Detection of synergistic activity of antibiotics in *Klebsiella pneumoniae* using MALDI-TOF MS

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ABSTRACT
Antibiotic resistance, and the increased incidence of multidrug-resistant bacteria, has become a major healthcare problem worldwide. Carbapenemase-producing *Klebsiella pneumoniae* can cause severe nosocomial infections that require combination therapy to increase the chances of effective treatment. So far no method has been developed to assess the effect of such antibiotic combinations for every-day use. A new method for rapid susceptibility testing is the MALDI Biotyper Antibiotic Susceptibility Test Rapid Assay (MBT-ASTRA), which uses specific software to make a quantitative comparison of MALDI-TOF MS (matrix-assisted laser desorption/ionization time-of-flight mass spectrometry) spectra of bacteria grown in the presence and absence of antibiotics. In this proof-of-concept study the visualization of susceptibility and synergy was performed using the MBT-ASTRA for different strains of *Klebsiella pneumoniae* with the antibiotics meropenem, colistin and rifampin. The results of this study showed that the susceptibility of different antibiotics can be visualized already after 1 hour of incubation. When combining these three antibiotics the synergistic effect previously shown using laborious time-kill experiments could be detected for the investigated strains. The MBT-ASTRA is thus a rapid method for visualizing antibiotic susceptibility and seems to have potential also for synergy testing.

*Keywords:* Klebsiella pneumoniae; Antibiotic resistance; MALDI-TOF MS; Carbapenem; Synergy
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<table>
<thead>
<tr>
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<th>Description</th>
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<tbody>
<tr>
<td>ATCC</td>
<td>American Type Culture Collection</td>
</tr>
<tr>
<td>AUC</td>
<td>Area under curve</td>
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<td>CCUG</td>
<td>Culture Collection University of Gothenburg</td>
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<tr>
<td>CST</td>
<td>Colistin</td>
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<tr>
<td>CTX-M</td>
<td>Cefotaximinase-Munich</td>
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<tr>
<td>ESBL</td>
<td>Extended spectrum β-lactamase</td>
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<tr>
<td>EUCAST</td>
<td>European Committee of Antimicrobial Susceptibility Testing</td>
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<tr>
<td>IMP</td>
<td>Imipenemase metallo-β-lactamase</td>
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<tr>
<td>KP</td>
<td><em>Klebsiella pneumoniae</em></td>
</tr>
<tr>
<td>KPC</td>
<td><em>Klebsiella pneumoniae</em> carbapenemase</td>
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<tr>
<td>MALDI-TOF MS</td>
<td>Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry</td>
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<td>MBT-ASTRA</td>
<td>MALDI Biotyper Antibiotic Susceptibility Test Rapid Assay</td>
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<td>MEM</td>
<td>Meropenem</td>
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<tr>
<td>MIC</td>
<td>Minimum inhibitory concentration</td>
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<tr>
<td>NDM</td>
<td>New Delhi metallo-β-lactamase</td>
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<td>OXA</td>
<td>Oxacillinase β-lactamase</td>
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<td>PCR</td>
<td>Polymerase chain reaction</td>
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<td>RG</td>
<td>Relative growth</td>
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<td>RIF</td>
<td>Rifampin</td>
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<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
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<tr>
<td>SHV</td>
<td>Sulphydryl variable</td>
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<tr>
<td>SIR</td>
<td>Sensitive, intermediate, resistant</td>
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<tr>
<td>UK NEQAS</td>
<td>United Kingdom National External Quality Assessment Service</td>
</tr>
<tr>
<td>VIM</td>
<td>Verona integron-encoded metallo-β-lactamase</td>
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INTRODUCTION

Antibiotic resistance

Antibiotic resistance, and the increased incidence of multidrug-resistant bacteria, has become a major healthcare problem worldwide [1]. Antibiotic multiresistance in Gram-negative rods belonging to Enterobacteriaceae is conferred primarily by extended spectrum β-lactamases (ESBLs), enzymes that hydrolyze the β-lactam ring of β-lactam antibiotics. The β-lactam family comprises penicillins, cephalosporins, monobactams and carbapenems, which exert their bactericidal effect by inhibiting cell wall synthesis. The broad-spectrum antibiotics belonging to the carbapenems is a group of antibiotics useful in the treatment of multiresistant Enterobacteriaceae, due to its stability to β-lactamase hydrolysis [2]. Unfortunately, the emergence of carbapenem-resistant Enterobacteriaceae is now becoming an increasingly serious public health problem [3]. Resistance to carbapenems can be acquired through mechanisms such as drug efflux, loss of porins, and carbapenemase-production [2], the latter of which is predominantly caused by the serine-carbapenemases Klebsiella pneumoniae carbapenemase (KPC) and oxacillinase β-lactamase (OXA), or the metallo-β-lactamases Verona integron-encoded metallo-β-lactamase (VIM), New Delhi metallo-β-lactamase (NDM) or imipenemase metallo-β-lactamase (IMP) [4].

Klebsiella pneumoniae is an encapsulated opportunistic pathogen responsible for urinary tract infections and severe nosocomial infections such as pneumonia and sepsis [2]. Carbapenemase-producing K. pneumoniae are usually extensively drug resistant due to the occurrence of many resistance genes on the same mobile genetic elements [5]. Severe infections with multiresistant carbapenemase-producing K. pneumoniae require combination therapy to enhance the antibacterial effects and increase the chances of effective treatment [6]. Some carbapenemase-producing isolates (mainly OXA) produce only discrete levels of carbapenemases and therefore cannot be reliably detected by the clinical cut-off values. This poses a problem from an infection control point-of-view, since it enables carbapenemase-producing strains to escape early detection and continue their dissemination [5].

