tubes and the sample was plated out directly with manual plating and spiral plating at the same time. So, the higher dilution tubes contained lower amount of bacteria although antibiotic concentrations were the same in every dilution tube. Finally, all the plates were incubated in a 37° C room for 24 hours and visible colonies were counted. For spiral plate counting, all the colonies of each sector were counted from outer to inner sectors of quarter pairs on the spiral plate using the counting grid until 20 colonies were found. Then, the colonies in an opposite quarter were counted until the sector where 20^{th} colonies were found in the first quarter. After adding both counts, CFU/ml was obtained by dividing the volume of the respective sector according to the manufacturer's guideline (Appendix 4). The experiments were performed duplicates.

- **Avoiding antibiotic carryover effect in counting the plates**

For manual plating, the sample was placed in one spot on the MH II agar plate and left for a short while to allow the antibiotics to sink in a little before glass beads were added and the bacteria spread out over the whole plate. If there was antibiotic carryover effect after 24 hours incubation, a clear area could be defined in the zone where the sample was put. After the plate had divided into sections (commonly four sections), the bacterial colonies in the other sections which were free from carryover effect were counted. Then, the numbers of bacterial colonies were multiplied by the numbers of the divided sections. This method was done for all experiments in this report and is always applied in our laboratory.

4. Static time-kill experiments

Mean steady state concentrations (Css) of unbound protein fraction of drug in human were applied as antibiotic concentrations used in these experiments. Css of the antibiotics were searched in the literature which had been calculated based on the formula of the area under the antibiotic concentration curve in serum or plasma over 24 hours divided by 24 (AUC_{0-}

$_{24h}$/24h). Since there was no relevant reference for rifampicin available, the mean concentration (Cmax+Cmin)/2 was used. The following concentrations (Css x fraction unbound drug) were used: colistin_ 1.18 mg/L[27](2.36 mg/L x 0.5), 2.12 mg/L[27], 2.54 mg/L and 4.24 mg/L, meropenem[61]_ 6.76 mg/L (6.9 mg/Lx 0.98[62]), rifampicin[60]_ 1.74 mg/L (8.7 mg/Lx 0.2) and fosfomycin[58,59]_ 83.25 mg/L (83.25 mg/Lx 1). The above concentrations of the antibiotics were obtained by diluting them in PBS from already prepared antibiotics (from method 2). Antibiotic regimens used in these experiments were colistin, fosfomycin, colistin+ rifampicin, colistin+meropenem, colistin+ fosfomycin and colistin+ rifampicin+ meropenem.

One colony was picked from strain stock MH II plates and incubated into MH II broth at 37°C shaking water bath for overnight culture. In order to achieve starting inocula in logarithmic growth phase, approximately 5x 10^9 cfu/ml bacteria were further inoculated in pre-warmed MH II broth and incubated for 1.5 hours. After that, the start culture was further inoculated into MH II broth to obtain starting inoculum of, approximately 5x 10^7 cfu/ml and serial dilution of bacteria was made. Then, samples were plated out manually at time 0 hour after serial dilution was done. The targeted amount of antibiotics was added to the test tube and the tube was incubated again in a 37°C shaking water bath until next sampling time. The sampling procedures were performed at 0, 1, 2, 4, 6 and 24 hour time points. All the plates were incubated in a 37° C room for 24 hours and visible colonies were counted. All the experiments were carried out at least two times. In this experiments, the higher dilution tubes were contained the lower amount of bacteria and antibiotic concentrations.

5. Kinetic time-kill experiments

The target concentrations of antibiotics used were as follow: meropenem[57]= 50 mg/L (50 mg/Lx 1) and rifampicin[60]= 4.35 mg/L (17.4mg/Lx 0.25) according to Cmax of unbound protein fraction of drug in human already calculated from literature. The above concentrations of the antibiotics were obtained by diluting them in PBS from already prepared antibiotics (from method 2). However, Css were used for colistin 1.18 mg/L[27] (2.36mg/Lx 0.5) because of its long half-life (14.4 hours[27]). Half-lives for rifampicin and meropenem were used as 2.2[63]and 1[61] hours respectively.

The experiments were run in the previously developed *in vitro* kinetic time-kill model[64,65] which mainly contains an air-tight, open bottomed spinner flask connected to a dilution pump and dosing system (syringe pump)[66]. At first, overnight culture was created and the starting inoculum was obtained after 1.5 hours. After a fresh media bottle was connected to one arm of

the culture flask containing 100 ml MH II broth, start culture was inoculated into that flask to yield a concentration of 1x 10^7 CFU/ml. Then, the rate of the fresh media was set at a constant rate of 69 ml/h ($F= V \times (\ln 2 / t_{1/2})$ in main dilution pump. The flow rate in the dilution pump should be of the drug that has the shortest half-life (in this experiment, meropenem) to compensate for the loss of other longer half-life drug (here, rifampicin) in the flask. For three antibiotics combination with different half-lives, a syringe pump was used connected with a Taflon tube to one arm of the flask controlled by the ARUDose 2.0 software. Bacteria were trapped in 0.45 μm pore sized filter and the stirrer prevented blockage of the filter. Then, samples were taken out with a syringe through the silicon capped arm of the flask and plated out manually at time 0 hour after serial dilution had made. Targeted amount of antibiotics were added to the spinner flask and media bottle and syringe according to experimental set up. The sampling procedures were performed at 0, 1, 2, 4, 6, 8 hour time points (for colistin+ rifampicin, colistin+ meropenem and colistin+ rifampicin+ meropenem combination) All the plates were incubated in a 37° C room for 24 hours and visible colonies were counted. All the experiments were carried out at least twice.

6. Population analysis profiles

Eight different colistin concentrations were prepared 0, 0.5, 1, 2.5, 5, 10, 20 and 40 x MIC in MH II agar for NDM-KP IR 20K (MIC_{CST}= 0.25 mg/L) and NDM-KP IR 62E (MIC_{CST}= 0.125 mg/L). The certain amount of colistin solution was added to each MH II melted agar according to eight different concentrations of colistin. The colistin agar was poured into the empty plates. The plates were cool down for a while and were stored.

- **Static time-kill experiments with colistin containing plates**

Overnight cultures were grown in MH II broth in a 37° C shaking water bath. Bacteria were further inoculated in pre-warmed MH II broth and incubated for 1.5 hours for letting them grow logarithmically. After serial dilutions had been created, the samples were spread onto MH II plates containing 0, 0.5, 1, 2.5, 5, 10, 20 and 40 x MIC colistin and all the plates were incubated for 24 hours. Here, colistin was contained only in the agar plates.

Bacteria were treated with 1.18 mg/L of colistin in the test tube and incubated in the 37° C shaking water bath for 24 hours. After 24 hours incubation in water bath, the samples (bacteria treated with colistin) were plated on MH II plates containing 0, 0.5, 1, 2.5, 5, 10, 20 and 40 x MIC colistin. All the plates were incubated in a 37° C room for 24 hours and counted visible colonies manually. The samples were saved for further experiments at - 80˚ C.

7. Data analysis

To determine MIC values, if the value reached the large marking point on the E test strip ladder scale, then it was read as a final MIC value. If the value was at the smaller marking point, it was read MIC value of above larger marking point. Final MIC values were selected from median values of separated experiments.

For analysis of all time-kill experiments, lower limit of detection for an individual bacterial count was < 1.0 $\log_{10}$ CFU/ml bacterial concentrations. Synergy was defined as ≥ 2 $\log_{10}$ decrease in CFU/ml between the combination and its most active constituent after 24 hours. Bactericidal effect was defined as ≥ 3 $\log_{10}$ decrease in CFU/ml after 24 hours compared with the starting inoculum. Bacteriostatic effect was defined as between ≥ 1 $\log_{10}$ and < 3 $\log_{10}$ reduction in CFU/ml after 24 h compared with the starting inoculum.

RESULTS

1. Antibiotic susceptibility tests

Among 7 antibiotics tested with all 9 NDM- and 2 VIM-producing *K. pneumoniae* strains, all strains were susceptible to colistin according to EUCAST clinical breakpoints. All strains were resistant to fosfomycin except the two VIM-KP strains, and they were also resistant to meropenem except for two of the NDM-KP strains (IR8 and IR20K) and one VIM-KP (ÖN 2211), moreover, most of the strains could grow at > 32 mg/L for rifampicin (Table 2).

2. Validation between spiral plating and manual beads plating

Two VIM-KP strains and NDM-KP IR 62E were used for validating spiral plating using a spiral plater (Easy Spiral Pro Automatic Spiral Plater, Interscience, Bois Arpents, France) and traditional manual bead plating methods. Bacteria were treated with a high amount of antibiotics in different amount of bacteria. In general, the differences between two plating methods were less than 0.5 $\log_{10}$ CFU/ml not only in the treatment with the triple antibiotic combination (figure 1, A) but in the control as well (figure 1, B). However, carryover effects of antibiotics (Figure 2) were found in first three dilutions of both the spiral and the manual plates (both approximately 10^7, 10^6 and 10^5 CFU/ml of bacteria) of ÖN 2211, which is sensitive to three antibiotics. The first four dilutions of manual plates (~ 10^7, 10^6, 10^5 and 10^4 CFU/ml of bacteria) and first two dilutions of spiral plates (~ $10^7$and 10^6 CFU/ml of bacteria) of IR 62E showed carryover effects of antibiotics but T 14789 strain were not found carryover effects (Table 6).

Furthermore, among static time-kill experiments using both plating methods, three NDM-KP strains were treated with a lower amount of antibiotics using a starting inoculum of nearly 10^7 CFU/ml. Because it is not possible to include all the graphs in this report, only one representative graph of static time-kill experiments for NDM-KP IR8, IR 20K and IR 62E in colistin, rifampicin and meropenem combination is shown (Figure 3). The results of both methods were found to be similar for all three strains however, there was some variation between the two methods (approximately 1 log difference) in some cases (e.g. IR 8 at 24 hours in Figure 2). Moreover, the carryover effect occurred in all three strains, especially in the highest concentration of bacteria loaded tubes.

3. Static time-kill experiments

- **Time-kill experiments using different concentrations of colistin in combination with rifampicin and meropenem** (Figures 6-8), (Tables 3 and 5)

First of all, time-kill experiments of three NDM-KP strains (IR 20K, IR 8 and IR 62E) with three different concentrations of colistin (1.18, 2.54 and 4.24 mg/L) in combinations including rifampicin and meropenem, will be discussed. All three strains were sensitive to colistin (MIC= 0.25 mg/L for IR20K, 0125 mg/L for the other two strains) and MIC values of rifampicin were >32 mg/L for IR 62E and IR 20K and 32 mg/L for IR 8. IR 20K was sensitive to meropenem (MIC=0.75 mg/L) whereas, IR 8 and IR 62 had an MIC of 4 mg/L (intermediate sensitivity) and >32 mg/L (resistant) respectively.

When IR 20K (figure 6 and table 4) was treated with three concentrations of colistin in combinations, the overall trend followed that higher colistin concentrations gave fast and high killing rate in first hours (approximately $\geq$ 4 $\log_{10}$ CFU/ml reduction) and reached the lowest CFU/ml (approximately $\geq$ 4 $\log_{10}$ CFU/ml reduction) in 24 hours comparing with other colistin concentration. Killing rate of colistin monotherapy was consistent during 24 hours in all colistin concentrations. Regrowth during the monotherapy occurred after 6 hours till 24 hours to 2-9 $\log_{10}$ CFU/ml. However, the effect of colistin was better when it was combined with rifampicin and meropenem. Four bactericidal effects and four bacteriostatic effects were found in different regimens (except- colistin monotherapy and colistin- 2.54 mg/L + rifampicin) of different colistin concentrations. The colistin + meropenem combination and the triple combination had bacteriostatic or bactericidal effect at all concentrations of colistin.

