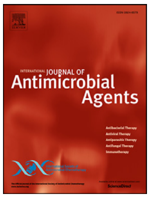
International Journal of Antimicrobial Agents 53 (2019) 698–702

Contents lists available at ScienceDirect

International Journal of Antimicrobial Agents

journal homepage: www.elsevier.com/locate/ijantimicag



Short Communication

Where should antibiotic gradient diffusion strips be crossed to assess synergy? A comparison of the standard method with a novel method using steady-state antimicrobial concentrations

Ana Motos[a,b], Lindsay M. Avery[a], Kailynn J. DeRonde[c], Elias M. Mullane[a], Joseph L. Kuti[a], David P. Nicolau[a,d,*]

[a] Center for Anti-Infective Research and Development, Hartford Hospital, Hartford, CT, USA
[b] Division of Animal Experimentation, Department of Pulmonary and Critical Care Medicine, Hospital Clinic, Barcelona, Spain
[c] Department of Pharmacy, Connecticut Children's Medical Center, Hartford, CT, USA
[d] Division of Infectious Diseases, Hartford Hospital, Hartford, CT, USA

ARTICLE INFO

Article history:
Received 6 November 2018
Accepted 9 March 2019

Editor: Jean-Marc Rolain

Keywords:
Synergy
Gradient diffusion strips
Pseudomonas aeruginosa
Multi-drug resistance

ABSTRACT

Multi-drug resistance among *Pseudomonas aeruginosa* in hospitals, and particularly intensive care units, has achieved alarming rates. Some combination antimicrobial therapies have demonstrated promising synergistic effects and an ability to overcome resistance without increasing drug-related toxicities. Nevertheless, rapid and feasible methods to identify synergy have not been routinely implemented in clinical microbiology laboratories.

Synergistic activity of meropenem plus tobramycin or levofloxacin against clinical *P. aeruginosa* isolates (N=21) was assessed by two different methods using gradient diffusion strips (GDSs). A 90° angle was created at the intersection of the minimum inhibitory concentration (MIC) of each drug by the standard method, and by a novel method, the cross was placed at clinically relevant steady-state concentrations (C_{ss}) based on recommended dosing regimens. Fractional inhibitory concentration indexes were determined to describe antibiotic interactions. Time-kill analyses were performed over 24 h in duplicate for instances of discordance between the standard cross method and the novel method.

Synergy between meropenem and tobramycin by the novel method was observed in one (4.8%) isolate and between meropenem and levofloxacin in two (9.5%) isolates. Agreement with the standard method was 86–100% for meropenem plus tobramycin and meropenem plus levofloxacin combinations, respectively. Time-kill studies resulted in agreement with GDSs crossed at C_{ss} in two of three instances of discordance between GDS methods.

This novel method of synergy testing that involves crossing GDSs at steady-state concentrations may be a rapid and feasible tool for routine practice. Further comparisons of this novel procedure with time-kill methods are needed.

1. Background

Multi-drug resistant (MDR) *Pseudomonas aeruginosa* is becoming increasingly pervasive [1]. One approach to combat elevations of the minimum inhibitory concentration (MIC) is to treat patients with synergistic antibiotic combinations, which have the potential to overcome resistance and decrease the risk of toxicity associated with high antimicrobial serum levels [2].

Synergy occurs when the combination of two or more antimicrobials results in activity greater than the sum of the actions of each antimicrobial alone [3]. Combination therapy with meropenem and tobramycin or levofloxacin has been routinely used in the setting of MDR *P. aeruginosa* infection. Identification of in vitro synergistic activity may improve the informed selection of antibiotic therapy, though rapid (<24 h after isolated pathogen) methods to detect these interactions have not been routinely adopted in clinical microbiology laboratories [4]. Crossing antibiotic gradient diffusion strips (GDSs) at 90° angles at MICs, instead of checkerboard or time-kill assay (TKA) methodologies, is an accepted, easily-applied technique used to identify in vitro synergistic activity [5]. In fact, this practice requires knowledge of MIC up front, and therefore takes ~48 h to complete once the pathogen

---

[*] Corresponding author. Center for Anti-Infective Research and Development, Hartford Hospital, 80 Seymour Street, Hartford, CT 06102, USA. Tel.: +1 860 972 3941; fax: +1 860 545 3992.