Antibiotic combinations and methods of synergy testing

The effect of antibiotic combinations is usually evaluated in vitro by methods such as gradient tests, the checkerboard method or by time-kill experiments. When using gradient tests the test strips can be applied in a 90° cross formation on the same medium to show synergistic effects.
when two antibiotics are combined, while the checkerboard method uses broth microdilution plates for combining different serially diluted antibiotics. For time-kill experiments samples of antibiotics alone and in different combinations are obtained at different time-points, plated and colony counts determined [7]. Synergy means that there is an enhanced bactericidal effect with the combination of antibiotics compared to the effects of the individual antibiotics after 24 h, e.g. a >2-log_{10} decrease in bacteria when using time-kill experiments [6], or by a fractional inhibitory concentration of <0.5. [7]. A few limitations for these methods of synergistic testing are that they have a limited number of antibiotics that can be tested (gradient tests), can be difficult to interpret and time-consuming, and do not always show good agreement [7].

Colistin, a polymyxin antibiotic and a last treatment option for severe infections with multidrug-resistant *Klebsiella* spp., is used in many combinations due to its ability to increase the permeability of the bacterial cell membrane to other antibiotics [2]. Combining colistin with other antibiotics using the checkerboard method has shown evidence of synergy against colistin-resistant KPC-producing strains of *K. pneumoniae*, with colistin plus rifampin being the most consistently synergistic combination [4]. *In vitro* time-kill experiments evaluating several two and three drug combinations have demonstrated synergy against carbapenemase-producing *K. pneumoniae*. Tängdén *et al.* found the combination of rifampin with meropenem and colistin to be the most effective regimen against both VIM- and NDM-producing strains *in vitro*, despite the fact that the bacteria were resistant to some of the antibiotics when tested individually [6]. Combining meropenem with colistin and rifampin enhances the bactericidal effect by using different mechanisms of action: meropenem inhibits peptidoglycan synthesis by binding to penicillin-binding proteins while rifampin prevents extension of RNA by binding to RNA-polymerase, and colistin interferes with the cytoplasmic membrane by acting as a detergent. This combination enhances the antibacterial activity for Enterobacteriaceae despite their intrinsic resistance to rifampin [2].

**Conventional susceptibility testing and detection of resistance mechanisms**

Antibiotic susceptibility testing is often performed using phenotypic tests, such as disc diffusion or gradient tests (e.g. Etest), which require bacteria to grow overnight [8]. Phenotypic tests for detecting resistance mechanisms are also available, such as the double-disc synergy test which can show ESBL_{A}-production (e.g. SHV-12 [sulfhydryl variable], CTX-M-15 [ceftaximinase-Munich]) [9]. The requirement for culturing bacteria limits the
speed to obtain results and more rapid methods are therefore continuously being developed. Bypassing the culturing of bacteria by using molecular biology is one way to increase the speed, e.g. by using real-time quantitative PCR (qPCR). Such methods are faster but only show genetic information, i.e. if resistance genes are present or not, which may not directly mirror the gene expression [8]. Simple methods are available for the detection of some resistance mechanisms, e.g. the detection of β-lactamase production by the Nitrocefin test, but these methods are few and limited to specific mechanisms and species [10].

MALDI-TOF MS principle
Finding new rapid methods for the identification of resistant bacteria is important to enable appropriate antimicrobial treatment. Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) is a widely used method implemented in clinical microbiology for the identification of microorganisms, such as bacteria. MALDI-TOF MS is a technique that uses laser excitation to ionize the sample material (figure 1), for example functional groups in bacterial ribosomal proteins. Bacteria are first applied onto a MALDI-plate and then overlaid with a chemical matrix. As the laser irradiates the sample the matrix absorbs the energy and transfers heat to the proteins, which results in desorption and ionization of the sample proteins. The ions are then separated in a flight tube according to their mass-to-charge ratio, where lighter ions will travel faster than larger ions. As the ions reach the detector a mass spectrum is created using the mass-to-charge ratio and signal intensity [2].

Figure 1. The principle of MALDI-TOF MS where a sample is ionized and the ions are separated according to their mass-to-charge ratio.

(http://www.mayomedicallaboratories.com/articles/communique/2013/01-maldi-tof-mass-spectrometry/)
Susceptibility testing using MALDI-TOF MS

Recently, it has been shown that MALDI-TOF MS can be used for the detection and characterization of β-lactamases, and during the last year a concept for antibiotic susceptibility testing has been described. The first methods detecting the activity of β-lactamases [11, 12] have also been shown to be able to detect β-lactam resistance directly from positive blood cultures [13, 14]. The primary limitation of this method is that only resistance due to the hydrolysis of β-lactam antibiotics (i.e. resistance caused by β-lactamases) can be detected, and thus another method for a more general susceptibility testing has been developed. This method uses heavy amino acids that are incorporated only by metabolically active bacteria [15, 16], but requires a specific culture medium without the amino acid that is used as a marker. Quite recently, a second and improved approach called MBT-ASTRA (MALDI Biotyper Antibiotic Susceptibility Test Rapid Assay) was presented that uses quantitative MALDI-TOF MS for detecting antibiotic resistance in a broth dilution system with no requirement for very specific culture medium [17].