In the treatment regimens of the IR 8 strain (figure 8 and table 4), colistin monotherapy behavior was similar to behavior seen with IR 20K. Most concentrations of colistin had a killing rate of approximately $\geq$ 3 $\log_{10}$ CFU/ml reduction in one hour (except 2.12 mg/L colistin regimens). After 2 hours, the bacteria grew back until 24 hours to 3-9 $\log_{10}$ CFU/ml. Interestingly, a colistin and meropenem combination with different colistin concentrations was found to be nearly 9 $\log_{10}$CFU/ml after 24 hours which was similar to colistin monotherapy. Only 4.24 mg/L colistin containing colistin+ rifampicin regimen showed a bactericidal effect for the IR 8 strain. Six bacteriostatic effects were found in different regimens (except colistin monotherapy and colistin 1.18/4.24 mg/L + meropenem) of different colistin concentrations (except 2.12 mg/L). The colistin+ rifampicin combination had bacteriostatic or bactericidal effect at all concentration of colistin.

For the IR 62E strain (figure 7 and table 4), as seen with the previous two strains, the effect of colistin alone seemed to be ineffective compared with other regimens. First hour killing effect (approximately more than 3 $\log_{10}$ CFU/ml reduction) was still found in this strain like with other two. After 4 hours, most of the combinations in different colistin concentrations showed bacterial regrowth until 24 hours to 2.7-9 $\log_{10}$ CFU/ml. Overall, for IR 62E, four bactericidal effects and four bacteriostatic effects were seen in different regimens (except for colistin monotherapy and colistin-4.24 mg/L + meropenem) of different colistin concentrations. The colistin+ rifampicin combination and the triple combination had a bacteriostatic or bactericidal effect at all concentrations of colistin.

Among these three strains with in different regimens containing three different concentrations of colistin, IR 20K (meropenem sensitive) was the most efficiently killed strain, with a colistin + meropenem regimen containing 4.24 mg/L colistin concentration and followed by colistin + rifampicin + meropenem regimen containing 4.24 mg/L colistin concentration against IR 20K.

- **Time-kill experiments using 1.18 mg/L colistin in combination with rifampicin and meropenem** (figures 4-8), (tables 3 and 5)

Generally, at least ≥ 3 $\log_{10}$ CFU/ml of bacteria were killed within one hour after adding antibiotics and considerably grew back mostly after 4 hours until 24 hours (figures 4-8 and tables 3 and 5). Without antibiotic treatment the stationary phase was reached after logarithmic growth and the bacterial count remained around 4.8 x 10^9 CFU/ml until 24 hours.

In colistin monotherapy, all bacteria were killed ≥ 3 $\log_{10}$ CFU/ml reduction at first hour. Re-growth occurred mostly after 2 hours up to 24 hours to around 9 $\log_{10}$ CFU/ml. Colistin monotherapy against all 9 strains had neither bactericidal nor bacteriostatic effects. The result of this therapy was similar to those of the control group at 24 hours.

For the colistin and rifampicin combination, most of the strains had an approximate 4 $\log_{10}$ reduction within 1 hour. Most of them grew back after 6 hours until 24 hours to 1-6 $\log_{10}$ CFU/ml, whereas, K 6 (MIC_{RIF}= 12) was below detection limit until 24 hours. Bacteriostatic and/or bactericidal effect was shown for all 9 strains and this combination seemed to be most effective against K 6 overall.

With regards to a colistin and meropenem regimen, around 4 $\log_{10}$ CFU/ml reduction was occurred within 1 hour. After 4 hours, all strains regrew until 24 hours to 4.5-9 $\log_{10}$ CFU/ml

at most. This combination was not very effective and, was found to be only bacteriostatic against IR 20K and IR 62E. This regimen appeared similar to colistin monotherapy at 24 hours.

For the triple combination (colistin, rifampicin and meropenem), at the 1 hour time point, the therapy decreased most of the strains to around 4-5 $\log_{10}$ CFU/ml. Most regrowth occurred after 4 hours, until 24 hours to around 2.7-6 $\log_{10}$ CFU/ml. Bactericidal effects were found in K 1, IR 62E and IR 20K. A bacteriostatic effect was found in all other strains except IR 18K.

For the colistin and fosfomycin combination for IR 8 (figure 19 and table 4), it reduced the bacterial concentration to > 3 $\log_{10}$ CFU/ml during the 1st hour. Fosfomycin alone showed a trend similar to the control from 1 hour to 24 hours at around 9 $\log_{10}$ CFU/ml. Not much difference between colistin alone and double combination was found except at 24 hours. Bactericidal or bacteriostatic effects were not found for this strain with this combination.

All in all, bactericidal and bacteriostatic effects after 24 hours were found for colistin + rifampicin combination against 3 and 6 strains and colistin + rifampicin + meropenem combination against 3 and 5 strains, respectively. Moreover, colistin and meropenem combination also showed bacteriostatic effect against 2 strains. However, among the 9 strains, colistin and rifampicin combination therapy had the highest bactericidal effect against NDM-KP K 6.

4. Kinetic time-kill experiments (figure 9 and table 4)

At the beginning of the experiments, the starting inoculum of the IR 8 strain was around 6.5 $\log_{10}$ CFU/ml (figure 9, A). For the static and kinetic time-kill tests, control group grew logarithmically for 6 hours, until the stationary phase was reached, after which the bacterial count remained at 9.2 x 10^8 CFU/ml until 24 hours. Amazingly, all four regimens killed the bacteria within 1st hour. Although colistin monotherapy and colistin and rifampicin treatments could not maintain bacterial killing effect after 2 hours, the other two regimens showed no detectable growth at 8 hours. The bactericidal effect after 8 hours was obvious in the double and triple combinations, whereas triple combination was the most effective followed by colistin + meropenem and colistin + rifampicin regimens respectively. At 24 hours, colistin monotherapy was similar to the control.

The kinetic profile, i.e. the concentrations of antibiotics during the experiments, were calculated and drawn as a graph (figure 9, B). After 4 hours, the amount of antibiotic was very

low in the system. In detail, rifampicin and meropenem concentrations reached <1 mg/L after 4.5 hours and 5.5 hours respectively.

5. Static time-kill experiments with colistin containing plates (figure 10)

During regular static time-kill experiments, MH II plates containing different concentrations of colistin were used in addition to antibiotic free MH II plates at time 0 and 24 hours. Two bacterial strains were tested, IR 20K and IR 62E. At 0 hour, bacteria grew to around 7 $\log_{10}$ CFU/ml until their own MIC. Very few bacteria were able to grow on further two or three higher MIC plates at 0 hour for each strain. 24 hours after adding 1.18 mg/L colistin to samples, they grew well, reaching around 9 $\log_{10}$ CFU/ml, even in high amount of colistin containing plates (40 x MIC).

Table 2. Antibiotic susceptibilities of NDM- and VIM-producing *K. pneumoniae* strains shown as minimal inhibitory concentration (MIC) values (mg/L) and classification according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST) clinical breakpoints

Antibiotic	NDM-KP K1		NDM-KP K6		NDM-KP K9		NDM-KP IR8*		NDM-KP IR15		NDM-KP IR18K		NDM-KP IR19K		NDM-KP IR20K		NDM-KP IR62E*		VIM-KP ÖN2211*		VIM-KP T14789*	
	MIC	SIR	MIC	SIR	MIC	SIR	MIC	SIR	MIC	SIR	MIC	SIR	MIC	SIR	MIC	SIR	MIC	SIR	MIC	SIR	MIC	SIR
Aztreonam	>256	R	0.125	S	0.25	S	>256	R	>256	R	>256	R	>256	R	>256	R	>256	R	4	I	2	I
Ciprofloxacin	32	R	32	R	32	R	>32	R	>32	R	>32	R	>32	R	>32	R	>32	R	4	R	>32	R
Colistin	0.125	S	0.125	S	0.125	S	0.125	S	0.25	S	0.25	S	0.125	S	0.25	S	0.125	S	0.125	S	0.125	S
Fosfomycin	64	R	256	R	1024	R	256	R	512	R	192	R	48	R	48	R	48	R	4	S	32	S
Meropenem	32	R	32	R	32	R	4	I	16	R	>32	R	>32	R	0.75	S	>32	R	2	S	>32	R
Rifampicin	32	-	12	-	32	-	32	-	16	-	>32	-	>32	-	>32	-	>32	-	>32	-	>32	-
Tigecycline	0.75	S	0.25	S	0.5	S	2	I	0.5	S	4	R	2	I	2	I	3	R	1	S	2	I

SIR classification according to EUCAST clinical breakpoints; S: susceptible, I: intermediate, R: resistant, -: not defined

* The MIC values of NDM-KP IR8, NDM-KP IR62E, VIM-KP ÖN 2211 and VIM-KP T14789 were kindly received from Thomas Tängden's Paper IV of his Ph.D thesis.

Table 3. Summary of mean bacterial concentrations and standard deviation (SD) at 0, 1 and 24 h and change in bacterial concentrations in log $_{10}$ cfu/ml (Δ) at 1 and 24 h compared with the starting inoculums (0 h) during static time-kill experiments against NDM-producing *K. pneumoniae* strain NDM-KP K1, NDM-KP K6, NDM-KP K9, NDM-KP IR15, NDM-KP IR18K and NDM-KP IR19K using 1.18 mg/L of colistin in the regimens. Bactericidal effect (≥ 3 $\log_{10}$ reduction in CFU/ml after 24 h) is shown in orange. Bacteriostatic effect (≥ 1 $\log_{10}$ - < 3 $\log_{10}$ reduction in CFU/ml after 24 h) is shown in yellow.

