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In vitro killing of multidrug/extensively drug-resistant *Pseudomonas aeruginosa* by fosfomycin alone or in combination with antipseudomonal antibiotics

Pseudomonas aeruginosa is a leading cause of nosocomial infections. Given the constant rise in resistance, adequate therapy is increasingly demanding. Fosfomycin recently became an appealing treatment option of bacterial infections due to multidrug-resistant bacteria (MDR). So far, fosfomycin synergy with other antibiotics has been assessed in studies, but only a limited number focused on MDR *P. aeruginosa* and on the effect of these combinations on the duration of the postantibiotic effect (PAE). We investigated synergy of fosfomycin with an array of antipseudomonal antibiotics using gradient diffusion strip cross method and time-kill method, and their effect on the duration of PAE against 51 variously resistant *P. aeruginosa* isolates. The highest rate of synergy was observed for combination with ceftazidime (23.4 %) and gentamicin (19.1 %). The PAE of antibiotic combinations was superior to that of the drugs alone. Our findings indicate that fosfomycin combination therapy may be a valuable treatment alternative.

Keywords: *Pseudomonas aeruginosa*, fosfomycin, antibiotic synergy, time-kill method, gradient diffusion strip method, postantibiotic effect

Introduction

Pseudomonas aeruginosa is an important hospital pathogen causing a variety of infections such as ventilator associated pneumonia, bloodstream infections, urinary tract infections, wound and burn infections. Therapy of infections with resistant isolates pose a serious challenge to clinicians because of its chromosomal, intrinsic, and acquired resistance traits.[1–3]

Acquired resistance to β-lactam antibiotics in *P. aeruginosa* is partly due to the production of extended-spectrum beta-lactamases (ESBLs) belonging to SHV, GES, VEB, BEL, PME and PER family and more frequently to the production carbapenemases of class A

(KPC, GES), B (VIM, IMP, DIM, NDM, AIM, FIM, AIM) and D.[1,4,5] Hyperexpression of chromosomal AmpC β-lactamases, upregulation of efflux systems (MexAB, MexCD, MexEF) and decreased outer membrane permeability caused by modification of OprD porin contributes to resistance.[5,6 1,7]Hyperexpression of chromosomal AmpC cephalosporinase in *P. aeruginosa* due to induction or derepression confers resistance to expanded-spectrum cephalosporins, but spares cefepime and carbapenems[4]. For that reason, clinicians rely on antibiotic combinations to treat infections associated with multidrug (MDR) or extensively drug resistant (XDR) *P. aeruginosa*. Fosfomycin does not exhibit cross resistance with other antibiotic classes and has been shown to exert synergy with antibiotics belonging to other classes. Fosfomycin monotherapy is not recommended, as rapid emergence of resistance has been reported[7–9], but the drug may increase membrane permeability for other antimicrobials, even those to which the bacteria are resistant to, rendering them susceptible, when administered in combination.[7,10,11] Because of its specific mechanism of action and preserved activity against MDR bacteria, there is growing interest in fosfomycin usage for the treatment of highly resistant microbial infections, including those associated with MDR *P. aeruginos*a. Synergy studies are a valuable tool to assess potential alternative treatment strategies especially against MDR and XDR pathogens and to aid clinicians in the optimal antibiotic combination choice in times of limited options. Data from studies for *in vitro* synergism of fosfomycin in combination with other antibiotics have been variable and, regarding some antibiotics, based on a limited number of isolates without defined resistance phenotypes.[7,8,11–21] Also, a substantial amount was published before year 2005 with very heterogeneous definitions of synergy. Recent studies have shown synergy of fosfomycin with different β-lactam antibiotics[11,15–17], aminoglycosides[16,18,19], quinolones[7,16,20,21], and polymyxins.[7] However, the rate of synergy depended on the method used to detect it. According to a 2020 review study, fosfomycin exhibited synergy mostly with chloramphenicol (53 %),

aminoglycosides (43 %) and cephalosporins (36 %).[22] Moreover, antagonism has been reported with β-lactam antibiotics, quinolones and aminoglycosides.[12–14,22] So far, evidence of synergy in MDR and XDR isolates, particularly those harbouring metallo-β-lactamases (MBLs), compromising therapy with antipseudomonal β-lactam antibiotics except of monobactams, are extremely scarce. Only a few studies evaluated the effect of combination therapy on MDR or carbapenem resistant *P. aeruginosa*, mostly using few antibiotic combinations.[7,8,11,15,23–27] Ceftazidime and imipenem displayed high rates of synergy, followed by cefepime, colistine and piperacillin/tazobactam.[8,11]Also, Walsh reported improvements in bacterial killings in combination with ciprofloxacin on three MDR of total four clinical isolates.[7] To our knowledge, no bibliographic data covering XDR isolates is available.

Analysis of *in vitro* synergy of antibiotics is usually performed by checkerboard method, time-kill technique (TK), and gradient diffusion strip (GDS) cross method, which demonstrate low or moderate level of concordance and questionable correlation with clinical studies.[28–30] The GDS cross method determines the bacteriostatic activity, while the time-kill method determines the bactericidal activity of antibiotics in combinations. Since the methods are based on different effects, discrepancy between results is expected.

The postantibiotic effect (PAE) is a parameter linked to the pharmacokinetics of an antimicrobial and could be used in modulation of dosage regimes.[31] Only a few studies have recorded a modest fosfomycin PAE (0,3-2,5 h with concentrations of 4 mg/L and 1.1 -5.5 h with concentrations of 256 mg/L) against clinical isolates of *P. aeruginosa in vitro.*[9,19] There are no reports on the effect of fosfomycin combination on the duration of PAE. This void in evidence calls for further investigation, as a less frequent dosage regime may be of value in regard of drug toxicity.

In this study we analysed the synergistic effect of fosfomycin in combination with other antibiotics active on *P. aeruginosa* using time-kill technique and GDS cross method. A considerable number of antibiotics of several classes have been included, some of which, to our knowledge, have not previously been tested either using these methods, or against MDR and XDR *P. aeruginosa* isolates. The aim was to determine the most successful antibiotics to be combined with fosfomycin against clinical isolates of *P. aeruginosa* with various resistance patterns and resistance genes content, including MDR and XDR isolates, and to determine the effect of different antibiotics on the duration of PAE induced by fosfomycin.