In this study, the MBT-ASTRA assay was utilized to investigate the antibiotic susceptibility of different strains of *Klebsiella pneumoniae*. The aim was to visualize (i) differences in MALDI-TOF MS spectra between sensitive and resistant strains and (ii) to see if synergistic activity of different combinations of antibiotics could be detected using this method.

**METHODS AND MATERIALS**

**Bacterial strains**

Clinical isolates of carbapenemase-producing *Klebsiella pneumoniae* strains were kindly provided by Thomas Tängdén, Uppsala University. Originally these were obtained from the Department of Clinical Microbiology, Akademiska University Hospital, Uppsala, Sweden. Two VIM-1-producing *K. pneumoniae* (VIM-KP) strains, T14789 and ÖN-2211, and two NDM-1-producing *K. pneumoniae* (NDM-KP) strains, IR8 and IR62E, were used. The VIM-producing isolates originated from patients transferred from Greek hospitals but were isolated in Scandinavia, while the NDM-producing isolates derived from Chennai, India. All strains have been previously described [18, 19]. *K. pneumoniae* control strains were obtained from the clinical microbiology laboratory at Örebro University Hospital. The isolate ATCC 25955 (American Type Culture Collection) represented a sensitive strain, CCUG 54718 (Culture Collection University of Gothenburg) represented an ESBL-producing strain (producing...
CTX-M-15), while the isolate called PS20 (from United Kingdom National External Quality Assessment Service [UK NEQAS]) produced both KPC and an ESBL (SHV-12). All isolates were confirmed to species level by MALDI-TOF MS, with scores above 2.0 being interpreted as confirmed species identification.

**Susceptibility testing**

Antibiotic susceptibility testing was performed to confirm the expected resistance patterns of the included isolates. Testing was performed using disc diffusion (Oxoid, Basingstoke, UK), according to recommendations by the European Committee for Antimicrobial Susceptibility Testing (EUCAST), and the Etest (BioMérieux, Marcy l’Etoile, France) according to the manufacturer’s instructions. For meropenem the M.I.C.Evaluator (Oxoid, Basingstoke, UK) was used. An inoculum equal to a 0.5 McFarland turbidity standard was prepared for each isolate and the suspension was inoculated on Mueller-Hinton II agar (BBL; BD, Le Point de Claix, France) by evenly distributing the suspension with a swab. The antibiotic discs and Etest strips were applied and plates were incubated at 35°C for 16-18 h under aerobic conditions. Disc diffusion was performed with the antibiotic discs piperacillin-tazobactam (30 µg), ceftazidime (10 µg), cefotaxime (5 µg), meropenem (10 µg), imipenem (10 µg), ciprofloxacin (5 µg), amikacin (30 µg), gentamicin (10 µg) and trimetoprim-sulfamethoxazole (25 µg), while the gradient test was performed with meropenem, colistin and rifampin. The zones of inhibition were measured for disc diffusion using a slide gauge, and SIR-classification (sensitive, intermediate, resistant) was performed using the clinical breakpoints provided by EUCAST [20]. The minimum inhibitory concentration (MIC) was interpreted as the intersection point between the Etest strip and the inhibition zone according to the manufacturer’s instructions.

The synergy of cephalosporins in combination with the β-lactamase inhibitor clavulanate was also investigated using the double-disc synergy test. Suspensions were prepared by transferring a cotton swab from a 0.5 McFarland solution to 1 ml 0.9% NaCl. A new swab was dipped into this inoculum and spread evenly on Mueller-Hinton II agar. The cephalosporin discs cefepime (30 µg), cefotaxime (5 µg) and ceftazidime (10 µg) were then applied at a defined distance (25 mm and 20 mm) around a central disc of amoxicillin-clavulanate (30 µg) according to a template. A disc of ceftoxitin (30 µg) was also included, and the zone diameter was measured for this antibiotic and for cefepime after the incubation
Synergy was interpreted as any increase or distortion in the inhibition zone towards the disc of amoxicillin-clavulanate.

**Antibiotics for MBT-ASTRA experiments**

The antibiotics meropenem (Hospira, Royal Leamington Spa, UK), colistin (Sigma-Aldrich, Steinheim, Germany) and rifampin (Sigma-Aldrich) were prepared by weighing the powder and dissolving it in MilliQ-water. Antibiotic concentrations used during experiments were close to optimum concentrations determined by Tängdén et al. [5] and were as follows: meropenem at 8 mg/L, colistin at 4 mg/L and rifampin at 2 mg/L. Aliquots of the antibiotic solutions were stored frozen at -80°C until use. Meropenem was prepared fresh on the day of experiments or analyzed via MALDI-TOF MS prior to its usage to confirm its activity.