Strain	Antibiotic Regimen	0h	SD (0h)	1h	SD (1h)	24h	SD (24h)	Δ (1h)	Δ (24h)
K1	CST	7.00	0.13	2.46	0.32	9.13	0.11	-4.54	2.13
	CST+RIF	6.92	0.08	2.40	0.66	4.95	1.08	-4.52	-1.97
	CST+MEM	7.00	0.08	2.72	0.36	9.26	0.10	-4.28	2.26
	CST+RIF+MEM	6.95	0.13	2.26	0.49	2.69	2.34	-4.69	-4.26
K 6	CST	6.93	0.13	3.51	0.67	9.23	0.01	-3.42	2.30
	CST+RIF	6.91	0.03	3.51	0.60	1.00	0.00	-3.40	-5.91
	CST+MEM	6.89	0.12	3.24	0.36	9.37	0.20	-3.65	2.48
	CST+RIF+MEM	6.90	0.13	3.08	0.76	5.43	0.46	-3.82	-1.47
K 9	CST	6.92	0.00	3.37	0.41	9.26	0.01	-3.55	2.34
	CST+RIF	7.04	0.10	2.87	0.68	5.31	0.71	-4.17	-1.73
	CST+MEM	6.97	0.17	3.25	0.31	9.39	0.19	-3.72	2.42
	CST+RIF+MEM	6.98	0.14	2.88	0.47	5.18	1.53	-4.10	-1.80
IR 15	CST	6.89	0.10	2.37	0.40	9.47	0.12	-4.52	2.58
	CST+RIF	6.87	0.03	2.22	0.66	4.53	1.75	-4.65	-2.35
	CST+MEM	6.91	0.05	2.50	1.44	9.34	0.01	-4.41	2.43
	CST+RIF+MEM	6.93	0.03	2.28	0.71	4.79	2.06	-4.65	-2.14
IR 18K	CST	6.80	0.15	3.02	0.14	8.55	0.16	-3.78	1.75
	CST+RIF	6.77	0.09	3.53	0.91	3.83	2.45	-3.24	-2.94
	CST+MEM	6.81	0.15	3.55	0.88	7.48	3.30	-3.26	0.67
	CST+RIF+MEM	6.62	0.21	2.81	0.28	6.22	0.55	-3.81	-0.40
IR 19K	CST	6.84	0.12	2.74	0.49	9.29	0.05	-4.10	2.45
	CST+RIF	6.75	0.01	2.30	0.85	2.79	2.53	-4.45	-3.96
	CST+MEM	6.81	0.05	2.71	0.24	9.24	0.06	-4.10	2.43
	CST+RIF+MEM	6.80	0.04	1.56	0.45	4.42	1.87	-5.24	-2.38

Table 4. Summary of mean bacterial concentrations and standard deviation (SD) at 0, 1, 8 and 24 h and change in bacterial concentrations in log $_{10}$ cfu/ml (Δ) at 1 and 24 h compared with the starting inoculums (0 h) during kinetic time-kill experiments against NDM-producing *K. pneumoniae* strain IR 8 using 1.18 mg/L of colistin in the regimens. Bactericidal effect (≥ 3 $\log_{10}$ reduction in CFU/ml after 8/24 h) is shown in orange. Bacteriostatic effect (≥ 1 $\log_{10}$ - < 3 $\log_{10}$ reduction in CFU/ml after 8/24 h) is shown in yellow.

Antibiotic Regimen	0h	SD (0h)	1h	SD (1h)	8h	SD (8h)	24 h	SD (24h)	Δ (1h)	Δ (8h)	Δ (24h)
CST	6.66	0.20	1.00	0.00	-	-	8.64	0.01	-5.66	-	1.98
CST+RIF	6.50	0.09	1.00	0.00	2.46	0.71	-	-	-5.50	-4.04	-
CST+MEM	6.48	0.11	1.00	0.00	1.00	0.00	-	-	-5.48	-5.48	-
CST+RIF+MEM	6.60	0.08	1.00	0.00	1.00	0.00	-	-	-5.60	-5.60	-

Table 5. Summary of mean bacterial concentrations and standard deviation (SD) at 0, 1 and 24 h and change in bacterial concentrations in log $_{10}$ cfu/ml (Δ) at 1 and 24 h compared with the starting inoculums (0 h) during static time-kill experiments against NDM-producing *K. pneumoniae* strain NDM-KP IR 8, NDM-KP IR 20K and NDM-KP IR 62E using different concentration of colistin in the regimens. Bactericidal effect and bacteriostatic effect are shown in orange and yellow respectively.

Strain	Colistin concentration (mg/L)	Antibiotic Regimen	0h	SD (0h)	1h	SD (1h)	24h	SD (24h)	Δ (1h)	Δ (24h)
IR 8	1.18	CST	6.70	0.02	3.57	0.85	9.20	0.04	-3.13	2.50
		CST+RIF	6.90	0.09	1.92	1.08	4.38	1.90	-4.98	-2.52
		CST+MEM	6.86	0.10	3.07	0.43	8.92	0.52	-3.79	2.06
		CST+RIF+MEM	6.87	0.10	2.17	1.08	5.33	0.73	-4.70	-1.54
	2.12	CST	7.24	0.47	4.05	0.43	9.17	0.07	-3.19	1.93
		FOF	7.13	0.72	7.24	1.04	9.02	0.03	0.11	1.89
		CST+FOF	7.06	0.55	3.21	0.56	6.49	1.62	-3.85	-0.57
	2.54	CST	6.81	0.04	1.93	0.04	9.16	0.05	-4.88	2.35
		CST+RIF	7.17	0.67	2.33	0.88	4.25	2.86	-4.84	-2.92
		CST+MEM	6.87	0.13	2.90	1.38	9.20	0.15	-3.97	-2.33
		CST+RIF+MEM	6.78	0.06	2.13	0.91	4.50	2.26	-4.65	-2.28
	4.24	CST	6.81	0.08	1.55	0.72	9.13	0.13	-5.26	2.32
		CST+RIF	6.95	0.06	1.39	0.55	3.03	2.87	-5.26	-3.92
		CST+MEM	6.87	0.08	2.92	0.06	9.22	0.06	-3.95	2.35
		CST+RIF+MEM	6.86	0.13	2.66	2.34	4.36	1.94	-4.20	-2.50
IR 20K	1.18	CST	7.18	0.67	2.55	0.45	9.18	0.18	-4.63	2.01
		CST+RIF	6.90	0.09	2.87	0.30	3.50	2.06	-4.03	-3.40
		CST+MEM	6.96	0.24	2.76	0.44	4.57	0.84	-4.20	-2.39
		CST+RIF+MEM	6.91	0.31	2.79	0.14	3.82	1.94	-4.12	-3.09
	2.54	CST	6.85	0.06	2.22	0.42	9.32	0.10	-4.63	2.47
		CST+RIF	6.80	0.04	2.03	1.03	5.82	1.10	-4.77	-0.98
		CST+MEM	6.71	0.14	2.66	0.45	4.17	0.97	-4.06	-2.54
		CST+RIF+MEM	6.83	0.02	2.08	1.52	4.69	1.01	-4.75	-2.14
	4.24	CST	6.89	0.12	1.76	0.40	9.18	0.05	-5.13	2.29
		CST+RIF	6.87	0.03	1.84	0.79	3.90	2.52	-5.03	-2.97
		CST+MEM	6.90	0.01	2.38	0.25	2.15	0.28	-4.52	-4.75
		CST+RIF+MEM	6.91	0.05	2.26	0.70	2.40	2.42	-4.65	-4.51
IR 62E	1.18	CST	6.74	0.17	2.60	0.88	9.25	0.15	-4.14	2.51
		CST+RIF	6.80	0.03	2.84	1.46	5.67	0.81	-3.96	-1.13
		CST+MEM	6.80	0.11	3.31	1.41	4.49	1.04	-3.49	-2.31
		CST+RIF+MEM	6.72	0.05	2.62	1.15	3.54	2.21	-4.10	-3.18
	2.54	CST	6.75	0.16	1.90	0.84	9.26	0.15	-4.85	2.51
		CST+RIF	6.89	0.09	3.06	2.00	4.89	1.09	-3.83	-2.00
		CST+MEM	6.89	0.01	1.99	1.72	3.51	2.66	-4.90	-3.38
		CST+RIF+MEM	6.83	0.02	1.81	1.15	3.71	0.09	-5.02	-3.12
	4.24	CST	6.82	0.15	1.61	1.05	8.72	1.24	-5.21	1.90
		CST+RIF	7.13	0.73	2.19	1.24	2.73	2.44	-4.94	-4.40
		CST+MEM	6.81	0.02	2.21	0.08	9.23	0.13	-4.60	2.42
		CST+RIF+MEM	6.79	0.06	1.56	0.78	5.16	1.27	-5.23	-1.63

Table 6. Summary of the amounts of bacteria plated on both spiral and manual plates showing carryover effect of antibiotics for NDM-KP öN 2211, IR 62E and T 14789

Bacterial strain	Carryover effect found at the amounts of bacteria plated on	
	Spiral plate	Manual plate
öN 2211	~ 10^7, 10^6 and 10^5 CFU/ml	~ 10^7, 10^6 and 10^5 CFU/ml
IR 62E	~ $10^7$and 10^6 CFU/ml	~ 10^7, 10^6, 10^5 and 10^4 CFU/ml
T 14789	-	-

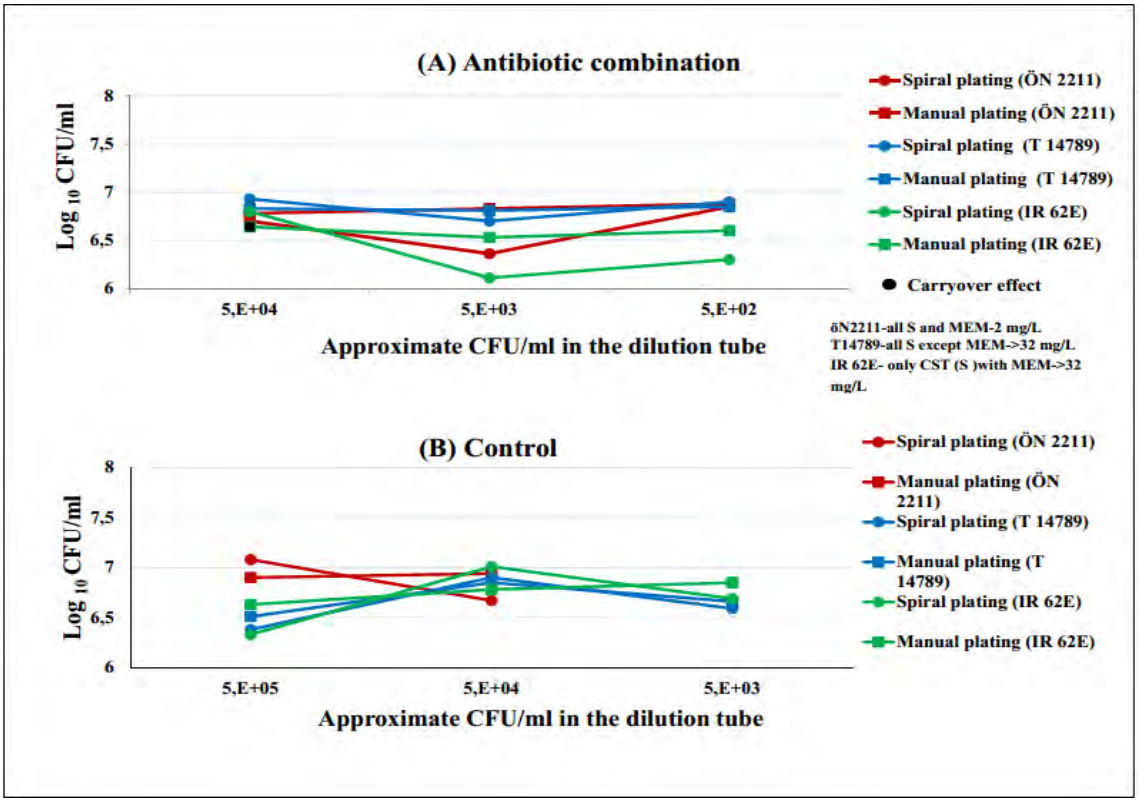

Figure 1. Results from validation between spiral and manual plating methods for *K. pneumoniae* VIM-KP ÖN 2211, VIM-KP T 14789 and NDM-KP IR 62E treating with colistin, fosfomycin and meropenem combination (A) and without antibiotics (B). X axis represents as amount of bacteria of the sample plated on each plate and Y axis shows the bacterial concentration of the initial suspension in logarithmic values. Different colors correspond to different bacterial strains and carryover effect of antibiotics is marked by black color.