E-mail address: david.nicolau@hhchealth.org (D.P. Nicolau).

https://doi.org/10.1016/j.ijantimicag.2019.03.006
0924-8579/

is isolated, limiting its applicability to promptly improve patient care [4]. Moreover, synergy may be identified at concentrations exceedingly higher than clinically achievable concentrations – albeit a noteworthy observation, but one of less usefulness to the treating clinician. In this context, we propose a novel and more rapid synergy method crossing GDS at clinically relevant concentrations, trying to mimic the in vivo exposures achieved with standard antibiotic dosing regimens and without the prerequisite of determining the MIC. In the study, the synergy agreement was assessed between a method crossing GDSs at steady state concentrations with the standard GDS method in a diverse sample of clinical *P. aeruginosa* isolates.

2. Materials and methods

2.1. Antimicrobial Susceptibility Testing

Clinical *P. aeruginosa* isolates exhibiting a wide range of meropenem MICs were selected for study. The isolates were previously collected from several hospital systems within the USA and indexed into the Center for Anti-Infective Research and Development (Hartford, CT, USA). The MICs of meropenem, tobramycin, and levofloxacin were determined by GDSs (Etest®, bioMérieux, Durham, NC, USA) as manufacturer's instructions recommend. An inoculum of ~8 $\log_{10}$ colony forming units per mL ($\log_{10}$ cfu/mL) was streaked onto Mueller–Hinton II agar (MHA, Becton Dickinson & Co., Sparks, MD, USA) and GDSs were placed. Colony counts on Trypticase™ Soy Agar II with 5% Sheep Blood (Becton Dickinson & Co., Sparks, MD, USA), performed to ensure appropriate inoculum concentration, and MIC results were read following 16–20 h of incubation at 37°C.

2.2. Synergy assessment by GDSs

Antibiotic interactions between meropenem and tobramycin or levofloxacin were both evaluated at the predetermined MICs as well as at approximated a priori average steady-state concentrations (C_{ss}) safely achievable in adult patients. The antibiotic C_{ss} were calculated by dividing the area under the free drug concentration time curve (*f*AUC) by 24 h. *f*AUC was determined by the ratio between recommended dosing regimens for critically ill patients (2 g of meropenem q8h, 7 mg/kg of tobramycin q24h, 750 mg of levofloxacin q24h) [6] and clearance in those patients with normal kidney function (creatinine clearance ≥50 mL/min) [7–9], and multiplying by the unbound fraction [9–11]. Using previous literature data, C_{ss} equal to 16 mg/L, 6 mg/L, and 3 mg/L were selected for meropenem, tobramycin, and levofloxacin, respectively. An inoculum of ~8 $\log_{10}$ cfu/mL was streaked onto MHA and GDSs were placed at 90° angles and intersected at the predetermined MICs or at the C_{ss} as defined above [5]. The MICs in combination were read following 16–20 h of incubation at 37°C as the values at which the inhibition zone intersected the scale on each GDS. The mean fractional inhibitory concentration index (FICI) was calculated by dividing the mean MIC of each drug in combination by the MIC of each drug alone and adding the results. The FICI results were interpreted as follows: ≤0.5 as synergy; >0.5 to ≤4 as no interaction; and >4 as antagonism [12].

2.3. TKA

When discordant results between GDS methods were recorded, TKA, the 'gold standard" synergy assessment [5], was performed in duplicate as previously described [13]. Briefly, free peak and trough concentrations of meropenem (65.7 and 4.3 mg/L) and tobramycin (21.2 and 0.2 mg/L) alone and in combination were tested for each discordant isolate during 24 h in 10 mL of an initial ~6 $\log_{10}$ cfu/mL bacterial culture in cation-adjusted Mueller–Hinton II broth. Antibiotic concentrations were chosen on the basis of previous recommended dosing regimens and the standard TKA procedure [6,13]. Positive control samples, a bacterial culture without any added antimicrobial, were concurrently conducted. At 0, 3, 6 and 24 h following inoculation, the bacterial burden in each sample was quantified (limit of detection was 2.70 $\log_{10}$ cfu/mL) using serial dilution and colony count techniques. The results were read after 16–20 h of incubation at 35°C. Synergy was defined as a reduction of ≥2 $\log_{10}$ cfu/mL among the antibiotic combinations tested compared with its most active single component after 24 h [3].