**Experimental design**

The antibiotics (meropenem, colistin and rifampin) were first tested separately for the reference strains ATCC 25955 and PS20. Synergy tests were then performed for the clinical strains VIM-KP T14789 and NDM-KP IR8 by using the antibiotics separately and by combining meropenem and colistin, as well as meropenem with colistin and rifampin. Technical replicates of experiments were performed either as one replicate or in triplicates. The experimental procedure follows Lange et al. [17] and uses analysis from culture plates. Fresh overnight cultures on blood agar (Columbia blood agar base [Oxoid, Basingstoke, UK] with 6% defibrinated horse blood [Håtunalab, Bro, Sweden]) were used for the experiments. A suspension of bacteria with a turbidity of 0.5 McFarland in brain-heart infusion (BHI)-broth (BBL; BD, Le Pont de Claix, France) was prepared for each isolate. From this suspension 200 µl was incubated in the absence and presence of an antibiotic at 36°C under agitation (125 rpm) for a defined time (0 h, 1 h, 3 h and 24 h). The volume of the tested antibiotic was 25 µl to begin with but was later altered to 5 µl, which was the volume used for the synergy tests. After the incubation, the suspensions were centrifuged for 2 min at 18890 x g (Hettich Zentrifugen Mikro 22, Tuttlingen, Germany) and the supernatant was carefully removed to avoid loss of cells. The cells were then washed with 100 µl MilliQ-water by centrifugation for 2 min at 18890 x g and the supernatant was carefully removed. After air-drying the pellet for a few minutes the cells were lysed by adding 10 µl of 70% formic acid (Sigma-Aldrich) and slightly tapping the tube for mixing. The same amount (10 µl) of 100% acetonitrile (Sigma-Aldrich) supplemented with RNase B (Sigma-Aldrich) as an internal standard (50+1 µl), was
added after a few minutes and the tube slightly tapped for mixing. The lysate was cleared by centrifugation and 1 µl of the supernatant was transferred to a polished steel target (Bruker Daltonics GmbH, Bremen, Germany), with each lysate spotted twice. After air-drying, spots were overlaid with 1 µl of portioned HCCA matrix (10 mg/ml α-cyano-4-hydroxy-cinnamic acid in 50% acetonitrile/2.5% trifluoroacetic acid [Bruker Daltonics]).

Detection with MALDI-TOF MS

MALDI-TOF MS analysis was performed using a Microflex LT benchtop mass spectrometer (Bruker Daltonics, Bremen, Germany) with the flexControl software (Bruker Daltonics). A visual analysis of peaks was performed using flexAnalysis software (Bruker Daltonics). Spectra of bad technical quality were removed. The spectra were baseline-subtracted and smoothed, and the spectra were subsequently compared using a Beta-version of the MBT-ASTRA software (kindly provided by Markus Kostrzewa and Christoph Lange, Bruker Daltonics). Pseudo-gel view representations were performed with the gel view display of the MALDI Compass Explorer (Bruker Daltonics).

Data analysis

The MBT-ASTRA software normalizes the spectra to the maximum peak and calculates the area under the curve (AUC) for the included spectra, as well as the relative growth (RG) of a tested strain with antibiotics to its reference without antibiotics, and display results as box plots. The AUC is a reflection of how well the bacteria have grown, with higher values representing better growth, i.e. a larger amount of proteins and peptides. The RG-values are calculated from the AUCs of identical samples via the formula below, where the AUC for spectra obtained in the presence of an antibiotic is divided by the AUC of spectra obtained in the absence of an antibiotic.

$$\text{Relative growth} = \frac{\text{AUC}_{\text{Antibiotic}}}{\text{AUC}_{\text{BHI}}}$$

An RG below 0.4 indicates a sensitive strain while resistant strains should have an RG close to 1.0. All values were displayed in box plot diagrams indicating the median by the bold black line, the 25th and 75th percentiles by the box and the lowest and highest observations by the whiskers. Dots represent outliers, i.e. extreme observations. No further statistical analyses were performed due to the limited number of tests. Synergy was defined as a significant
decrease in RG (well separated 95% confidence intervals) between the combination and the most active individual antibiotic after 24 h.

**Bacterial counts**

Plate counts of colony-forming units were performed for the first triplicate synergy test to confirm that it was indeed viable bacteria that were analyzed using MALDI-TOF MS. Plating was performed after the incubations by spreading 1 μl of the suspensions on blood agar and incubating at 36°C for 20-24 h.

**Ethical considerations**

The different strains of bacteria were not subject to any ethical considerations since they were not associated with any patient information.

**RESULTS**

**Antibiotic susceptibility testing**

The results of the antibiotic susceptibility testing (table I) showed that the reference strain ATCC 25955 was susceptible to all tested antibiotics, while CCUG 54718 showed a more resistant profile, e.g. to the penicillin and the cephalosporins. PS20 on the other hand was classified as resistant to most of the tested antibiotics, which was also the case for the four clinical strains (T14789, ÖN-2211, IR8 and IR62E). The strain IR62E was classified as susceptible to the carbapenems, despite the fact that it should be a carbapenemase-producing strain. All strains were susceptible to colistin (MIC <2 mg/L), while this varied for meropenem. While ÖN-2211 and IR8 would be classified as sensitive according to EUCAST breakpoints, T14789 would be classified as resistant. IR62E showed variable results due to two subpopulations with different expression of resistance. When isolated on a non-selective medium the bacteria were susceptible to meropenem, which was also true for the disc diffusion, but when grown on a selective medium for ESBL detection (i.e. containing cephalosporin antibiotics) with a disc of meropenem and subsequently retested, the strain was classified as resistant to meropenem. CCUG 54718 was the only strain that showed any interaction with the clavulanate in the double-disc synergy test, showing synergy for cefepime, cefotaxime and ceftazidime indicating ESBLₐ-production.
Table I. The antibiotic susceptibility of control strains and VIM- and NDM-producing *K. pneumoniae* strains with disc diffusion, Etest and the double-disc synergy test. Classification according to clinical breakpoints defined by EUCAST\(^a\) for disc diffusion.