Material and methods

Bacterial isolates

In total 51 *P. aeruginosa* isolates included in the study, were collected from various clinical specimens in five hospital centres in Croatia: University Hospital Centre Zagreb, University Hospital Centre Osijek, University Hospital Centre Split, General Hospital Pula, General Hospital Bjelovar and General Hospital Slavonski brod. The isolates were identified by Vitek 2 or MALDI-TOF MS (matrix assisted laser desorption ionization-time of flight mass spectrometry) (Bruker, Illinois, USA). Six isolates from Split were identified as positive for VIM-2 in previous studies.[32] The isolates harbouring PER-1 and coharbouring PER-1 and VIM-2 were kindly provided by G. M. Rossolini (Microbiology and Virology Unit, Careggi University Hospital, 50134 Florence, Italy.).

Antibiotic susceptibility testing

Antibiotic susceptibility testing to antipseudomonal antibiotics was performed by disk-diffusion and broth microdilution method in 96 well microtiter plates and Mueller-Hinton broth (MHB) according to Clinical & Laboratory Standards Institute (CLSI).[33] Antibiotic

powders were purchased from Sigma-Aldrich, prepared as stock solutions (5120 µg /mL) and diluted in sterile water. The range of antibiotic concentrations 0.12 to 256 µg/mL was prepared stepwise, doubling dilutions in Mueller Hinton broth. The dilutions (50 µL) were dispensed into wells of the microtiter plates with a multichannel pipette. Overnight broth culture of the tested strain was diluted to correspond to 0.5 McFarland and then diluted 1:100 to reach an inoculum size of $5x10^5$ CFU/mL. The samples were added to the microtiter plates in the amount of 50 µL and incubated overnight at 37 °C. The MIC was read as the lowest antibiotic concentration which prevented visible growth of bacteria.

The minimum inhibitory concentration (MIC) of fosfomycin was determined by agar dilution test and E-test. A breakpoint value of >64 µg/mL was used to define resistance.[34] The isolates were classified as susceptible (S), multidrug (MDR), extensively drug resistant (XDR) and pandrug resistant (PDR) according to Magiorakos et al.[35] MDR isolates are resistant to at least one antibiotic in three different antibiotic classes, whereas XDR isolates are susceptible to only two antibiotics belonging to different classes, usually to amikacin and colistin. S isolates are wild type strains without acquired resistance mechanisms. PDR isolates are resistant to all available antibiotics.

Detection of AmpC hyperexpression n

Isolates resistant to ceftazidime, but susceptible to cefepime were subjected to detection of the hyperexpression of AmpC β-lactamase. The AmpC disk test was performed on Mueller Hinton agar (MHA) plates inoculated with a suspension of 0.5 McFarland (10^8 CFU/mL) of the tested organism. Ceftazidime (30 µg) and cefepime (30 µg) discs were placed on the plate alone and in combination with 400 µg of phenylboronic acid (PBA). Plates were incubated overnight at 35 °C. A ≥ 5 mm increase of the zone diameter in combination with PBAwas considered as AmpC overexpression.

https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4385569/

Coudron PE. Inhibitor-based methods for detection of plasmid-mediated AmpC β-lactamases in *Klebsiella* spp., *Escherichia coli* and *Proteus mirabilis*. *J Clin Microbiol* 2005;43:416-7.

Phenotypic detection of carbapenemases

The modified Hodge test

The modified Hodge test was performed on Mueller Hinton agar (MHA) plates inoculated with a suspension of 0.5 McFarland of the indicator strain *E. coli* ATCC 25922. A 10 µg meropenem disc was placed in the centre of the plate and a straight line of the tested isolate was streaked from the edge of the disk to the edge of the plate. Plates were incubated overnight at 35 °C. Growth of the indicator strain in a clover leaf-like indentation along the tested organism was considered a positive result and the absence of growth was labelled as a negative one.[36]

The carbapenem inactivation method (CIM)

Ten µg meropenem discs were immersed in a thick suspension of tested organisms and incubated for 2 h at 35 °C. MHA plates were inoculated with *E. coli* ATCC 25922. Meropenem discs were removed from the suspension, placed on the inoculated MHA plates, and incubated at 35 °C for 24 h. The test was considered positive if the zone diameter was ≤15 mm or if there were colonies growing inside the inhibition zone.[37]

Combined disk (CD) test for detection of metallo-β-lactamases (MBL)

Two imipenem and two meropenem disks were placed on a MHA plate previously inoculated with a 0.5 McFarland suspension of the tested isolate. Ten µL of a 0.5 M EDTA solution was

added to one imipenem and one meropenem disk. The plates were incubated overnight at 36 °C. Augmentation of the inhibition zone of ≥7 mm around EDTA containing disks compared to control disks without EDTA indicated possible MBL production.[38,39]

Molecular detection of carbapenemase genes

The presence of genes encoding metallo-β-lactamases belonging to VIM, IMP and NDM family was determined by polymerase chain reaction (PCR) as previously described.[40–43] DNA was extracted by boiling method. Briefly, five colonies were suspended in 500 µL of ultrapure water, boiled at 95 °C for 10 minutes in the heat block, and spun in the bench centrifuge to remove the pellet. Clear supernatant was used as the DNA template. Master mix in the volume of 25 µL (EmeraldAmp Max PCR Master Mix, Takara), containing DNA polymerase, buffer and nucleotides, was mixed with 20 µL of ultrapure water, 1 µL of each primer and 3 µL of the DNA template. A total volume of 50 µL was obtained. Primers VIM-F (5'-CAG-ATT-GCC-GAT-GGT-GGT-TGG-3') and VIM-R (5'-AGG-TGG-GCC-ATT-CAG-CCA-GA-3'), IMP-F (5'-GAA-GGY-GTT-TAT-GTT-CAT-AC-3') and IMP-R (5'-GTA-MGT-TTC-AAG-AGT-GAT-GC-3'),he and NDM-F (5'-AAT-GGA-ATT-GCC-CAA-TAT-TAT-GC-3') and NDM-R (5'-CGA-AAG-TCA-GGC-TGT-GTT-GC-3') were used to amplify *bla*$_{\text{VIM}}$, *bla*$_{\text{IMP}}$ genes, and *bla*$_{\text{NDM}}$ gene, respectively. The amplification was done in Alpha AC Thermal cycler, Ac-196, (Cole-Palmer, Ltd, Staffordshire, UK). PCR products were detected by agarose gel electrophoresis, at UV illuminator, after staining with ethidium bromide. The size of the PCR amplicon was determined using DNA ladder (DNA MOL. WEIGHT MARKER XIV, Medical Intertrade, Zagreb, Croatia).