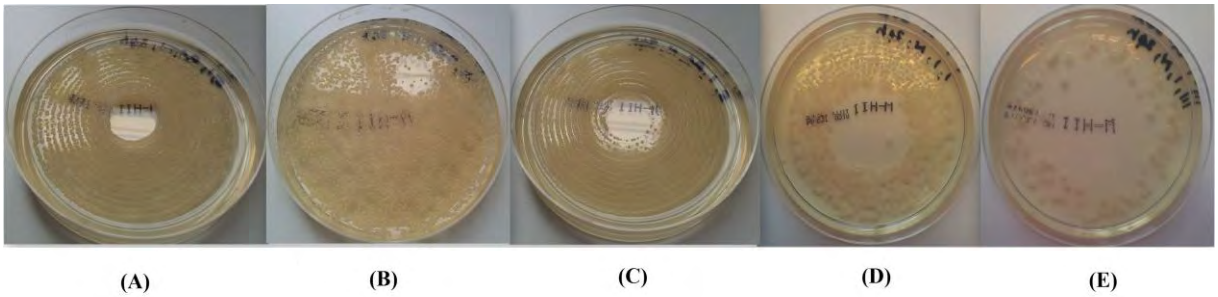

Figure 2. Bacteria were grown on MH II agar treated with antibiotics by spiral and manual plating methods showing spiral plate without carryover effect of antibiotics on spiral plate (A), carryover effect at upper right corner of manual plate (B), carryover effects on spiral plates at inner-most sectors (C), at inner sectors (D) and at most of the sectors (E).

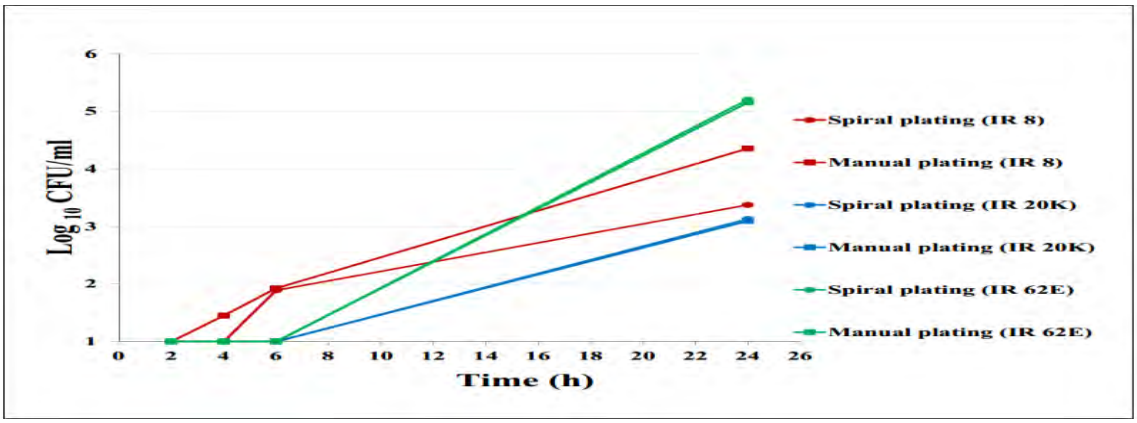

Figure 3. Results from validation between spiral and manual plating methods for *K.pneumoniae* NDM-KP IR 8, NDM-KP IR 20K and NDM-KP IR 62E treating with colistin, fosfomycin and meropenem combination. Different amount of bacterial concentration of the samples in logarithmic values are presented along with time in hours. Different colors correspond to different bacterial strains. Lower limit of detection for bacterial count is 1 $\log_{10}$ CFU/ml. All the results are described in mean values.

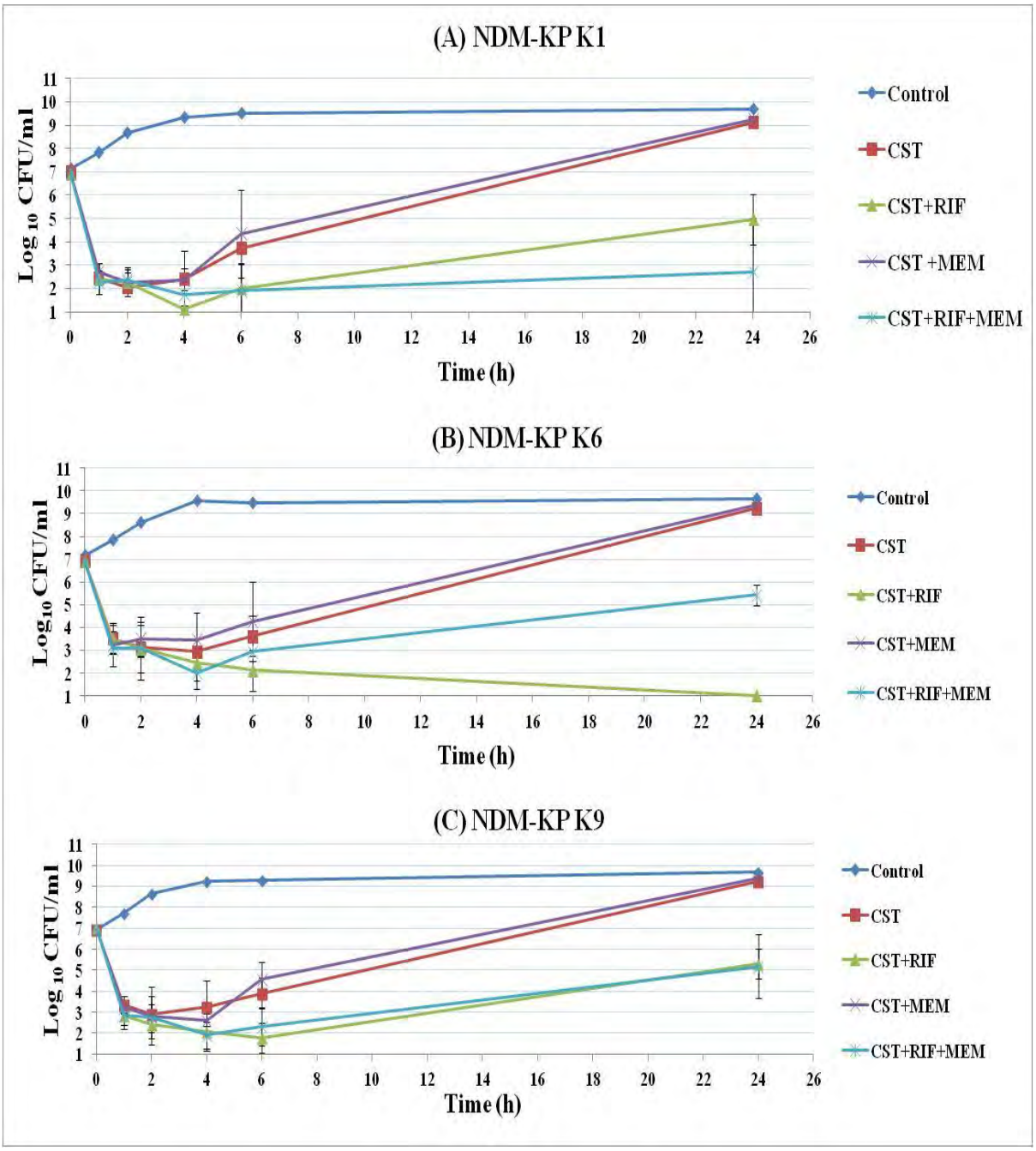

Figure 4. Results from static time-kill experiments for NDM-producing *K. pneumoniae* NDM-KP K1 (A), K6 (B) and K9 (C) treating with four regimens containing colistin concentration 1.18 mg/L. Different amount of bacterial concentration of the samples in logarithmic values are presented along with time in hours. Different colors correspond to different regimens. Lower limit of detection for bacterial count is 1 $\log_{10}$ CFU/ml. All the results are described in mean values and included standard deviation (SD). CST- colistin, RIF- rifampicin, MEM- meropenem

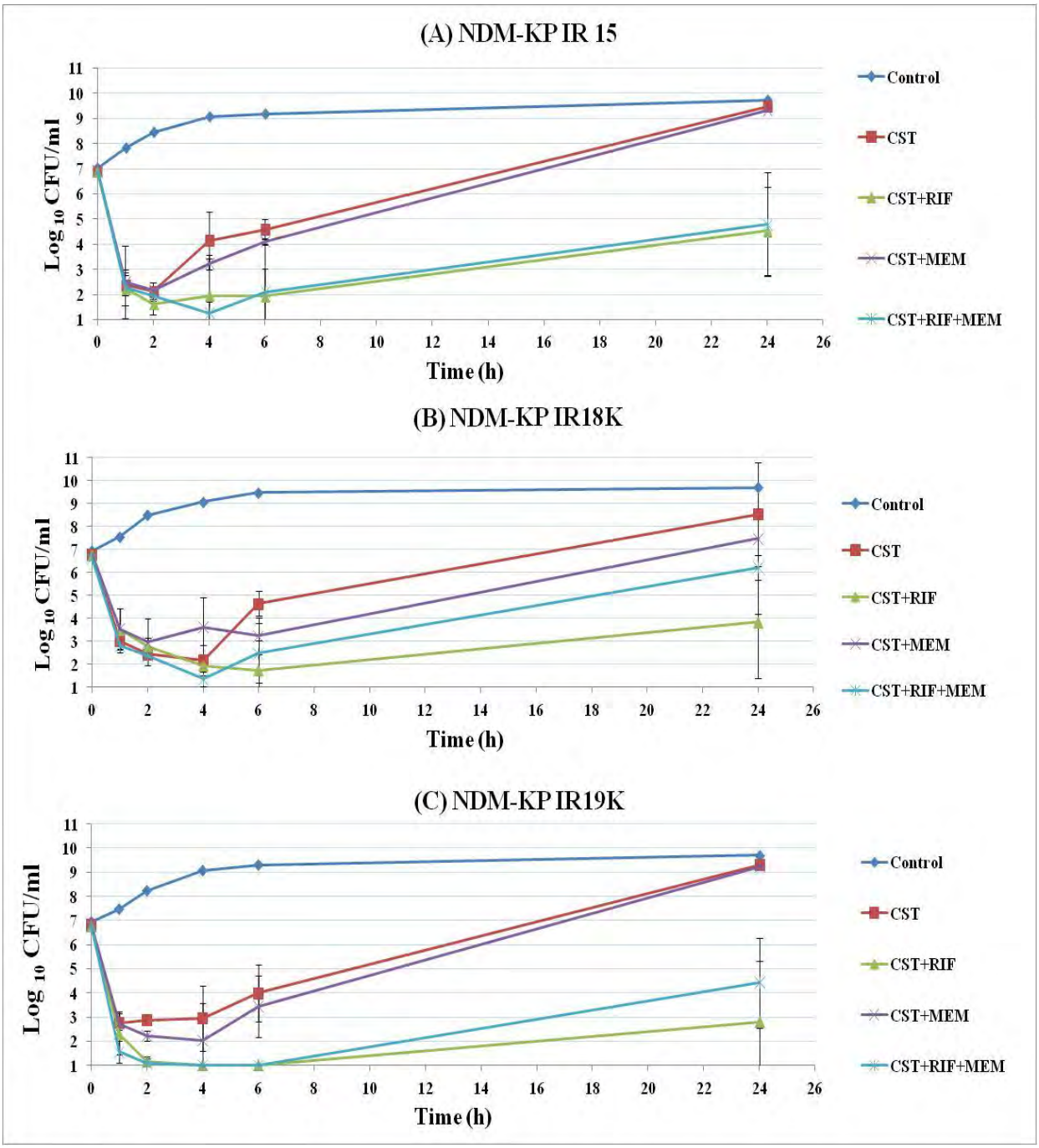

Figure 5. Results from static time-kill experiments for NDM-producing *K. pneumoniae* NDM-KP IR15(A), IR18K (B) and IR19K (C) treating with four regimens containing colistin concentration 1.18 mg/L. Different aratmount of bacterial concentration of the samples in logarithmic values are presented along with time in hours. Different colors correspond to different regimens. Lower limit of detection for bacterial count is 1 $\log_{10}$ CFU/ml. All the results are described in mean values and included standard deviation (SD). CST- colistin, RIF- rifampicin, MEM- meropenem