<table>
<thead>
<tr>
<th>Antibiotic susceptibility</th>
<th>ATCC 25955</th>
<th>CCUG 54718</th>
<th>KPC (PS20)</th>
<th>VIM-KP T14789</th>
<th>VIM-KP ÖN-2211</th>
<th>NDM-KP IR8</th>
<th>NDM-KP IR62E</th>
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<tbody>
<tr>
<td><strong>Disc diffusion</strong></td>
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<td>S</td>
<td>R</td>
<td>R</td>
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<td>R</td>
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<tr>
<td>Ceftazidime</td>
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<td>R</td>
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<td>R</td>
<td>R</td>
<td>R</td>
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<td>Cefotaxime</td>
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<td>R</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>R</td>
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<tr>
<td>Meropenem</td>
<td>S</td>
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<td>R</td>
<td>R</td>
<td>R</td>
<td>S(^b)</td>
<td></td>
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<td>Imipenem</td>
<td>S</td>
<td>S</td>
<td>R</td>
<td>R</td>
<td>I</td>
<td>S(^b)</td>
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<td>Ciprofloxacin</td>
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<td>R</td>
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<td>Amikacin</td>
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<td>R</td>
<td>I</td>
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<tr>
<td>Gentamicin</td>
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<td>S</td>
<td>S</td>
<td>R</td>
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<tr>
<td>Trimetoprim-sulfamethoxazole</td>
<td>S</td>
<td>R</td>
<td>S</td>
<td>R</td>
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<tr>
<td><strong>Etest (mg/L)</strong></td>
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<tr>
<td>Meropenem</td>
<td>0,015</td>
<td>2</td>
<td>&gt;32</td>
<td>2</td>
<td>2</td>
<td>0,5(^b)</td>
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<tr>
<td>Colistin</td>
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<td>0,125</td>
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<td>Rifampin</td>
<td>&gt;32</td>
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<td><strong>Double-disc synergy test</strong></td>
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<tr>
<td>Cefoxitin</td>
<td>S</td>
<td>S</td>
<td>R</td>
<td>R</td>
<td>R</td>
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<td>Cefepime</td>
<td>S</td>
<td>I</td>
<td>R</td>
<td>R</td>
<td>R</td>
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<td>R</td>
</tr>
<tr>
<td>Synergy(^c)</td>
<td>NI</td>
<td>FEP+</td>
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<td>CTX+</td>
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</tbody>
</table>

\(^a\) SIR classification according to EUCAST definitions: S, susceptible; I, intermediate; R, resistant.

\(^b\) NDM-KP IR62E initially showed a meropenem MIC of 0,5 mg/L but for isolates retrieved on ESBL-selective media close to a meropenem disc the MIC was >32 mg/L.

\(^c\) Abbreviations: FEP, cefepime; CAZ, ceftazidime; CTX, cefotaxime; NI, no interaction.

Analysis of reference strains

The results of the AUC for reference strains ATCC 25955 and the KPC-producing PS20 incubated with and without meropenem (figure 2) showed that there was a difference between the two strains. While the AUCs for PS20 were very similar in both setups, the ATCC 25955 incubated with meropenem showed a decrease in AUC.
Figure 2. A comparison of box plots for the area under the curve (AUC) for strains PS20 (resistant to meropenem) and ATCC 25955 (susceptible) incubated in the presence and absence of meropenem. Results for strains are after 1 h incubation, using one replicate.

From the AUCs, which are calculated from spectra normalized to the internal standard, the MBT-ASTRA software also calculated RG-values. Since the two AUCs for PS20 were very similar the resulting quotient, the RG-value, was close to 1.0 (figure 3). The fact that this strain grows equally well in both setups, regardless of the inclusion of meropenem, shows that the growth of this strain is not inhibited by a concentration of meropenem several times higher than the clinical S-breakpoint and is thus considered resistant to meropenem. The dissimilar AUCs for ATCC 25955, on the other hand, resulted in a low RG below the breakpoint of 0.4, which indicates that this is a strain susceptible to meropenem. Another way of viewing the spectra obtained from different incubation setups is by displaying them in a gel view mode (figure 4). The similarity of the gel view spectra for PS20 is evident, while the gel view spectra for ATCC 25955 was much weaker when meropenem was included.
Figure 3. A relative growth (RG) comparison for strains ATCC 25955 and PS20 representing the ratio of the area under the curve in the presence and absence of meropenem, using one replicate. A RG below 0.4 (red line) indicates a sensitive strain.

Figure 4. Gel view of spectra for PS20 and ATCC 25955 in the presence and absence of meropenem.
Figure 5 shows a comparison of the reference strains PS20 and ATCC 25955 incubated in the presence and absence of colistin for 1, 3 and 24 h. While both strains showed low RG-values after 1 h, the values were much higher after 3 and 24 h incubation indicating a regrowth of bacteria. When the same reference strains were incubated in the presence and absence of rifampin (figure 6) the resulting RG-quotients were all around 1.0, regardless of strain and incubation time.

**Figure 5.** Relative growth comparison for ATCC 25955 and the KPC strain PS20 incubated for 1, 3 and 24 h in the presence and absence of colistin, using one replicate.
Figure 6. Relative growth comparison for ATCC 25955 and the KPC strain PS20 incubated for 1, 3 and 24 h in the presence and absence of rifampin, using one replicate.