Synergy testing

Synergy was determined by gradient diffusion strips cross method (GDC) and time-kill assays. GDC was performed on MHA plates. The strips were set at a 90º with the strip

crossing point at the MIC of each antibiotic determined separately. MHA plates with inoculated bacterial suspensions and strips with an antibiotic concentration gradient were incubated overnight at 36 °C. MIC of antibiotic A in combination with antibiotic B and the MIC of antibiotic B in combination with antibiotic A were determined to calculate the fractional inhibitory concentration index (FICI). FICI is defined as $\Sigma FIC = FIC_A + FIC_B = C_A^{combined}/MIC_A^{alone} + C_B^{combined}/MIC_B^{alone}$. FICI values ≤ 0,5 denote synergism, values from 0,5 to ≤ 1 denote an additive effect, from 1 to ≤ 4 are considered indifferent and values > 4 are considered antagonistic.[29,44] The following antibiotics were tested for synergy effect: ceftazidime, cefepime, piperacillin/tazobactam, gentamicin, amikacin, ciprofloxacin, imipenem and colistin.

Time-kill assays were done in MHB as previously described. For the time-kill assay four isolates were selected, according to their resistance phenotype, presence of MBL's and GDSC method results: P14 as a VIM positive XDR isolate, P32 and P45 are MDR isolates without detected carbapenemases, and P36 as a MDR isolate which exhibited synergy in five combinations when GDSC was preformed: ceftazidime, cefepime, piperacillin/tazobactam, gentamicin and ciprofloxacin. An overnight broth culture was diluted to achieve a 0,5 McFarland optical density. The obtained culture was once again diluted 1:100, incubated for 2 h at 37 °C to reach the starting inoculum of 10^6-10^7 CFU/mL and exposed to fosfomycin alone or combined with other antibiotics for 24 h. Antibiotic concentrations used during time-kill experiments represented peak concentrations of non-protein bound drug in human body fluids according to the bibliographic data for ceftazidime (170 mg/L) [45], cefepime (131 mg/L), piperacillin/tazobactam (210/24 mg/L), ciprofloxacin (2,8 mg/L), fosfomycin (395 mg/L) [23], gentamicin (9 mg/L)[46], amikacin (38 mg/L)[23,47], imipenem (55 mg/L)[48] and colistin (2,9 mg/L).[49–51] An unexposed control was run in parallel. Bacterial counts at times 0, 2, 4, 6, 8 and 24 h were determined by viable counting. The experiments were done in duplicate.

Synergy was defined as ≥ 2 log10 decrease in colony count at 24 h with the antimicrobial combination compared to the most active single agent. Indifference was defined as a decrease or increase < 2 log10 CFU/mL after 24 h, and antagonism as an increase ≥ 2 log10 CFU/mL after 24 hours in comparison to the most potent antibiotic alone. Bactericidal effect was defined as ≥ 3 log10 decrease (> 99.9 %) in the colony count after 24 h compared with the starting inoculum.[29,52] Bacteriostatic activity was defined as maintenance of the original inoculum concentration or a reduction of less than 99.9 % (< 3 log10) of the total number of CFU/mL in the original inoculum.[53,54]

Postantibiotic effect (PAE)

PAE was determined by a standard viable counting method for the four isolates used in the time-kill study.[55,56] Strains were incubated overnight, diluted 1:100 in prewarmed MHB and incubated in the shaking water bath for 2 h to reach the logarithmic phase of growth before addition of an antibiotic or a combination of antibiotics. The starting inoculum was adjusted to 10^6-10^7 CFU/mL. After 2 h antibiotics were removed by centrifugation and washed twice in saline solution. The pellet was resuspended in 5 mL of prewarmed MHB and further incubated with shaking for 24 h. The samples were withdrawn at time zero, immediately after centrifugation, and then every two hours at 4, 6 and 8 h and after 24 h. Fifty µL of 10-fold dilutions were spread on MacConkey agar plates. Antibiotic carryover was prevented by dilution of the sample and spreading it over the whole plate. Plates were read after incubation of 18 h at 37 °C. A growth control without antibiotic exposure was performed in the same way. PAE was calculated according to the following formula: PAE = T - C, where T is the time required for the viable counts of the antibiotic exposed cultures to increase by 1 log10 above the counts observed immediately after dilution, and C is the corresponding time for the unexposed culture.[55,57] PAE values of 0-2 hours were considered short, 2-4 hours moderately

long, and prolonged if they were >4 h.[58] When the PAE induced by the combination of drugs was at least 1 h longer than the sum of the PAEs of individual antibiotics, the combination was considered synergistic. When the PAE was similar to or shorter than the sum of the effect of each antibiotic individually, it was categorized as additive or antagonistic, respectively. Addition is considered as an effect roughly similar to the sum of individual effects, and indifference as a combination effect no different from the longest individual PAE. An antagonistic effect produced by a combination is defined to be at least 1 h shorter than the longest effect of individual antibiotics of the specified combination.[59,60]

Statistical analysis

To address the differences in synergy between isolates with and without acquired β-lactamases statistical analysis using chi-squared test was performed. Time kill data were expressed as means ± standard deviations (SD) of duplicate determinations. To determine significant differences ($p < 0.05$) among the means, one way analysis of variance (ANOVA) was performed.

Results

Antibiotic susceptibility and detection of β-lactamases

The isolates showed variable levels of susceptibility to antipseudomonal antibiotics. There were 39.2 % (n=20) multidrug-resistant (MDR) and 49 % (n=25) extensively drug-resistant (XDR) isolates. None of the isolates belonged to the PDR category. The rate of susceptibility to fosfomycin was 47.1 % (n=24). The rates of susceptibility to other antibiotics were as follows: colistin 88.2 % (n=45), amikacin 43.1 % (n=22), piperacillin/tazobactam 39.2 % (n=20), ciprofloxacin 21.6 % (n=11), cefepime 19.6 % (n=10), gentamicin 19.6 % (n=10), meropenem 17.6 % (n=9), imipenem 13.7 % (n=7), ceftazidime 11.8 % (n=6). In total 53.8 %

of VIM positive strains were susceptible to fosfomycin (n=14). MIC values of tested antibiotics are shown in Table 1.