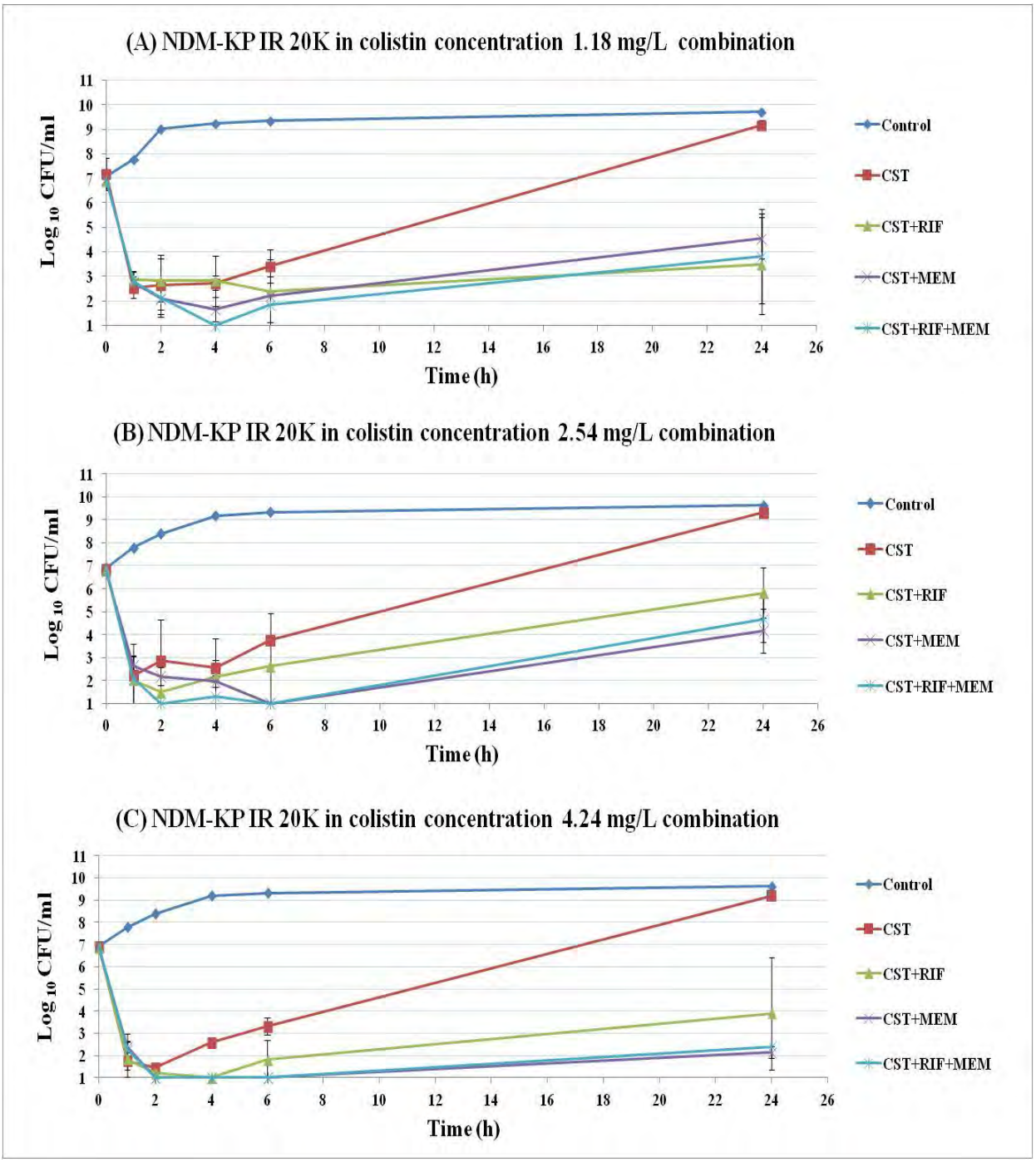

Figure 6. Results from static time-kill experiments for NDM-producing *K. pneumoniae* NDM-KP IR20K treating with four regimens containing colistin concentrations 1.18 mg/L(A), 2.54 mg/L(B) and 4.24 mg/L (C). Different amount of bacterial concentration of the samples in logarithmic values are presented along with time in hours. Different colors correspond to different regimens. Lower limit of detection for bacterial count is 1 $\log_{10}$ CFU/ml. All the results are described in mean values and included standard deviation (SD). CST- colistin, RIF- rifampicin, MEM- meropenem

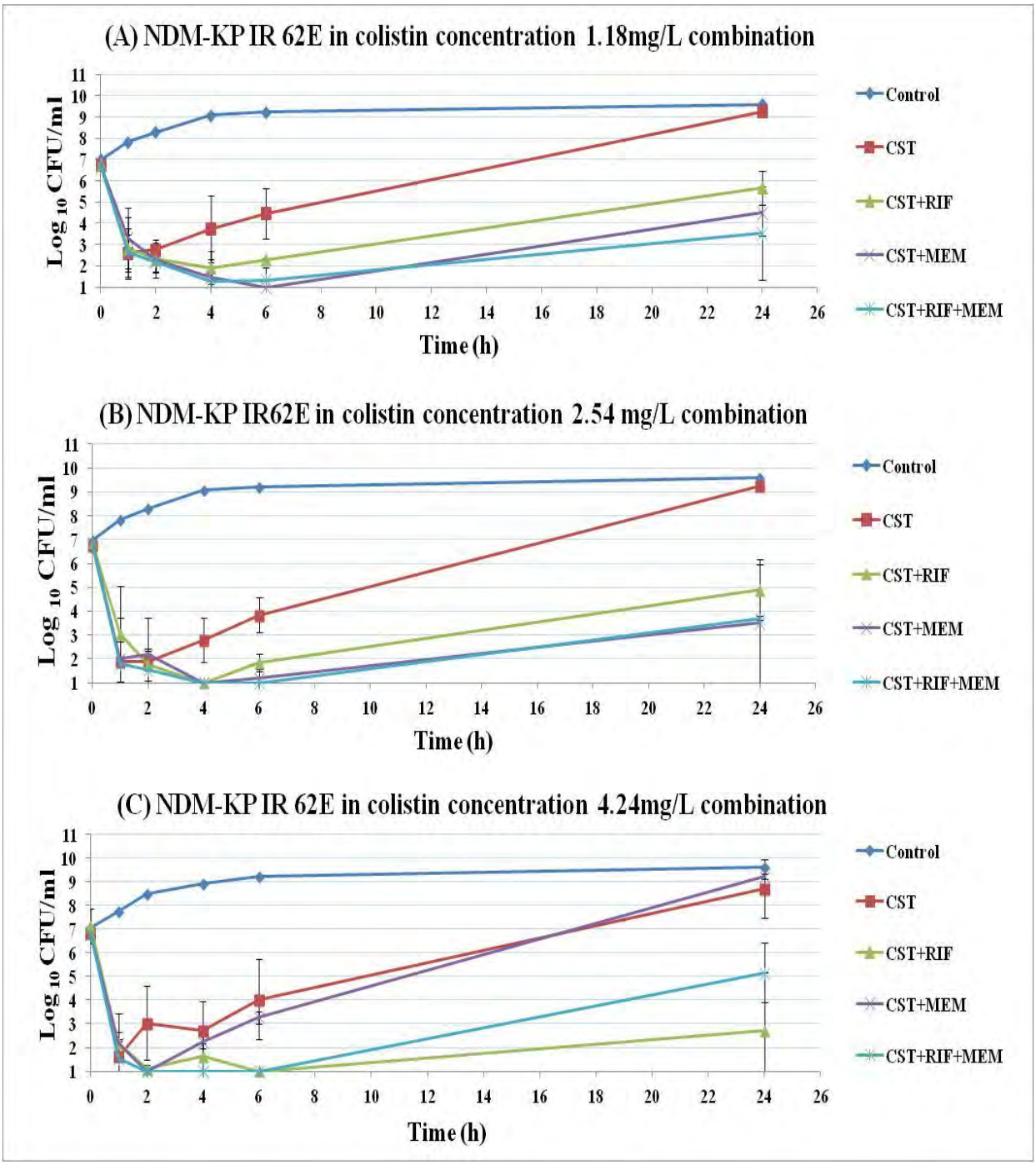

Figure 7. Results from static time-kill experiments for NDM-producing *K. pneumoniae* NDM-KP IR62E treating with four regimens containing colistin concentrations 1.18 mg/L(A), 2.54 mg/L(B) and 4.24 mg/L(C). Different amount of bacterial concentration of the samples in logarithmic values are presented along with time in hours. Different colors correspond to different regimens. Lower limit of detection for bacterial count is 1 $\log_{10}$ CFU/ml. All the results are described in mean values and included standard deviation (SD). CST- colistin, RIF- rifampicin, MEM- meropenem