3. Results

3.1. Antimicrobial Susceptible Testing

MICs of meropenem, tobramycin and levofloxacin varied widely among the 21 tested isolates, ranging from 1.5 to >32, 0.25 to 192, and 0.5 to >32 mg/L, respectively (Supplementary Table S1).

3.2. Synergy assessments and agreement of both GDS methods

Classification of antibiotic interactions according to FICI agreed in 39 of 42 (92.9%) combinations between GDS crossed at the MIC and at the C_{ss}. The 39 agreements were classified as synergy in two isolates and no interaction in 37 isolates (Fig. 1). Agreement between both methods ranged from 86% in meropenem-tobramycin assessments, to 100% in meropenem-levofloxacin assessments (Table 1). Two out of three discordant isolates showed synergy when meropenem and tobramycin GDSs were crossed at each individual MIC and no interaction at C_{ss}, while the other instance was the opposite.

Using the novel method, a low proportion of synergy was found for both combinations, meropenem plus tobramycin (4.8%) and meropenem plus levofloxacin (9.5%). Nevertheless, the combination of tobramycin or levofloxacin with meropenem demonstrated increased potency, with ≥2-fold reductions in meropenem MIC in combination against eight and two isolates, respectively (Supplementary Fig. S1).

3.3. TKAs

TKAs were conducted for *P. aeruginosa* C27-3, C1-1, and C39-9 isolates (Fig. 2) to assess meropenem and tobramycin interactions. None of the concentration combinations yielded synergy, therefore agreeing with the result from the novel method in two of three scenarios.

4. Discussion

Clinical microbiology laboratories can not yet depend on a rapid in vitro method that precisely detects synergistic interactions between two antibiotics. The TKA has been considered the gold standard technique, but unfortunately it is not workable because it is labor-intensive and time-consuming [4]. Moreover, some authors have already highlighted the limitations of TKA, concluding that a true gold standard is still missing [14], In fact, static concentrations may not even translate to in vivo synergy, especially in critically ill patients with changing pharmacokinetic parameters. In order to shorten the assessment, we compared crossing GDS at fixed C_{ss} versus at the MIC of each agent.

Overall, the categorical agreement according to the FICI between both methods was 92.9%. Importantly, TKAs tipped the scale in favor of the novel GDS method in the instances of disagreement.