Analysis of the clinical strain VIM-KP T14789
Results of the MBT-ASTRA synergy test for the clinical strain VIM-KP T14789 are shown in figures 7-9, where the antibiotics meropenem, colistin and rifampin are investigated alone and in different combinations. When only one replicate was used (figure 7), the RG-values were below 0.4 after the 1 h incubation for colistin alone, the combination of meropenem with colistin, as well as for the triple combination also adding rifampin to the combination. After incubating the suspensions for 3 h, only the triple combination had an RG below 0.4, while after 24 h colistin on its own was the only one with an RG below 0.4.
Figure 7. Synergy test showing the relative growth for VIM-KP T14789 with single, double and triple combinations of meropenem, colistin and rifampin incubated for 1, 3 and 24 h, using one replicate.

The synergy test was also performed for VIM-KP T14789 using triplicates (figure 8), but with only 1 h and 24 h incubation, and with a BHI control at 0 h. The BHI at 0 h showed a very low AUC, and at the same level as several of the investigated antibiotics. After the 1 h incubation colistin alone, meropenem and colistin, as well as meropenem, colistin and rifampin showed low AUCs, as well as RG-values (figure 9) below the 0.4 breakpoint. After 24 h the AUC was only low for the triple combination of meropenem, colistin and rifampin, and with an RG close to being included by the 0.4 breakpoint.
**Figure 8.** Synergy test showing the area under the curve for VIM-KP T14789 with single, double and triple combinations of meropenem, colistin and rifampin incubated for 1 and 24 h, as well as a BHI control at 0 h, using three replicates.

**Figure 9.** Synergy test showing the relative growth for VIM-KP T14789 with single, double and triple combinations of meropenem, colistin and rifampin incubated for 1 and 24 h, in triplicates.
Bacterial counts were also performed where suspensions from one triplicate experiment were plated after the different incubations. This experiment showed results with many colonies from all suspensions. The results (figure 10) also showed that there were much fewer colonies from suspensions incubated with the combination of meropenem, colistin and rifampin compared to suspensions without any antibiotics. No difference was seen in bacterial counts when comparing suspensions incubated in the presence of meropenem and those with only BHI.

**Figure 10.** Bacterial counts from suspensions incubated for 1 h with the combination of meropenem, colistin and rifampin (left) and in the absence of an antibiotic (right).

**Analysis of the clinical strain NDM-KP IR8**

The results of the synergy experiment using the clinical strain NDM-KP IR8 (figure 11), showed results near identical to those of VIM-KP T14789. For IR8 the RG-values were below or close to the breakpoint of 0.4 after the 1 h incubation for colistin alone, the combination of meropenem and colistin, as well as for the triple combination of meropenem, colistin and rifampin. After 24 h the triple combination was the only one below the breakpoint.
**Figure 11.** Synergy test showing the relative growth for NDM-KP IR8 with single, double and triple combinations of meropenem, colistin and rifampin incubated for 1 and 24 h, using triplicates.

**DISCUSSION**

With increasing resistance to carbapenems and other antibiotics worldwide [1, 3], and with severe conditions such as sepsis requiring quick treatment, it is of vital importance to develop the area of rapid detection of antibiotic resistance and to investigate the potential of combination therapy. MALDI-TOF MS is an instrument that has been implemented in clinical microbiology for the identification of microorganisms, but has also shown great promise in the field of antibiotic susceptibility testing [11-16]. Recently, Lange *et al.* [17] have developed a method called MBT-ASTRA that allows the amount of peptides and proteins within a MALDI-TOF MS spectrum to be quantified by incorporating an internal standard, which correlates to the number and hence growth of bacteria. Comparing the spectra of different bacteria grown in the presence or absence of antibiotics thus allows an interpretation of susceptibility. In addition to this new method by Lange *et al.*, the basis of this study relies on the research of Tängdén *et al.* [6], who evaluated different double and triple antibiotic combinations and demonstrated the highest synergy for the combination of meropenem with...
colistin and rifampin – three antibiotics using different mechanisms of action. This is a proof-of-concept study investigating the visualization of susceptibility and synergy with the MBT-ASTRA by using quantitative MALDI-TOF MS. While this method allows the susceptibility of different antibiotics to be investigated, this is the first attempt to visualize synergy.

**Antibiotic susceptibility testing**

According to the results of the disc diffusion, SIR-classification was performed [20] which confirmed the anticipated results for the different strains. The reference strain ATCC 25955 was indeed sensitive to the tested antibiotics, while the ESBL-producing strain CCUG 54718 was resistant to the penicillin and cephalosporins, but sensitive to the carbapenems. The fact that this was an ESBL-producing strain was also confirmed by the results of the double-disc synergy test. The KPC- and ESBL-producing strain PS20 showed a high level of resistance, with the KPC breaking down the carbapenems, which ESBL-production alone does not. In the double-disc synergy test the activity of the clavulanate, inhibiting the ESBL, is masked by the activity of the KPC, which is why no synergy is visible. The susceptibility of the clinical strains showed a high level of resistance, which was to be expected of these different carbapenemase-producing strains. According to the MIC-values all strains were susceptible to colistin, while the results varied for meropenem. No MIC-classification breakpoints of rifampin are available for species of Enterobacteriaceae [20] since they are intrinsically resistant, but the MIC-values of 32 mg/ml and above do suggest that the antibiotic virtually had no effect on the bacteria.