None of the tested isolates exhibited hyperexpression of AmpC β-lactamase.

In total 54.9 % (28/51) isolates displayed a positive modified Hodge and positive CIM test, indicating production of carbapenemases. There were 58.8 % (30/51) isolates demonstrating positive combined disk test with EDTA test indicating the presence of MBL (Table 1.).

VIM metallo-β-lactamase was identified in 51 % (26/51) isolates. Seven isolates tested positive for *bla*$_{VIM2}$ and two for *bla*$_{PER-1}$ extended-spectrum β-lactamase in the previous studies (Table 1.).[1,32] Ona isolate coharboured both genes encoding VIM-2 and PER-1.

[Table 1 near here]

Synergy testing

GDS cross method

The highest rate of synergy was obtained for fosfomycin combination with ceftazidime (21.6 %, n=11), followed by gentamicin (17.7 %, n=9). Other combinations yielded low rates of synergy, for instance ciprofloxacin (9.8 %, n=5), piperacillin/tazobactam (7.8 %, n=4), cefepime (7.8 %, n=4), amikacin (5.9 %, n=3) and imipenem (5.9 %, n=3). In combination with colistin, synergy was not observed.

The additive effect was observed mostly for combinations with ceftazidime (45.1 %, n=23) and gentamicin (43.1 %, n=22). High rates of indifference were observed in combination with colistin (88.2 %, n=45). No antagonism was observed in this study (Table 2.). *[Table 2 near here]*

No statistically significant difference in synergy between isolates with and without acquired β-lactamases was observed.

A fosfomycin MIC lowering effect was observed in this study. Ceftazidime, gentamicin, cefepime, amikacin, imipenem, piperacillin/tazobactam, ciprofloxacin and colistin lowered the fosfomycin MIC for at least one dilution in 68.6 % (n=35), 62.7 % (n=32), 56.9 % (n=29), 41.2 % (n=21), 41.2 % (n=21), 37.3 % (n=19), 37.3 % (n=19) and 27.5 % (n=14) of the isolates, respectively. Out of 26 fosfomycin resistant strains, a category change to sensitive was observed in 53.8 % (n=14) with gentamicin, 50 % (n=13) with imipenem and ceftazidime, 46.2 % (n=12) with cefepime, 42.3 % (n=11) with piperacillin/tazobactam, 38.5 % (n=10) with amikacin and 34.6 % (n=9) with ciprofloxacin and colistin.

Time-kill assays

The XDR isolate *P. aeruginosa* P. 14 positive for VIM MBL did not show any significant differences in the time-kill kinetics between any of the tested antibiotics alone and in combination with fosfomycin. Neither synergistic nor antagonistic interactions were noticed. Moreover, no bactericidal effect was observed (Figure 1).

P. aeruginosa P32 with MDR phenotype and without carbapenemase, showed a reduction of >2 log10 CFU/mL after 24 h for combinations including colistin, amikacin, piperacillin/tazobactam, gentamicin and ceftazidime compared to each single antibiotic, indicating synergy. Gentamicin combination exhibited the most pronounced and the fastest synergistic and bactericidal effect, compared to single gentamicin, already after 4 h. A bactericidal effect was also noticed in combination with colistin after 6 h, amikacin alone and in combination after 8 h, and imipenem after 8 h, respectively, but regrowth occurred after 24 h (Figure 1). Although synergy was observed for five antibiotic combinations, a statistically significant difference was determinated among combinations with piperacillin/tazobactam, gentamicin and amikacin, due to intra-strain variability of the tested organism.

Time-kill kinetics of MDR P36 isolate showed a synergistic effect in combinations with gentamicin, ciprofloxacin, imipenem and colistin. Combinations with gentamicin and imipenem expressed synergism already after 2 h. A bactericidal effect was also observed with imipenem after 24 h. Colistin alone and in combination exerted a bactericidal effect already after 2 h, but unlike when used alone, no bacterial growth after 24 h was observed when colistin was used in combination. Bacterial growth after 24 h was not detectable also in combination with gentamicin, with a bactericidal effect after 6 h. Combinations with amikacin, cefepime, ceftazidime and piperacillin/tazobactam demonstrated no significant differences (Figure 1). Statistical analysis confirmed synergy in combinations with gentamicin and colistin.

Isolate *P. aeruginosa* P45 with MDR phenotype showed a reduction of 2 log10 CFU/ml after 24 h for combinations with cefepime, piperacillin/tazobactam, gentamicin, imipenem and colistin, of which combinations with cefepime, piperacillin/tazobactam and colistin were statistically significant. A bactericidal effect was exhibited in combinations with imipenem and gentamicin after 6 h and 24 h, respectively. Combinations with piperacillin/tazobactam resulted in bacterial killing with no detectable growth after 24 h, while the same effect was demonstrated in combination with colistin already after 8 h with no regrowth at 24 h. A synergistic effect was noticed in combination with ceftazidime after 6 h, but regrowth occurred after 24 h. Combinations with ciprofloxacin and amikacin demonstrated no significant differences in the time-kill kinetics (Figure 1).

Overall, combinations with gentamicin and colistin mostly resulted in synergy and presented a strong bactericidal effect after 24 h. A strong bactericidal effect was also observed in combinations with imipenem (Figure 1). *[Figure 1 near here]*

The two different synergy assays showed different levels of correlation. Correspondence was observed in 1 of 4 isolates in combination with ceftazidime,

piperacillin/tazobactam, imipenem and colistin, 2 of 4 isolates in combination with gentamicin, and mostly in combination with cefepime, amikacin and ciprofloxacin in 3 of 4 isolates (Table 3). It should be noted that GDS cross method offers determination of the additive effect, which cannot be assessed with TK method. *[Table 3 near here]*

PAE

PAE values were determined for the same isolates for which time-kill kinetics were assessed. Fosfomycin produced a short PAE in all tested strains. PAE values are shown in Table 4.

A short PAE was observed with imipenem ranging from 0.15 to 0.99, while the longest was observed with ciprofloxacin with duration up to 4.45 h. Combinations with fosfomycin mostly prolonged the PAE, with a switch from short to moderate in 1 of 4 isolates with imipenem, amikacin and gentamicin. In combination with colistin the effect was prolonged from moderate to prolonged in half of the isolates. The longest PAE was observed in combination with ciprofloxacin (>6 h).