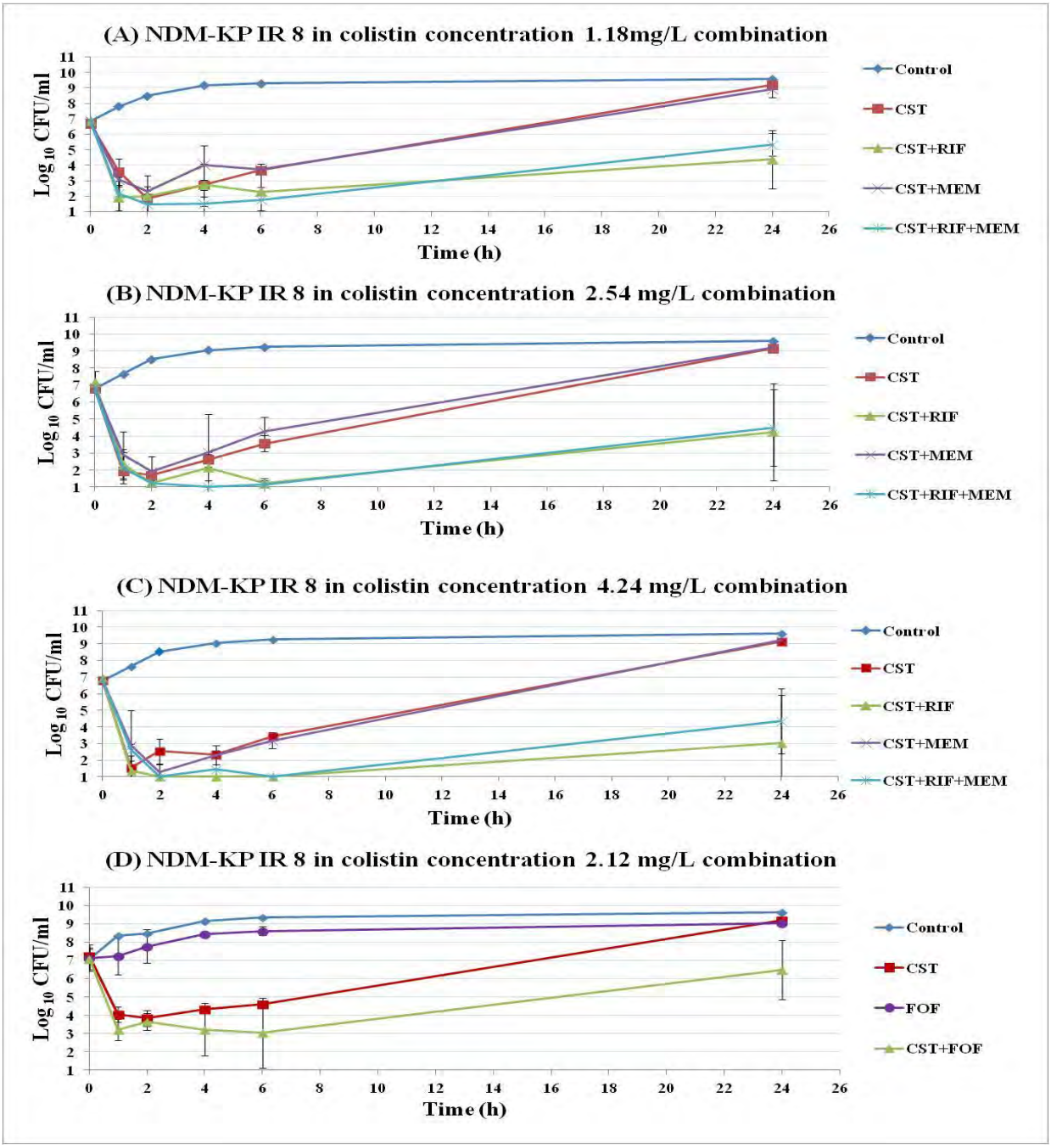

Figure 8. Results from static time-kill experiments for NDM-producing *K. pneumoniae* NDM-KP IR8 treating with different regimens containing colistin concentrations 1.18 mg/L (A), 2.54 mg/L (B), 4.24 mg/L (C) and 2.12 mg/L (D). Different amount of bacterial concentration of the samples in logarithmic values are presented along with time in hours. Different colors correspond to different regimens. Lower limit of detection for bacterial count is 1 $\log_{10}$ CFU/ml. All the results are described in mean values and included standard deviation (SD). CST- colistin, RIF- rifampicin, MEM- meropenem, FOF- fosfomycin

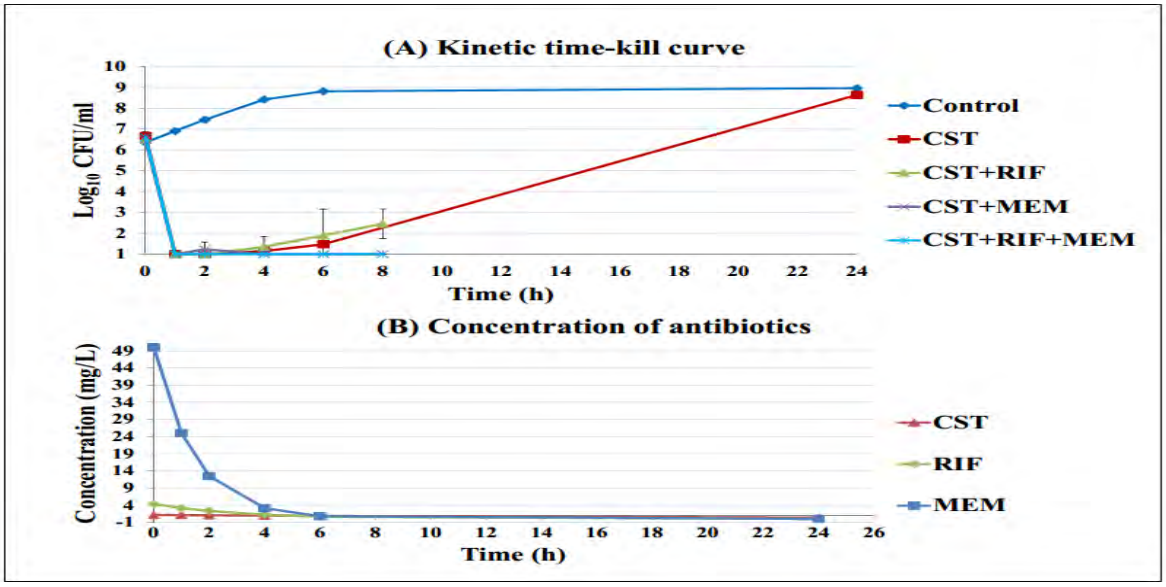

Figure 9. Results from kinetic time-kill experiments for NDM-producing *K.pneumoniae* NDM-KP IR 8 treating with colistin concentration 1.18 mg/L, rifampicin and meropenem. Different amount of bacterial concentration of the samples in logarithmic values are presented along with time in hours. Different colors correspond to different regimens. Lower limit of detection for bacterial count is 1 $\log_{10}$ CFU/ml. All the results are described in mean values and included standard deviation (SD) (A). Antibiotic concentrations were changed along the time in hours. Amount of the antibiotic concentrations were closed to zero along the time until 24 hours (B). CST- colistin, RIF- rifampicin, MEM- meropenem

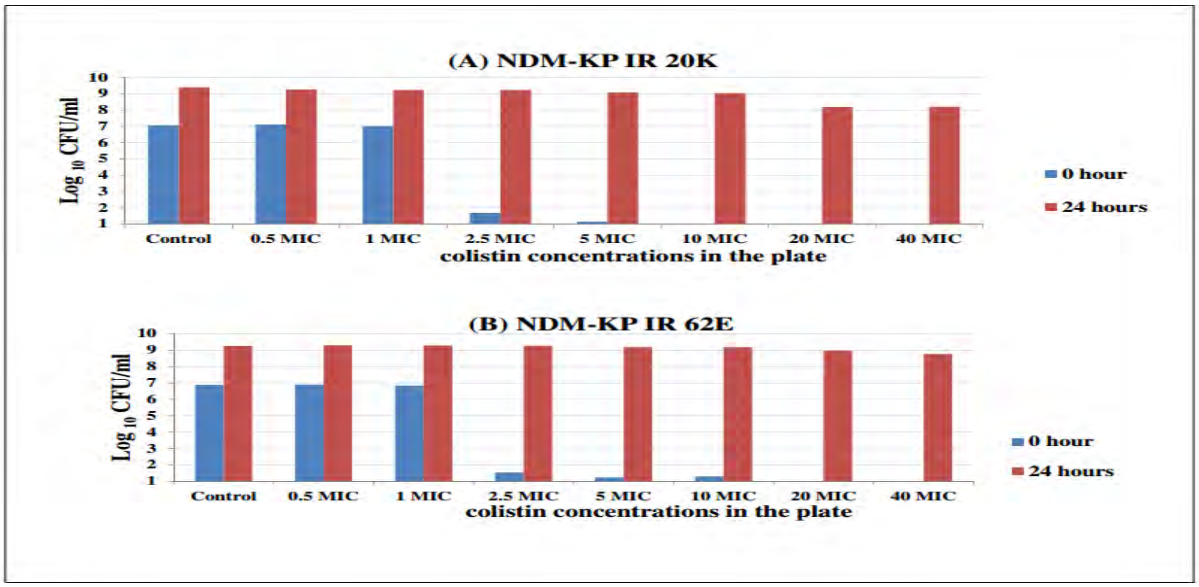

Figure 10. Results from static time-kill experiments for NDM-producing *K.pneumoniae* NDM-KP IR 20K (A) and IR62E (B) treating with colistin concentration 1.18 mg/L in the tube and different amounts of colistin in the agar. Different amount of bacterial concentration of the samples in logarithmic values are presented at different time points, 0 hour (Blue) and 24 hours (Red). All the results are described in mean value.

DISCUSSION

In this report, a total of 9 NDM-producing *K. pneumoniae* strains were used for static time-kill experiments with four different antibiotic regimens all of which included colistin concentration 1.18 mg/L in combination with rifampicin and/or meropenem. In addition, three of the strains (IR 20K, IR 8 and IR 62E) were tested with three different concentrations of colistin (1.18, 2.54, 4.24 mg/L) with the four different antibiotic regimens. IR 8 growth patterns were also investigated with colistin (2.12 mg/L) in combination with fosfomycin. Furthermore, kinetic time-kill experiments were performed with IR 8. Moreover, colistin resistance development in IR 20K and IR 62E were examined with colistin (1.18 mg/L) monotherapy and subsequent plating on 8 different sets of colistin concentration in the agar, as well, as part of a population analysis.

First, spiral and manual plating methods were compared using two VIM-KP strains (ÖN 2211 and T 14789) and one NDM-KP strain (IR 62E) with colistin + meropenem + fosfomycin in combination. We also compared both methods in the regular static time-kill experiments. The results of both methods were very similar and it appeared to be that the spiral plater can, in most cases, be relied on for the bacterial enumeration, as stated by Gilchrist *et al* in 1972[38]. However, carryover effects of antibiotics may interfere with the reliable acquisition of actual number of bacteria. We found a clear carryover effect in some of the first dilution samples of two strains (ÖN 2211 and IR 62E strains), strangely enough only on plates with a high amount of bacteria (even though the antibiotic concentration was kept the same). With such a clear carryover effect in some of the plates we did not dare to use the spiral plating method for the time-kill experiments.

Overall the results from the static time-kill experiments show that the double combination of colistin+ rifampicin regimen was the most effective one, with a bactericidal effect against 3 and a bacteriostatic effect against 6 out of 9 strains. Previous time-kill study also showed that this regimen was effective in carbapenemase-producing *K.pneumoniae* strains[67]. Moreover, Urban *et al* showed that the colistin + rifampicin combination was 25% effective among CR-KP strains in ¼ MIC concentration of antibiotics[68].

It is not surprising that K 6 showed the highest sensitivity to the colistin + rifampicin regimen due to its MIC of rifampicin 12 mg/L and colistin 0.125 mg/L which were among the lowest in all the strains. Although IR 15 also had second lowest rifampicin value of 16, the colistin + rifampicin combination was only bacteriostatic against this strain. The reason may be that IR 15

had a higher MIC of colistin (0.25) compared to K 6 although they were both sensitive to colistin. The colistin + rifampicin regimen seemed to be less effective to IR 20K in 2.54 mg/L colistin combination despite that a bactericidal effect was shown with 1.18 mg/L colistin treatment. This effect is possibly due to experimental error or variation of bacterial concentration in the start inoculum.

Bacteriostatic and bactericidal effects were seen against the meropenem-sensitive IR 20K using the combination of colistin+ meropenem. Comparing this combination effect between IR 20K (MIC_{MEM}= 0.75) and IR 8 (MIC_{MEM}= 4), the effect in IR 20K was more pronounced than in IR 8 due of MIC value of meropenem. Surprisingly this combination also had bacteriostatic effect against the meropenem resistant strain IR 62E (MIC_{MEM} > 32). The triple combination regimen was also bactericidal against the meropenem-sensitive IR 20K, and two meropenem-resistant strains, K1 and IR 62E, all of which are sensitive to colistin and with an MIC > 32 to rifampicin. It has previously been shown that mortality rates were reduced in patients suffering from carbapenemase-producing *K. pneumoniae* taking meropenem combination including colistin or aminoglycoside or tigecycline when meropenem MIC is < 4 mg/L[32]. A previous study in this lab, however, revealed no bactericidal or –static effects of a colistin + meropenem regimen against the two VIM-KP strains although for one of VIM-KP strains, the meropenem MIC was 2 [69]. The reason might be that the previous study and ours used different strains and different enzymes. Surprisingly, the colistin + meropenem combination was bactericidal against the meropenem-resistant IR 62E at lower concentrations (2.54 mg/L colistin) but not at the highest colistin concentration. This may be due to experimental errors or the strain itself.