The clinical strain NDM-KP IR62E showed variable results for the carbapenems in the confirmatory antibiotic susceptibility testing. The results of the disc diffusion showed that the strain was sensitive to carbapenems which, together with the low MIC of meropenem, was not in agreement with the results of Tängdén *et al.* [5]. However, when the strain was retrieved from the freezer on an ESBL-agar with a meropenem disc and then colonies close to the meropenem disc retested using the Etest, results showed a resistant profile in accordance with Tängdén. These aberrant results were probably caused by the strain first being cultured on a non-selective medium after freezing, which enabled bacteria that had lost their KPC-gene (which can happen during freezing) to grow, which they actually did a lot better than those who still retained their resistance. And when the strain was later cultured on a selective medium with a meropenem disc, only those that expressed the resistance gene were able to grow close to the disc. It is important to note this duality since results of subsequent
experiments performed for frozen bacteria may be affected if they do not show the expected expression of resistance. In the future, strains should be cultured on a selective medium to ensure that the bacteria investigated express the expected pattern of resistance.

**Analysis of reference strains**

From the results of the quantitative MALDI-TOF MS analysis, the spectra were compared using the MBT-ASTRA software. For suspensions incubated in the presence and absence of meropenem this comparison resulted in an RG-value below the breakpoint of 0.4 for ATCC 25955 (figure 3), which confirmed that this strain was sensitive to meropenem. This was also shown with the gel view display (figure 4) showing a very low intensity spectrum when incubated in the presence of meropenem, compared to a spectrum with high intensity when grown in BHI only. This correlated very well with the antibiotic susceptibility testing, where the strain was shown to be sensitive to meropenem, as well as to the other antibiotics tested. The KPC-producing strain PS20 was used as a comparison to visualize the result of a strain resistant to meropenem, which was shown by an RG-value of 1.0 (figure 3), clearly above the 0.4 breakpoint. The gel view image also showed that this strain had similar spectra regardless of sample incubation in the presence or absence of meropenem (figure 4).

When these reference strains were incubated in the presence of colistin they were inhibited after 1 hour of incubation, though only the KPC strain (PS20) was completely below the 0.4 breakpoint (figure 5). Colistin is an antibiotic that is used as a last-line treatment against many multiresistant bacteria [2], and was therefore expected to have an effect against both strains. Colistin did, however, show regrowth already after the 3 h incubation and subsequently also after the 24 h incubation, which is also what Tängdén observed after 24 h for this clinical strain [6]. While this regrowth might deem this antibiotic as poor choice of treatment, the clinical relevance must be evaluated on a more complex basis, including dosing scheme and pharmacokinetic and pharmacodynamic data, since this is an *in vitro* experiment using static antibiotic concentrations. While meropenem had no effect on the KPC-producing PS20, the sensitive ATCC 25955 was inhibited after 1 h, but also showed regrowth after 3 h and 24 h (results not shown).

The results for rifampin confirmed that this antibiotic did not have any effect on the reference strains tested (figure 6), since RG-values were clearly above the breakpoint of 0.4. This was expected since rifampin is not effective against Enterobacteriaceae due to intrinsic resistance.
This was also in accordance with the MIC-results for ATCC 25955 and PS20 showing values of >32 mg/L, a clear indication of resistant isolates.

Analysis of the clinical strain VIM-KP T14789

The clinical strain VIM-KP T14789 was chosen as the first test strain for the synergy experiments since it had shown the second to highest synergy for Tångdén et al. [6] and not any variable results in the susceptibility testing (table I). Synergy experiments were performed in different attempts, first using one replicate and then using triplicates. While the results for experiments using one replicate were as expected after 1 h incubation (figure 7), this was not the case after the 24 h incubation, so further experiments using more replicates were performed. Due to methodological difficulties during a first triplicate experiment, e.g. with the pellet not holding together possibly due to the centrifuge running hot, and difficulties for the MALDI-TOF MS finding spectra, this experiment was repeated. The incubation time-points were adjusted to include only 1 h and 24 h, since no comparative values were available at 3 h for results of experiments performed by Tångdén et al. [6]. Rifampin was also excluded since its lack of effect had already been confirmed. However, triplicates of BHI at 0 h were included to establish a reference point of the starting inoculum. Experiments were repeated after the MALDI-TOF MS had been calibrated using a bacterial test standard and the intensity of the laser adjusted, according to standard procedure normally performed at regular intervals.

The results of the synergy experiment performed in triplicate showed that meropenem alone was not an effective antibiotic against the clinical VIM-KP strain T14789 (figure 9). This is because the strain produces a carbapenemase (VIM) that degrades this antibiotic, rendering the strain resistant. Colistin however, did have a clear effect on the strain after incubation for 1 h, showing an RG below 0.4, but after 24 h the strain showed regrowth. The occurrence of regrowth seems to be an event associated with colistin, since this was also visible for the reference strains (also sensitive to this drug). The combination of meropenem with colistin, as well as the combination of meropenem, colistin and rifampin, also had an effect on the strain after 1 h. Colistin is often a component of antibiotic combinations since it acts as a detergent, which increases the permeability of the bacteria to other antibiotics [2]. This enables other antibiotics to exert their effect; sometimes antibiotics that are usually not a treatment option, for example by counteracting resistance mechanisms due to porin loss. By combining meropenem with colistin and rifampin the RG of T14789 was also kept low after the 24 h incubation, which seems to confirm the results of Tångdén et al. [6] who showed synergy for
this combination when using time-kill experiments. The result of the triple combination in this study seems to demonstrate how colistin helps rifampin to penetrate and exert an effect on the intrinsically resistant *K. pneumoniae*. On its own, rifampin showed no effect against T14789 (figure 7) and its expected insusceptibility to the used concentration of 2 mg/L had been confirmed *a priori* using the Etest, showing an MIC of >32 mg/L. The results of the antibiotics alone and in different combinations were all in accordance with Tängdén *et al.* [6] for the VIM-KP strain T14789. Tängdén *et al.* also found synergy for the combination of colistin and rifampin when using time-kill experiments. However, this combination was excluded since synergy was only visible for the NDM-KP strains, and was also lower than with the addition of rifampin in the Tängdén study.