PAE synergism was observed in 1 of 4 combinations with colistin. Other combinations with colistin resulted in addition. Combinations with gentamicin, ciprofloxacin and imipenem resulted in addition and indifference in half of the isolates, respectively. All combinations with amikacin were indifferent. *[Table 4 near here]*

Discussion

In this study we tried to assess the lack in evidence of synergy regarding MDR, XDR, as well as isolates harbouring β-lactamases, particularly MBLs, as last-resort carbapenem utility is vastly impacted by their presence[61], especially due to the possibility of restoring antibiotic susceptibility in combination.[11] For all antibiotic combinations synergy was assessed comparing the dynamic time-kill method, as the gold standard for synergy testing,

and GDS cross method, as a static single concentration method. Since broth dilution methods are not recommended for fosfomycin MIC determination, checkerboard method was not used in this study.[33]The main finding of our study is that synergy and the additive effect were most frequently observed in combination with ceftazidime and gentamicin, utilizing GDS cross method, regardless of the presence of acquired β-lactamases. Employing the time-kill method, we noted synergy in one of four isolates in combination with both, ceftazidime and gentamicin, and an additive effect in two of our isolates in combination with gentamicin. Similar data concerning *P. aeruginosa,* regardless of the resistance phenotype, were published in a recent review paper.[22] The mechanism suggested for synergy with ceftazidime is that fosfomycin, inhibiting an earlier enzymatic step in cell wall synthesis, is boosting β-lactam activity, increasing membrane permeability for β- lactam antibiotics.[10] It is plausible that fosfomycin also increases cellular uptake of aminoglycosides, resulting in increased protein synthesis inhibition and death.[18]

Substantial bacterial killing of more than 99.9 % (> 3 log10) was revealed in combinations with gentamicin, imipenem and colistin in three of four isolates. Our data also indicate bacteriostatic activity of fosfomycin against *P. aeruginosa*, which is in accordance with previously published data.[9] In our study, the most prominent difference in synergy results among the two methods used, was observed in combinations with colistin. We observed high levels of indifference when GDS cross method was used, while according to our time-kill assay results, colistin demonstrated synergy in three out of four isolates with a substantial bactericidal effect. Discrepancy in the levels of synergy with colistin depending on the method used is recorded in literature.[8,62] Furthermore, with time-kill method, only the XDR isolate P14, harbouring VIM MBL exhibited neither bactericidal effect, nor synergy. When we assessed synergy using the other three MDR isolates lacking carbapenemases, synergy was observed in combinations with β-lactam, as well as other antipseudomonal

antibiotics. One explanation could be antibiotic hydrolysis in the presence of VIM metallo-β-lactamase, diminishing the synergistic effect of the combinations. It should be taken into account that the isolates positive for certain resistance trait, for instance, VIM-2 or PER were clinical isolates very likely, possessing other resistance mechanisms such as hyperexpression of efflux pumps or porin loss, which also contribute to resistance, and thus it is very difficult to estimate the relationship between the resistance traits and pharmacodynamics response to an antibiotic or combination. Isolates positive for MBL were in most cases XDR, whereas those without the carbapenemase were MDR. Moreover, there were only two isolates with ESBLs included in the study which is far too less to make comparisons with those harbouring MBLs and to establish correlation between the resistance traits and antibiotic response.

Additionally, a MIC lowering effect using GDS cross method in all antibiotic combinations was observed, mostly again with ceftazidime and gentamicin. Lowering of the fosfomycin MIC by the antibiotic used in combination could render fosfomycin susceptible and usable in the therapy in case that the MIC of the tested isolate is slightly above the breakpoint value. This is also the case in all additive interactions, where the FICI values from > 0.5 to ≤ 1 indicate that the MIC values of used antibiotics are lowered in the presence of each other, which may be of interest to clinicians in marginal susceptibility reports. Moreover, GDS cross method is a quick technique easy to implement in clinical laboratories.

As far as we know, this is the first report describing the *in vitro* PAE of the here used antimicrobial combinations with fosfomycin: ceftazidime, cefepime, piperacillin/tazobactam, gentamicin, amikacin, ciprofloxacin, imipenem and colistin. The PAE of the tobramycin/fosfomycin combination was reported to be superior to the effect of antimicrobials alone.[19] We observed a short fosfomycin PAE of the four tested isolates, roughly around 1 h with peak serum levels of the drug. .Combinations with aminoglycosides prolonged the PAE in one of four isolates. Also, combinations with gentamicin demonstrated

addition in half of the isolates. Although no major PAE synergism was noticed in our study, the effect of antibiotic combinations was superior to that of the drugs alone. This was especially observed in combinations with colistin.

Our findings indicated that a substantial proportion (52.9 %) of clinical isolates are already resistant to fosfomycin. In our study synergy of fosfomycin with other antibiotics appears to be strain dependent, related to the specific properties of a particular strain and dependent on the method chosen for synergy analysis. Further evaluation of both time-kill and GDS method using a larger group of organisms could provide a more accurate assessment of synergy potential and a more accurate statistical analysis. Nevertheless, our *in vitro* results demonstrate potential benefits of using fosfomycin in combination with other antibiotics against MDR *P. aeruginosa* isolates where therapy options are extremely scarce and narrowing by day. Moreover, GDS cross method may prove to be a feasible method in a clinical laboratory when MIC values are intermediate or slightly above the resistance breakpoint value, in order to assist in the optimal combination therapy choice. The limitation of the study is a small number of isolates included in the study, and the fact that the only detected carbapenemase was VIM-2. There were no other carbapenemases included because they are not available. The number of ESBL positive organisms included in the study is also very small as they are rarely detected in this species. The experiments were performed in duplicate, which presents a limitation of statistical analysis.

Contributors

Mia Slade Vitković: experiment performance, data and laboratory analysis, manuscript preparation; Branka Bedenić: study design, supervision of laboratory work and manuscript preparation; Luka Bielen: study design, manuscript preparation; Ivanka Batarilo: manuscript preparation; Sara Kiebel: laboratory analysis; Gordana Maravić Vlahoviček: study design,

result analysis and manuscript preparation

Ethical approval

The Ethical Committee permission was the obtained from University Hospital Center Zagreb Ethical Committee, class 8.1-19/242-2, number 02/21 AG.

Conflict of interest

The authors report no competing interests to declare.