Moreover, the overall bactericidal effect on different strains, meropenem-sensitive IR 20K was killed most by meropenem combination therapies which included colistin and, colistin + rifampicin. Colistin monotherapy showed no bactericidal or bacteriostatic effect in this report. Similarly, many studies show that colistin monotherapy is not as effective as combination therapy containing colistin[67,70,68,69]. Another issue is that the killing rate during the 1st hour did not correlate to any bactericidal or bacteriostatic effects after 24 hours for all strains (e.g. triple combinations against IR 19K and colistin+ meropenem combination against IR 15 and colistin+ rifampicin combination against IR 8 in 1.18 mg/L colistin). In a clinical situation however, where the immune system of the patient is also present, the outcome might be different.

The colistin and fosfomycin combination had a synergistic effect against the IR 8 strain if synergy was defined as ≥ 2 $\log_{10}$ reduction after 24 hours compared with one of the most effective individual drugs. Similarly, a previous study showed this combination had a synergistic

effect against some of NDM-1 *K. pneumoniae* strains[70,69], and the combination has been shown to have a bactericidal effect as well[69]. Surprisingly, when the results for IR 8 and IR 62E in last year's study[69] are compared with this report, the result (compared under 4.24 mg/L colistin combination) with the same strains, differences were found. Bactericidal effects of colistin + rifampicin combination were found only in IR 8 and IR 62E in this report, whereas, the former report found that the colistin + rifampicin + meropenem combination was effective on those two strains. Further similar investigations are needed in future.

A kinetic time-kill study, mimicking human pharmacokinetics of the drug was performed for NDM-KP IR 8 using a higher dose (Cmax concentrations) of antibiotics than in the static time-kill experiments. IR 8 was sensitive to colistin (MIC= 0.125 mg/L), intermediately sensitive to meropenem (MIC= 4mg/L), and resistant to rifampicin (MIC > 32 mg/L). Although it was not susceptible to meropenem and rifampicin, bacteria were killed completely within 1 hour in all regimens. Apart from some variation, colistin monotherapy behaved very similar to the trend in the static time-kill study. This was expected since the colistin concentration was constant in this experiment. After 24 hours, bacterial regrowth reached nearly 9 $\log_{10}$ CFU/ml. As in the static time-kill experiments, colistin had no bactericidal or bacteriostatic effects. The colistin + rifampicin combination showed a regrowth after 2 hours until 8 hours which was similar to the static time-kill experiments. The triple combination and the combination of colistin + rifampicin efficiently reduced the bacteria down to the detection limit until 8 hours. The combination experiments in the kinetic model were only run for 8 hours, however, we expect that they will grow back some points after 8 hours. Additional experiments should be done, looking at a longer time-period and including repeated dosing of antibiotics as they would be dosed in patients.

0 and 24 hour samples from the static time-kill experiment of NDM-KP IR 20K and IR 62E, treated with colistin 1.18 mg/L were plated on agar plates containing 8 different amounts of colistin in the plate. Before adding colistin to the samples, at 0 hour, we examined which amount of colistin concentration inhibits bacterial growth on the plates. Both strains were unable to grow on the plates with colistin concentrations at around 1-2.5x MIC of the respective strains. After 24 hours treatment of the sample with colistin, regrowth occurred up to approximately 9 $\log_{10}$ CFU/ml. Plating showed that after a 24 hour exposure to colistin most of the bacteria in the sample could grow on plates containing even the highest MIC colistin. So, colistin monotherapy is not effective for clinical therapy due to rapid selection of colistin resistant subpopulations. For full population analyses further studies must be done, including other antibiotic regimens to evaluate whether or not combination therapy may prevent the emergence of resistance.

All in all, double and triple combinations of colistin, rifampicin and meropenem were more effective than colistin monotherapy as seen in the static as well as kinetic time-kill experiments. This report is expected to give information about possible combination therapies against NDM-producing *Klebsiella pneumonia* that might be used in clinical practice in the future.

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REFERENCES

1.Paterson, D. L. and Bonomo, R. A. Extended-Spectrum ?-Lactamases: a Clinical Update. *Clin. Microbiol. Rev.* **18,** 657–686 (2005).

2.Kumarasamy, K. K. *et al.* Emergence of a new antibiotic resistance mechanism in India, Pakistan, and the UK: a molecular, biological, and epidemiological study. *Lancet Infect. Dis.* **10,** 597–602 (2010).

3.Rice, L. and Bonomo, R. A. Murray, P.R., Jorgenson, J.H., Pfaller, M.A., et al (Ed) In: Manual of Clinical Microbiology **1,** 1114–1145 (2007).

4.Kumarasamy, K., Toleman, M. A. and Walsh, T. R. Emergence of a new antibiotic resistance mechanism in India, Pakistan and the UK: a molecular, biological and epidemiological study. *Lancet Infect. Dis.* **10,** 597–602 (2010).

5.Falagas, M. E. and Karageorgopoulos, D. E. Extended-spectrum beta-lactamase-producing organisms. *J. Hosp. Infect.* **73,** 345–354 (2009).

6.Ledent, P., Raquet, X., Joris, B., Van Beeumen, J. and Frere, J. M. A comparative study of class-D beta-lactamases. *Biochem. J.* **292,** 555–562 (1993).

7.Nordmann, P., Cuzon, G. and Naas, T. The real threat of *Klebsiella pneumoniae* carbapenemase-producing bacteria. *Lancet Infect. Dis.* **9,** 228–236 (2009).

8.Yigit, H. *et al.* Carbapenem-Resistant Strain of *Klebsiella oxytoca* Harboring Carbapenem-Hydrolyzing ?-Lactamase KPC-2. *Antimicrob. Agents Chemother.* **47,** 3881–3889 (2003).

9.Cagnacci, S. *et al.* Bloodstream infections caused by multidrug-resistant *Klebsiella pneumoniae* producing the carbapenem-hydrolysing VIM-1 metallo-beta-lactamase: first Italian outbreak. *J. Antimicrob. Chemother.* **61,** 296–300 (2008).

10. Giakkoupi, P. *et al.* VIM-1 Metallo-?-Lactamase-Producing *Klebsiella pneumoniae* Strains in Greek Hospitals. *J. Clin. Microbiol.* **41,** 3893–3896 (2003).

11. Yong, D. *et al.* Characterization of a New Metallo-β-Lactamase Gene, blaNDM-1, and a Novel Erythromycin Esterase Gene Carried on a Unique Genetic Structure in *Klebsiella pneumoniae* Sequence Type 14 from India. *Antimicrob. Agents Chemother.* **53,** 5046–5054 (2009).

12. Lytsy, B. *et al.* The first major extended-spectrum β-lactamase outbreak in Scandinavia was caused by clonal spread of a multiresistant *Klebsiella pneumoniae* producing CTX-M-15. *APMIS* **116,** 302–8 (2008).

13. Hawser, S. P. *et al.* Emergence of High Levels of Extended-Spectrum-β-Lactamase-Producing Gram-Negative Bacilli in the Asia-Pacific Region: Data from the Study for Monitoring Antimicrobial Resistance Trends (SMART) Program, 2007. *Antimicrob. Agents Chemother.* **53,** 3280–3284 (2009).

14. Sanchez, G. V. *et al. Klebsiella pneumoniae* Antimicrobial Drug Resistance, United States, 1998–2010. *Emerg. Infect. Dis.* **19,** 133–136 (2013).

15. Samuelsen, Ø. *et al.* Emergence of clonally related *Klebsiella pneumoniae* isolates of sequence type 258 producing plasmid-mediated KPC carbapenemase in Norway and Sweden. *J. Antimicrob. Chemother.* **63,** 654–658 (2009).

16.Magiorakos, A.-P. *et al.* The rise of carbapenem resistance in Europe: just the tip of the iceberg? *Antimicrob. Resist. Infect. Control* **2,** 6 (2013).

17. Leavitt, A., Navon-Venezia, S., Chmelnitsky, I., Schwaber, M. J. and Carmeli, Y. Emergence of KPC-2 and KPC-3 in Carbapenem-Resistant *Klebsiella pneumoniae* Strains in an Israeli Hospital. *Antimicrob. Agents Chemother.* **51,** 3026–3029 (2007).

18. Hawkey, P. M. and Livermore, D. M. Carbapenem antibiotics for serious infections. *BMJ* **344,** (2012).

19. Tenover, F. C. *et al.* Carbapenem Resistance in *Klebsiella pneumoniae* Not Detected by Automated Susceptibility Testing. *Emerg. Infect. Dis.* **12,** 1209–1213 (2006).

20. Livermore, D. M. and Williams, J. D. β-lactams: mode of action and mechanisms of bacterial resistance. In: Lorian Victor (Ed) Antibiotics in Laboratory Medicine. 4th Ed. 498–578. Williams and Wilkins, Maryland, USA (1996).

21. Castañeda-García, A., Blázquez, J. and Rodríguez-Rojas, A. Molecular Mechanisms and Clinical Impact of Acquired and Intrinsic Fosfomycin Resistance. *Antibiotics* **2,** 217–236 (2013).

22. Shrestha, K. and Tomford, J. fosfomycin: a review. *Infectious Diseases in Clinical Practice* **10,** 255–260 (2001).

23. Burman, D. W. J., Gallicano, K. and Peloquin, C. Comparative Pharmacokinetics and Pharmacodynamics of the Rifamycin Antibacterials. *Clin. Pharmacokinet.* **40,** 327–341 (2001).

24. Wehrli, W. and Staehelin, M. Actions of the rifamycins. *Bacteriol. Rev.* **35,** 290–309 (1971).

25. Barnett, M., Bushby, S. R. M. and Wilkinson, S. Sodium Sulphomethyl Derivatives of Polymyxins. *Br. J. Pharmacol. Chemother.* **23,** 552–574 (1964).

26. Plachouras, D. *et al.* Population pharmacokinetic analysis of colistin methanesulfonate and colistin after intravenous administration in critically ill patients with infections caused by gram-negative bacteria. *Antimicrob. Agents Chemother.* **53,** 3430–3436 (2009).

27. Garonzik, S. M. *et al.* Population Pharmacokinetics of Colistin Methanesulfonate and Formed Colistin in Critically Ill Patients from a Multicenter Study Provide Dosing Suggestions for Various Categories of Patients. *Antimicrob. Agents Chemother.* **55,** 3284–3294 (2011).

28. Chen, S. *et al.* Independent Emergence of Colistin-Resistant Enterobacteriaceae Clinical Isolates without Colistin Treatment. *J. Clin. Microbiol.* **49,** 4022–4023 (2011).

29. Kontopidou, F. *et al.* Colonization and infection by colistin-resistant Gram-negative bacteria in a cohort of critically ill patients. *Clin. Microbiol. Infect. Off. Publ. Eur. Soc. Clin. Microbiol. Infect. Dis.* **17,** E9–E11 (2011).

30. Paul, M. *et al.* Effectiveness and safety of colistin: prospective comparative cohort study. *J. Antimicrob. Chemother.* **65,** 1019–1027 (2010).