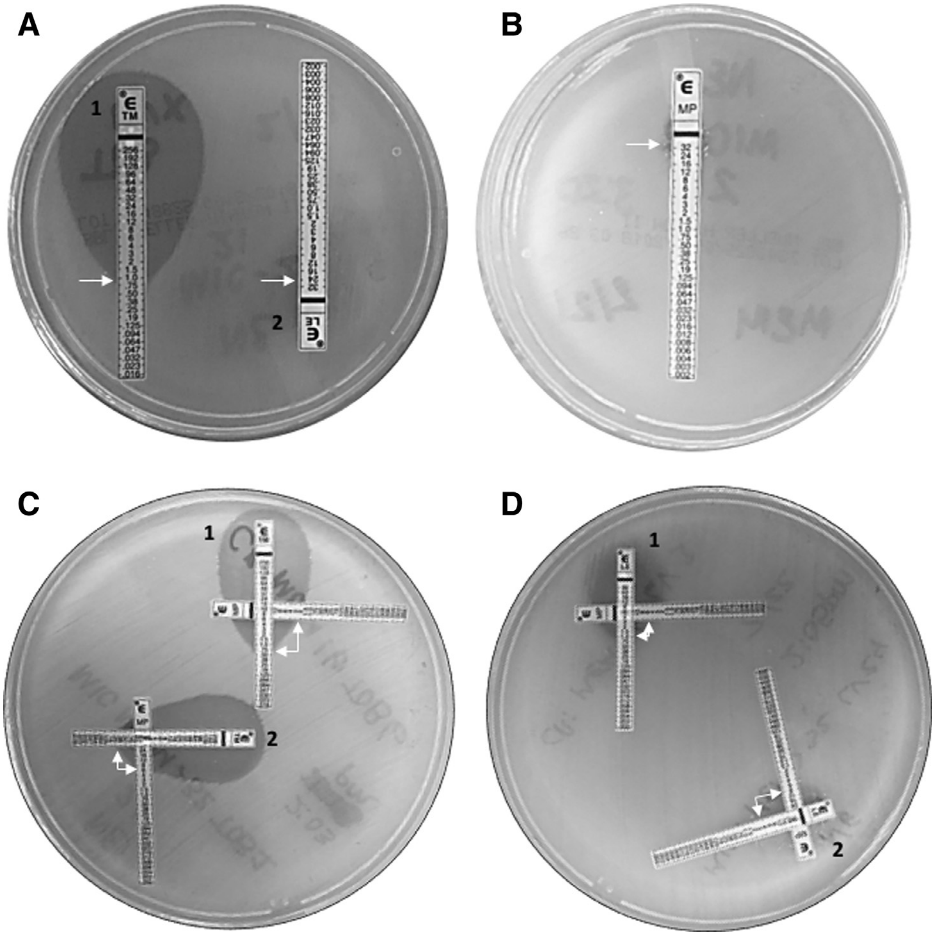


Fig. 1. Images of gradient diffusion strip (GDS) results for minimum inhibitory concentration (MIC) of tobramycin (A(1), 1 mg/L) and levofloxacin (A(2), 24 mg/L) alone, MIC of meropenem (B, >32 mg/L) alone, synergy test of meropenem plus tobramycin crossed at C_{ss} (C(1)) and at MIC (C(2)), and synergy test of meropenem plus levofloxacin crossed at C_{ss} (D(1)) and at MIC (D(2)) with *P. aeruginosa* C11-14. White arrows indicate the MICs. The results were concordant with no interaction between meropenem and tobramycin (fractional inhibitory concentration index (FICI) of 0.51 and 0.80, respectively) and synergistic activity between meropenem and levofloxacin (FICI of 0.44 and 0.27, respectively)

Table 1
Summary of antimicrobial synergy testing of *Pseudomonas aeruginosa* isolates by both gradient diffusion strip methods.

Pseudomonas aeruginosa isolates	Meropenem–tobramycin		Meropenem–Levofloxacin	
	FICI GDS at MIC	FICI GDS at C_{ss}	FICI GDS at MIC	FICI GDS at C_{ss}
C12-18	1.25	1.00	1.50	3.00
C32-15	1.42	1.43	1.17	2.00
C3-5	1.51	2.27	0.88	1.38
C3-18	1.01	1.38	1.50	2.50
C39-13	0.75	1.06	1.50	2.50
C50-5	1.50	2.00	1.58	2.25
C8-21	1.04	1.09	1.17	2.33
C27-3	0.50[a,b]	0.79[b]	0.88	0.94
C3-2	1.50	1.52	2.00	2.00
C3-30	1.13	1.13	1.17	2.00
C11-14	0.51	0.80	0.44[a]	0.27[a]
C15-20	0.63	1.00	1.13	0.78
C17-2	0.71	0.89	1.67	3.33
C21-8	0.88	0.69	2.00	1.67
C1-1	0.44[a,b]	0.83[b]	2.00	2.00
C16-26	0.71	0.88	0.94	1.03
C31-7	0.69	1.06	1.25	1.19
C39-9	0.52[b]	0.38 [a,b]	0.38[a]	0.47[a]
C12-8	0.88	1.00	1.13	2.50
C15-1	0.76	1.50	1.25	0.92
C3-6	0.71	1.08	0.75	0.75

FICI, fractional inhibitory concentration index; MIC, minimum inhibitory concentration.

[a] Synergistic interaction.

[b] Discordant results.