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References

1. Pagani L, Mantengoli E, Migliavacca R, Nucleo E, Pollini S, Spalla M, et al. Multifocal Detection of Multidrug-Resistant *Pseudomonas aeruginosa* Producing the PER-1 Extended-Spectrum β-Lactamase in Northern Italy. J Clin Microbiol 2004;42:2523–9.
2. Tümmler B. Emerging therapies against infections with *Pseudomonas aeruginosa*. F1000Research 2019;8:1371.
3. Aloush V, Navon-Venezia S, Seigman-Igra Y, Cabili S, Carmeli Y. Multidrug-Resistant *Pseudomonas aeruginosa*: Risk Factors and Clinical Impact. Antimicrob Agents Chemother 2006;50:43–8.
4. Livermore DM. Multiple Mechanisms of Antimicrobial Resistance in *Pseudomonas aeruginosa*: Our Worst Nightmare? Clin Infect Dis 2002;34:634–40.
5. Poole K. *Pseudomonas aeruginosa*: resistance to the max. Front Microbiol 2011;2:65.

6. Pournaras S, Maniati M, Spanakis N, Ikonomidis A, Tassios PT, Tsakris A, et al. Spread of efflux pump-overexpressing, non-metallo-β-lactamase-producing, meropenem-resistant but ceftazidime-susceptible *Pseudomonas aeruginosa* in a region with blaVIM endemicity. J Antimicrob Chemother 2005;56:761–4.
7. Walsh CC, Landersdorfer CB, McIntosh MP, Peleg AY, Hirsch EB, Kirkpatrick CM, et al. Clinically relevant concentrations of fosfomycin combined with polymyxin B, tobramycin or ciprofloxacin enhance bacterial killing of *Pseudomonas aeruginosa*, but do not suppress the emergence of fosfomycin resistance. J Antimicrob Chemother 2016;71:2218–29.
8. Samonis G, Maraki S, Karageorgopoulos DE, Vouloumanou EK, Falagas ME. Synergy of fosfomycin with carbapenems, colistin, netilmicin, and tigecycline against multidrug-resistant *Klebsiella pneumoniae, Escherichia coli*, and *Pseudomonas aeruginosa* clinical isolates. Eur J Clin Microbiol Infect Dis 2012;31:695–701.
9. Walsh CC, McIntosh MP, Peleg AY, Kirkpatrick CM, Bergen PJ. *In vitro* pharmacodynamics of fosfomycin against clinical isolates of *Pseudomonas aeruginosa*. J Antimicrob Chemother 2015;70:3042–50.
10. Yousef Memar M, Adibkia K, Farajnia S, Kafil HS, Khalili Y, Azargun R, et al. *In-vitro* Effect of Imipenem, Fosfomycin, Colistin, and Gentamicin Combination against Carbapenem-resistant and Biofilm-forming *Pseudomonas aeruginosa* Isolated from Burn Patients. Iran J Pharm Res 2021;20:286–96.
11. Avery LM, Sutherland CA, Nicolau DP. Prevalence of *in vitro* synergistic antibiotic interaction between fosfomycin and nonsusceptible antimicrobials in carbapenem-resistant *Pseudomonas aeruginosa*. J Med Microbiol 2019;68:893–7.
12. Buisson Y, Bercion R, Mauclère P, Hugard L, Schill H. Preliminary study of the antagonistic effects between fosfomycin and beta-lactams on *Pseudomonas aeruginosa*

observed on the antibiogram. Pathol Biol (Paris) 1988;36:671–4.

13. Reguera JA, Baquero F, Berenguer J, Martinez-Ferrer M, Martinez JL. Beta-lactam-fosfomycin antagonism involving modification of penicillin-binding protein 3 in *Pseudomonas aeruginosa*. Antimicrob Agents Chemother 1990;34:2093–6.

14. Pruekprasert P, Tunyapanit W. *In vitro* activity of fosfomycin-gentamicin, fosfomycin-ceftazidime, fosfomycin-imipenem and ceftazidime-gentamicin combinations against ceftazidime-resistant *Pseudomonas aeruginosa*. Southeast Asian J Trop Med Public Health 2005;36:1239–42.

15. Cuba GT, Rocha-Santos G, Cayô R, Streling AP, Nodari CS, Gales AC, et al. *In vitro* synergy of ceftolozane/tazobactam in combination with fosfomycin or aztreonam against MDR *Pseudomonas aeruginosa*. J Antimicrob Chemother 2020;75:1874–8.

16. Kastoris AC, Rafailidis PI, Vouloumanou EK, Gkegkes ID, Falagas ME. Synergy of fosfomycin with other antibiotics for Gram-positive and Gram-negative bacteria. Eur J Clin Pharmacol 2010;66:359–68.

17. Drusano GL, Neely MN, Yamada WM, Duncanson B, Brown D, Maynard M, et al. The Combination of Fosfomycin plus Meropenem Is Synergistic for *Pseudomonas aeruginosa* PAO1 in a Hollow-Fiber Infection Model. Antimicrob Agents Chemother 2018;62:e01682-18.

18. MacLeod DL, Velayudhan J, Kenney TF, Therrien JH, Sutherland JL, Barker LM, et al. Fosfomycin Enhances the Active Transport of Tobramycin in *Pseudomonas aeruginosa*. Antimicrob Agents Chemother 2012;56:1529–38.

19. MacLeod DL, Barker LM, Sutherland JL, Moss SC, Gurgel JL, Kenney TF, et al. Antibacterial activities of a fosfomycin/tobramycin combination: a novel inhaled antibiotic for bronchiectasis. J Antimicrob Chemother 2009;64:829–36.

20. Yamada S, Hyo Y, Ohmori S, Ohuchi M. Role of Ciprofloxacin in Its Synergistic

Effect with Fosfomycin on Drug-Resistant Strains of *Pseudomonas aeruginosa*. Chemotherapy 2007;53:202–9.

21. Figueredo VM, Neu HC. Synergy of ciprofloxacin with fosfomycin *in vitro* against *Pseudomonas* isolates from patients with cystic fibrosis. J Antimicrob Chemother 1988;22:41–50.

22. Antonello RM, Principe L, Maraolo AE, Viaggi V, Pol R, Fabbiani M, et al. Fosfomycin as Partner Drug for Systemic Infection Management. A Systematic Review of Its Synergistic Properties from *In Vitro* and *In Vivo* Studies. Antibiotics 2020;9:500.