The BHI analyzed at 0 h helps establish a reference point for the bacterial growth, since it represents the starting inoculum without any additional growth. When compared with the antibiotics that did show an effect on the strain it is evident that these are on the same level (figure 8), which indicates that the bacteria were inhibited from any further growth by the antibiotic or antibiotic combinations. The RG for the triple combination after 24 h showed a result across the 0.4 breakpoint (figure 9), while the AUC that was on the same level as the BHI at 0 h (figure 8). Perhaps as a step in further method development, the fixed breakpoint value of 0.4 that has been previously decided on could be adjusted according to a BHI reference at 0 h to ensure a correct classification. Synergy-specific cut-off values may also be required. In fact, a new RG cut-off of 0.5 has been suggested for a more reliable detection of resistant *K. pneumoniae* strains [21], so further optimization is ongoing.

The results of the bacterial counts showed a great amount of colonies equivalent to the trend of RG-values, e.g. meropenem and BHI showing similar amounts, while the triple combination showed much fewer colonies compared to BHI only (figure 10). The results confirmed that the proteins analyzed by the MALDI-TOF MS were indeed from viable bacteria. Due to the great amounts of bacteria only an estimation of the number and equivalency was made, and for more accurate numbers subsequent dilutions and viable count experiments would be necessary.

**Analysis of the clinical strain NDM-KP IR8**

The results of the synergy experiment for a second clinical strain, NMD-KP IR8, were near identical to those of VIM-KP T14789. However, in the antibiotic susceptibility testing
T14789 had an MIC of >32 mg/L for meropenem while IR8 only had an MIC of 2 mg/L (table I). This means that IR8 would be classified as susceptible according to EUCAST breakpoints [20] but probably resistant according to results of the synergy experiment. A subtle difference between the two strains could be interpreted, since the RG for IR8 with meropenem was slightly below 1.0 instead of above at both 1 h and 24 h incubation, which would suggest a stronger inhibition. However, if this difference is of significance or a mere variation between attempts is difficult to say. Since the concentration of meropenem in the experimental solutions should be 8 mg/L, one would expect IR8 to show a much lower RG given that it shows susceptibility at 2 mg/L. While the results of the synergy experiments were mostly in accordance with the time-kill experiments performed by Tängdén et al. [6], the results when using meropenem were not. After the 1 h incubation in the presence of meropenem Tängdén et al. could see a clear reduction in the viable counts of NDM-KP IR8 compared to the starting inoculum, while after 24 h there was regrowth. In this experiment, the initial effect of reduction was not visible and there was instead an increase in the AUC of meropenem after 1 h compared to the starting inoculum at 0 h (results not shown). However, the AUC was lower with meropenem than with only BHI, indicating fewer bacteria. Since this is a biological system, it is possible that there was an initial decrease with an occurrence prior to 1 h, and that the bacteria were analyzed during the phase of regrowth. It is also possible that the concentration in the experimental solution was lower than the expected 8 mg/L which might further reduce the effect of meropenem.

**Limitations and future perspectives**

One of the limitations of this study is that performing the protocol with tubes includes several steps requiring the removal of the supernatant, and this method is quite sensitive to any loss of cells. To better ensure that no cells are lost a second technique suggested by the manufacturer using plates and filters should be used in any future clinical setting to ensure a correct interpretation on susceptibility is made. It would then also be easier to standardize the method, and potentially incorporate antibiotic powders directly in the plates. A limitation of the software (which Bruker seem to be working on improving) is that there is a risk of nonsense spectra becoming a result since the spectra are normalized to the maximum peak regardless of the quality of spectra. Continued optimization of the method and software is therefore needed, since the method must be reliable to ensure a correct classification of susceptibility and thus appropriate treatment of patients. However, in the future, the MBT-ASTRA has the potential to be used as a rapid method of resistance detection, as well as an
alternative method for synergy testing instead of the time-consuming viable counts of time-kill experiments.

This is the first time the MBT-ASTRA is used to detect synergy. However, a larger study including viable counts is needed to provide results of greater reliability and statistical significance. In addition, using the plate technique and more replicates would be helpful, but more studies are necessary to evaluate this method and confirm its potential clinical use for antibiotic susceptibility testing as well as for synergistic testing. In future studies, further investigations on other antibiotic combinations may also be of interest.

Conclusions
In conclusion, the MBT-ASTRA is a rapid method for visualizing antibiotic susceptibility and seems to have potential for synergy testing. According to the results presented in this study the antibiotic combination of meropenem with colistin and rifampin shows a synergistic effect after 24 h that can be visualized using the MBT-ASTRA software.
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