31. Qureshi, Z. A. *et al.* Treatment outcome of bacteremia due to KPC-producing *Klebsiella pneumoniae*: superiority of combination antimicrobial regimens. *Antimicrob. Agents Chemother.* **56,** 2108–2113 (2012).

32. Daikos, G. L. & Markogiannakis, A. Carbapenemase-producing *Klebsiella pneumoniae*: (when) might we still consider treating with carbapenems? *Clin. Microbiol. Infect. Off. Publ. Eur. Soc. Clin. Microbiol. Infect. Dis.* **17,** 1135–1141 (2011).

33. Hirsch, E. B. & Tam, V. H. Detection and treatment options for *Klebsiella pneumoniae* carbapenemases (KPCs): an emerging cause of multidrug-resistant infection. *J. Antimicrob. Chemother.* **65,** 1119–1125 (2010).

34. Tascini, C. *et al.* Synergistic activity of colistin plus rifampin against colistin-resistant KPC-producing *Klebsiella pneumoniae*. *Antimicrob. Agents Chemother.* **57,** 3990–3993 (2013).

35. Song, J. Y. *et al. In vitro* activities of carbapenem/sulbactam combination, colistin, colistin/rifampicin combination and tigecycline against carbapenem-resistant *Acinetobacter baumannii*. *J. Antimicrob. Chemother.* **60,** 317–322 (2007).

36. Miyakis, S., Pefanis, A. & Tsakris, A. The challenges of antimicrobial drug resistance in Greece. *Clin. Infect. Dis. Off. Publ. Infect. Dis. Soc. Am.* **53,** 177–184 (2011).

37. Rosenblatt, J,E. Lorian Victor (Ed) Susceptibility testing of antimicrobials in liquid media. In: Antibiotics in Laboratory Medicine. 4th Ed. 112-126. Williams & Wilkins, Maryland, USA (1996).

38. Gilchrist, J. E., Campbell, J. E., Donnelly, C. B., Peeler, J. T. & Delaney, J. M. Spiral Plate Method for Bacterial Determination. *Appl. Microbiol.* **25,** 244–252 (1973).

39. Lounis, N. *et al.* Prevention of Drug Carryover Effects in Studies Assessing Antimycobacterial Efficacy of TMC207. *J. Clin. Microbiol.* **46,** 2212–2215 (2008).

40. Dankert, J., Holloway, Y., Joldersma, W. & Hess, J. Importance of minimizing carry-over effect at subculture in the detection of penicillin-tolerant viridans group streptococci. *Antimicrob. Agents Chemother.* **23,** 614–616 (1983).

41. Mueller Hinton II Broth Brochure. Becton, Dickinson and Company (2011).

42. Mueller Hinton II Agar Brochure. Becton, Dickinson and Company (2006).

43. Aztreonam Product Information. Sigma-Aldrich, Inc. (2003).

44. NCBI-PubChem compound databases. Colistin compound summary - PubChem. (2013).

45. Phosphomycin disodium salt-Material safety data sheet. *Sigma-Aldrich, Inc.* (2012).

46. Rifampicin Product Information. Sigma-Aldrich, Inc. (1997).

47. Merrem IV-Meropenem-Product Information. AstraZeneca Pty Ltd. (2010).

48. Sodium chloride Product Information. Merck KGaA company (2013).

49. Dimethyl sulfoxide- Material safety data sheet. Sigma-Aldrich, Inc. (2013).

50. Karvanen, M., Malmberg, C. Mohamed, A. Lagerbäck, P. Friberg, L. and Cars, O. Colistin is extensively lost during normal experimental conditions. Abstract D-690 In: program and

abstract of the 51th interscience conference on antimicrobial agents and chemotherapy (sept 17-20). Chicago, IL: American Society for Microbiology. (2011).

51. Easy Spiral Pro Automatic Spiral Plater-Installation and user guide-version 1.5. Interscience

52. Acar JF, Goldstein FW. Disk Susceptibility Test. In: Lorian V (eds), Antibiotic in Laboratory Medicine, 4th ed, ch-1: 18-27 Williams & Wilkins, Maryland, USA (1996).

53. Meyers, B. R. *et al.* Pharmacokinetics of Aztreonam in Healthy Elderly and Young Adult Volunteers. *J. Clin. Pharmacol.* **33,** 470–474 (1993).

54. Burgess, D. S., Summers, K. K. & Hardin, T. C. Pharmacokinetics and pharmacodynamics of aztreonam administered by continuous intravenous infusion. *Clin. Ther.* **21,** 1882–1889 (1999).

55. Michalopoulos, A. S. & Falagas, M. E. Colistin: recent data on pharmacodynamics properties and clinical efficacy in critically ill patients. *Ann. Intensive Care* **1,** 1–6 (2011).

56. Markou, N. *et al.* Colistin serum concentrations after intravenous administration in critically ill patients with serious multidrug-resistant, gram-negative bacilli infections: A prospective, open-label, uncontrolled study. *Clin. Ther.* **30,** 143–151 (2008).

57. Kelly, H. C., Hutchison, M. & Haworth, S. J. A comparison of the pharmacokinetics of meropenem after administration by intravenous injection over 5 min and intravenous infusion over 30 min. *J. Antimicrob. Chemother.* **36,** 35–41 (1995).

58. Matzi, V. *et al.* Extracellular concentrations of fosfomycin in lung tissue of septic patients. *J. Antimicrob. Chemother.* **65,** 995–998 (2010).

59. Roussos, N., Karageorgopoulos, D. E., Samonis, G. & Falagas, M. E. Clinical significance of the pharmacokinetic and pharmacodynamic characteristics of fosfomycin for the treatment of patients with systemic infections. *Int. J. Antimicrob. Agents* **34,** 506–515 (2009).

60. Rifampicin package insert. Bredford company. (2003).

61.Conte Jr, J. E., Golden, J. A., Kelley, M. G. & Zurlinden, E. Intrapulmonary pharmacokinetics and pharmacodynamics of meropenem. *Int. J. Antimicrob. Agents* **26,** 449–456 (2005).

62. Meropenem products information. WWW document: www.fass.se. Date visited on 1st, March, 2013.

63. Ruslami, R. *et al.* Pharmacokinetics and Tolerability of a Higher Rifampin Dose versus the Standard Dose in Pulmonary Tuberculosis Patients. *Antimicrob. Agents Chemother.* **51,** 2546–2551 (2007).

64. 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.* **40,** 2478–2482 (1996).

65. Gustafsson, I., Löwdin, E., Odenholt, I. & Cars, O. Pharmacokinetic and Pharmacodynamic Parameters for Antimicrobial Effects of Cefotaxime and Amoxicillin in an *in vitro* Kinetic Model. *Antimicrob. Agents Chemother.* **45,** 2436–2440 (2001).

66. Lignell, A., Johansson, A., Löwdin, E., Cars, O. & Sjölin, J. A new *in vitro* kinetic model to study the pharmacodynamics of antifungal agents: inhibition of the fungicidal activity of amphotericin B against *Candida albicans* by voriconazole. *Clin. Microbiol. Infect.* **13,** 613–619 (2007).

67. Bratu, S. *et al.* Carbapenemase-producing *Klebsiella pneumoniae* in Brooklyn, NY: molecular epidemiology and *in vitro* activity of polymyxin B and other agents. *J. Antimicrob. Chemother.* **56,** 128–132 (2005).

68. Urban, C., Mariano, N. & Rahal, J. J. *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.* **54,** 2732–2734 (2010).

69. Tangden, T. Evaluation of double and triple antibiotic combinations for VIM- and NDM-producing *Klebsiella pneumoniae* by *in vitro* time-kill experiments. Ph.D thesis paper IV (2012).

70. Berçot, B., Poirel, L., Dortet, L. & Nordmann, P. *In vitro* evaluation of antibiotic synergy for NDM-1-producing Enterobacteriaceae. *J. Antimicrob. Chemother.* **66,** 2295–2297 (2011).

APPENDIX

1. Culture Media

BD BBL ™ Mueller Hinton II Broth (Cation-Adjusted)[41]

Reagents per liter purified water-

3 g of Beef extract, 17.5 g of Acid Hydrolysate of Casein and 1.5 g of Starch

It is already adjusted required 20-25 mg/L of calcium and 10-12.5 mg/L of magnesium salts according to CLSI standard.

BD BBL TM Mueller Hinton II Agar (Cation-Adjusted)[42]

Reagents per liter purified water-

2 g of Beef extract, 17.5 g of Acid Hydrolysate of Casein, 1.5 g of Starch and 17 g of Agar

It is already adjusted required 20-25 mg/L of calcium and 10-12.5 mg/L of magnesium salts according to CLSI standard.

2. Summary of E test Interpretive Criteria according to EUCAST (European Committee on Antimicrobial Susceptibility Testing) Clinical breakpoint version 3.1 (www.eucast.org)

Antibiotic	Interpretive Criteria	
	Sensitive (S≤)	Resistance (R>)
Aztreonam	1	4
Ciprofloxacin	0.5	1
Colistin	2	2
Fosfomycin	32	32
Meropenem	2	8
Rifampicin	-	-
Tigecycline	1	2

Intermediate category is not listed in this table. It is interpreted as the values between the S and the R breakpoints. '-' indicates that susceptibility testing is not recommended as the species is a poor target with the drug. Isolates may be reported as R without prior testing.

3. Easy Spiral Pro Automatic Spiral Plater

Figure. Easy spiral pro automatic spiral plater (Interscience, Bois Arpents, France)

4. Volume constants in exponential mode by pairs of sectors Easy Spiral plater

Pairs of sectors	90 mm Petri dish exponential mode 50 µl plated
1	1
2	2.58
3	5.07
4	9
5	15.21
6	25
Complete Petri dish	50

5. Kinetic time-kill model

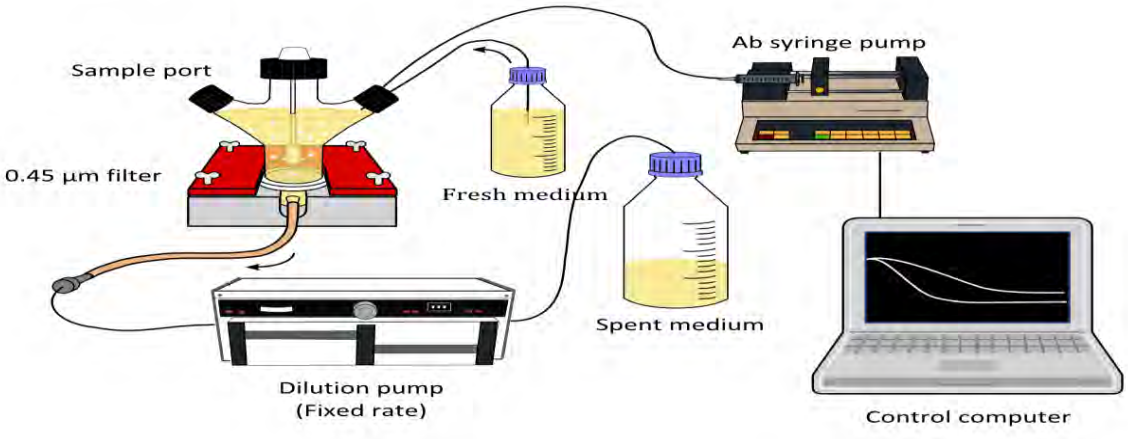

Figure B. The in vitro kinetic model with syringe pump with two different pharmacokinetic profiles (Image credit to Christer Malmberg)

Ab – Antibiotic