23. Monogue ML, Nicolau DP. Antibacterial activity of ceftolozane/tazobactam alone and in combination with other antimicrobial agents against MDR *Pseudomonas aeruginosa*. J Antimicrob Chemother 2018;1:942–52.

24. Jahan S, Davis H, Ashcraft DS, Pankey GA. Evaluation of the *in vitro* interaction of fosfomycin and meropenem against metallo-β-lactamase–producing *Pseudomonas aeruginosa* using Etest and time-kill assay. J Investig Med 2021;69:371–6.

25. Mikhail S, Singh NB, Kebriaei R, Rice SA, Stamper KC, Castanheira M, et al. Evaluation of the Synergy of Ceftazidime-Avibactam in Combination with Meropenem, Amikacin, Aztreonam, Colistin, or Fosfomycin against Well-Characterized Multidrug-Resistant *Klebsiella pneumoniae* and *Pseudomonas aeruginosa*. Antimicrob Agents Chemother 2019;63:e00779-19.

26. Papp-Wallace KM, Zeiser ET, Becka SA, Park S, Wilson BM, Winkler ML, et al. Ceftazidime-Avibactam in Combination With Fosfomycin: A Novel Therapeutic Strategy Against Multidrug-Resistant *Pseudomonas aeruginosa*. J Infect Dis 2019;220:666–76.

27. Olsson A, Wistrand-Yuen P, Nielsen EI, Friberg LE, Sandegren L, Lagerbäck P, et al.

Efficacy of Antibiotic Combinations against Multidrug-Resistant *Pseudomonas aeruginosa* in Automated Time-Lapse Microscopy and Static Time-Kill Experiments. Antimicrob Agents Chemother 2020;64:e02111-19.

28. Avery LM, Nicolau DP. Feasibility of routine synergy testing using antibiotic gradient diffusion strips in the clinical laboratory. J Antimicrob Chemother 2018;73:2264–5.
29. White RL, Burgess DS, Manduru M, Bosso JA. Comparison of three different *in vitro* methods of detecting synergy: time-kill, checkerboard, and E test. Antimicrob Agents Chemother 1996;40:1914–8.
30. Brennan-Krohn T, Kirby JE. Antimicrobial Synergy Testing by the Inkjet Printer-assisted Automated Checkerboard Array and the Manual Time-kill Method. J Vis Exp 2019;e58636.
31. Giamarellos-Bourboulis EJ, Kentepozidis N, Antonopoulou A, Plachouras D, Tsaganos T, Giamarellou H. Postantibiotic effect of antimicrobial combinations on multidrug-resistant *Pseudomonas aeruginosa*. Diagn Microbiol Infect Dis 2005;51:113–7.
32. Sardelic S, Bedenic B, Colinon-Dupuich C, Orhanovic S, Bosnjak Z, Plecko V, et al. Infrequent Finding of Metallo-Lactamase VIM-2 in Carbapenem-Resistant *Pseudomonas aeruginosa* Strains from Croatia. 2012;56:2746–9.
33. CLSI. Performance Standards for Antimicrobial Susceptibility Testing, document M100, 30th edition. Wayne, PA: Clinical and Laboratory Standards Institute; 2020.
34. López-Montesinos I, Horcajada JP. Oral and intravenous fosfomycin in complicated urinary tract infections. Rev Esp Quimioter 2019;32:37–44.
35. Magiorakos AP, Srinivasan A, Carey RB, Carmeli Y, Falagas ME, Giske CG, et al. Multidrug-resistant, extensively drug-resistant and pandrug-resistant bacteria: an international expert proposal for interim standard definitions for acquired resistance.

Clin Microbiol Infect 2012;18:268–81.

36. CLSI. Performance Standards for Antimicrobial Susceptibility Testing, document M100, 27th edition. 2017.

37. van der Zwaluw K, de Haan A, Pluister GN, Bootsma HJ, de Neeling AJ, Schouls LM. The Carbapenem Inactivation Method (CIM), a Simple and Low-Cost Alternative for the Carba NP Test to Assess Phenotypic Carbapenemase Activity in Gram-Negative Rods. PLoS One 2015;10:e0123690.

38. Franklin C, Liolios L, Peleg AY. Phenotypic Detection of Carbapenem-Susceptible Metallo-Lactamase-Producing Gram-Negative Bacilli in the Clinical Laboratory. J Clin Microbiol 2006;44:3139–44.

39. Sachdeva R, Sharma B, Sharma R. Evaluation of different phenotypic tests for detection of metallo-β-lactamases in imipenem-resistant *Pseudomonas aeruginosa*. J Lab Physicians 2017;9:249–53.

40. Mazzariol A, Mammina C, Koncan R, Di Gaetano V, Di Carlo P, Cipolla D, et al. A novel VIM-type metallo-beta-lactamase (VIM-14) in a *Pseudomonas aeruginosa* clinical isolate from a neonatal intensive care unit. Clin Microbiol Infect 2011;17:722–4.

41. Bośnjak Z, Bedenić B, Mazzariol A, Jarža-Davila N, Šuto S, Kalenić S. VIM-2 beta-lactamase in *Pseudomonas aeruginosa* isolates from Zagreb, Croatia. Scand J Infect Dis 2010;42:193–7.

42. Bubonja-Sonje M, Matovina M, Skrobonja I, Bedenic B, Abram M. Mechanisms of Carbapenem Resistance in Multidrug-Resistant Clinical Isolates of *Pseudomonas aeruginosa* from a Croatian Hospital. Microb Drug Resist 2015;21:261–9.

43. Poirel L, Nordmann P. Acquired Carbapenem-Hydrolyzing Beta-Lactamases and their Genetic Support. Curr Pharm Biotechnol 2005;3:117–27.

44. Doern CD. When Does 2 Plus 2 Equal 5? A Review of Antimicrobial Synergy Testing. J Clin Microbiol 2014;52:4124–8.

45. FDA. Ceptaz (ceftazidime for injection) [Internet]. England, GlaxoSmithKline, Research Triangle Park, NC 27709; Available from: https://www.accessdata.fda.gov/drugsatfda_docs/label/2002/050646s014lbl.pdf

46. FDA. Gentamicin injection [Internet]. Fresenius Kabi USA, LCC. 2013. Available from: https://www.accessdata.fda.gov/drugsatfda_docs/label/2014/062366s033lbl.pdf

47. FDA. Amikacin Sulfate [Internet]. Abbott laboratories, North Chicago, IL 60064, USA; Available from: https://www.accessdata.fda.gov/drugsatfda_docs/nda/97/64146AP.PDF

48. FDA. Primaxin (imipenem and cilastatin) for injection, for intravenous use [Internet]. Merck&Co., Inc.Whitehouse Station, NJ. USA. 2016. Available from: https://www.accessdata.fda.gov/drugsatfda_docs/label/2016/050587s074lbl.pdf

49. Car H. In vitro synergy and postantibiotic effect of colistin combinations with meropenem and vancomycin against gram negative bacteria with multiple carbapenem resistance mechanisms. Josip Juraj Strossmayer Univ Osijek 2020;198.

50. Markou N, Markantonis SL, Dimitrakis E, Panidis D, Boutzouka E, Karatzas S, 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 2008;30:143–51.

51. Moni M, Sudhir S, Dipu TS, Mohamed Z, Prabhu BP, Edathadathil F, et al. Clinical efficacy and pharmacokinetics of colistimethate sodium and colistin in critically ill patients in an Indian hospital with high endemic rates of multidrug-resistant Gram-negative bacterial infections: A prospective observational study. Int J Infect Dis 2020;100:497–506.

52. Tängdén T, Hickman RA, Forsberg P, Lagerbäck P, Giske CG, Cars O. Evaluation of Double-and Triple-Antibiotic Combinations for VIM-and NDM-Producing *Klebsiella pneumoniae* by *In Vitro* Time-Kill Experiments. 2014;58:1757–62.

53. Silva F, Lourenço O, Queiroz JA, Domingues FC. Bacteriostatic versus bactericidal activity of ciprofloxacin in *Escherichia coli* assessed by flow cytometry using a novel far-red dye. J Antibiot (Tokyo) 2011;64:321–5.

54. Jacqueline C, Caillon J, Le Mabecque V, Miègeville AF, Donnio PY, Bugnon D, et al. *In vitro* activity of linezolid alone and in combination with gentamicin, vancomycin or rifampicin against methicillin-resistant *Staphylococcus aureus* by time-kill curve methods. J Antimicrob Chemother 2003;51:857–64.

55. Bundtzen RW, Gerber AU, Cohn DL, Craig WA. Postantibiotic Suppression of Bacterial Growth. Clin Infect Dis 1981;3:28–37.

56. Craig WA. The postantibiotic effect. Clin Microbiol Newsl 1991;13:121–4.

57. Munckhof WJ, Borlace G, Turnidge JD. Postantibiotic Suppression of Growth of Erythromycin A-Susceptible and-Resistant Gram-Positive Bacteria by the Ketolides Telithromycin (HMR 3647) and HMR 3004. Antimicrob Agents Chemother 2000;44:1749–53.

58. Bedenić B, Beader N, Godič-Torkar K, Prahin E, Mihaljević L, Ćačić M, et al. Postantibiotic effect of colistin alone and combined with vancomycin or meropenem against *Acinetobacter spp.* with well defined resistance mechanisms. J Chemother 2016;28:375–82.

59. Li RC, Tang MC. Post-antibiotic effect induced by an antibiotic combination: influence of altered susceptibility to individual components. J Antimicrob Chemother 2005;55:583–6.

60. Ferrara A, Santos C Dos, Cimbro M. Postantibiotic effect of meropenem in

combination with gentamicin or sparfloxacin on Gram-positive and Gram-negative organisms. Clin Microbiol Infect 1998;4:431–5.

61. Codjoe FS, Donkor ES. Carbapenem Resistance: A Review. Med Sci 2018;6:1.

62. Di X, Wang R, Liu B, Zhang X, Ni W, Wang J, et al. *In vitro* activity of fosfomycin in combination with colistin against clinical isolates of carbapenem-resistant *Pseudomas aeruginosa*. J Antibiot (Tokyo) 2015;68:551–5.

Table 1. Susceptibility of *P. aeruginosa* isolates and carbapenemase detection

[a] MIC determined by agar dilution. MIC FOM ≤64 mg/L was considered susceptible, MIC FOM >64 mg/L resistant

[b] MDR (multidrug resistant): non-susceptible to ≥1 agent in ≥3 antimicrobial categories, XDR (extensively drug-resistant): non-susceptible to ≥1 agent in all but ≤2 categories[35]

Abbreviations: CIM - carbapenemase inhibition test, HODGE – modified Hodge test BL - β-lactamase, CD – combined disc with EDTA for detection of MBLs, nt - not tested

Table 2. GDS cross method rates of fosfomycin in combination with various antibiotics

[a] Fractional inhibitory concentration index (FICI)

Abbreviations: fosfomycin (FOM), cefepime (FEP), piperacillin/tazobactam (TZP), imipenem (IMI), ciprofloxacin (CIP), ceftazidime (CAZ), gentamicin (GM), colistin (COL) amikacin (AM); SIN - synergy, AD – additive effect, IND – indifferent effect, ANT – antagonism

Table 3. Comparison of synergy tests: GDS cross method (results in FICI) and time kill assay (results in Δ LOG10) for P. aeruginosa P14, P32, P36 and P45.

Abbreviations: fosfomycin (FOM), cefepime (FEP), piperacillin/tazobactam (TZP), imipenem (IMI), ciprofloxacin (CIP), ceftazidime (CAZ), gentamicin (GM), colistin (COL) and amikacin (AM). SIN – synergy, AD – additive effect, IND – indifference, ANT – antagonism

Table 4. PAE values (hours) for *P. aeruginosa* P14, P32, P36 and P45.

Abbreviations: fosfomycin (FOM), cefepime (FEP), piperacillin/tazobactam (TZP), imipenem (IMI), ciprofloxacin (CIP), ceftazidime (CAZ), gentamicin (GM), colistin (COL) and amikacin (AM).

Figure 1. Time kill kinetics for P14, P32, P36 and P45: unexposed, exposed to single antibiotics and in combination

Abbreviations: K – unexposed control, Fosfomycin (FOM), cefepime (FEP), piperacillin/tazobactam (TZP), imipenem (IMI), ciprofloxacin (CIP), ceftazidime (CAZ), gentamicin (GM), colistin (COL) and amikacin (AM)

Error bars represent standard deviations of duplicate determinations