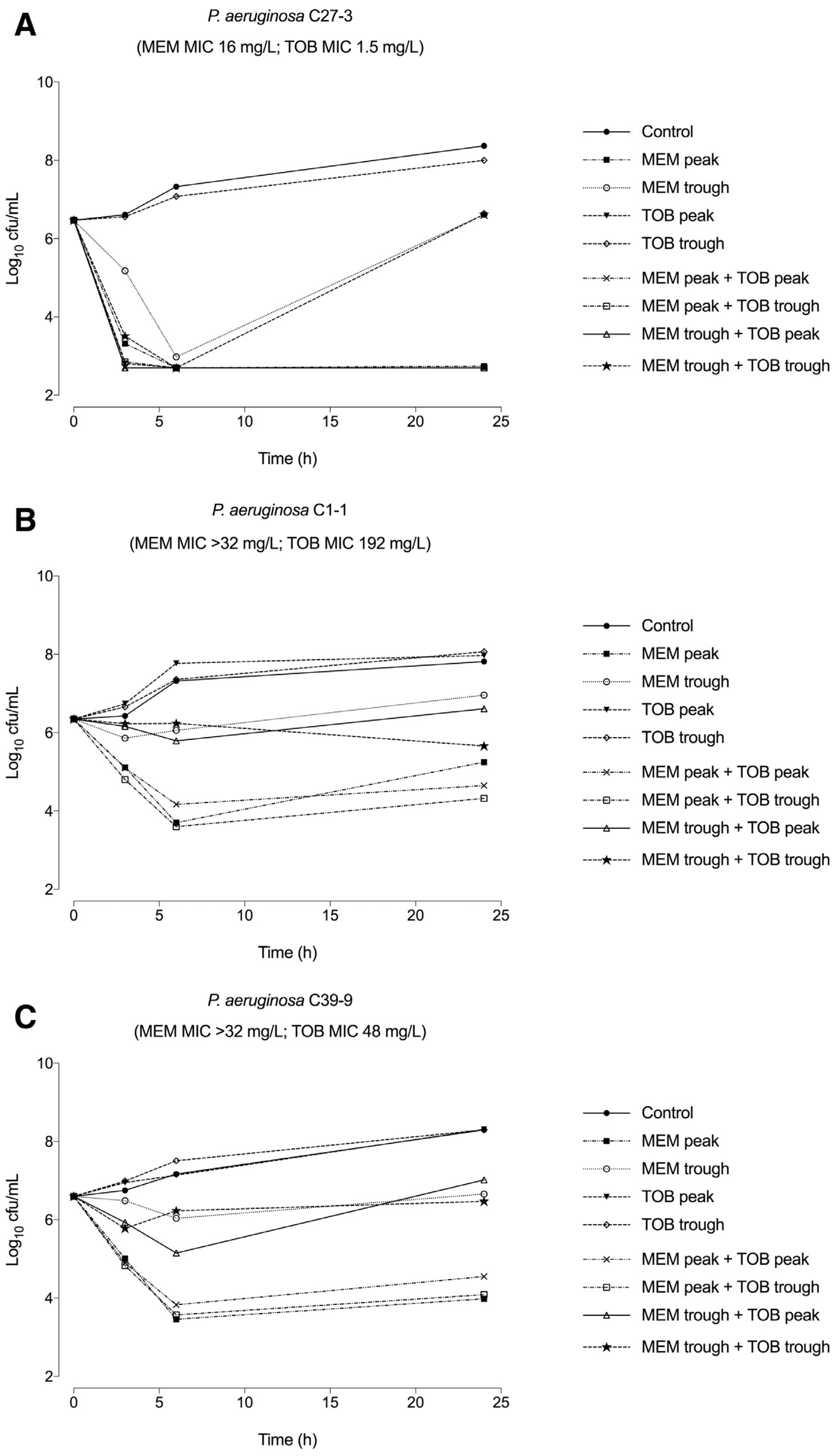


Fig. 2. Results of the time-kill experiments depicting the activity of meropenem and tobramycin alone and in combination at peak and trough concentrations against *P. aeruginosa* C27-3 (A), C1-1 (B), and C39-9 (C) isolates. MEM, meropenem; MIC, minimum inhibitory concentration; TOB, tobramycin.

The low proportion of synergy detected is consistent with previous studies that assessed antimicrobial interactions in *P. aeruginosa* isolates using the same antimicrobial combinations [15,16]; moreover, significant and encouraging decreases in meropenem MICs were detected when using the combination despite producing FICI scores that fell just below the synergistic threshold.

The results of this novel GDS method for testing synergy appear fairly well correlated with the results from the classical GDS synergy method. Several studies have compared synergy testing methods and report a wide range of concordance rates. Balke et al. observed >90% agreement when comparing the checkerboard method and a direct overlay GDS method [15], while White and colleagues reported an agreement from 63 to 75% for GDS methods when comparing obtained FICI scores with TKA results [5].

The key potential benefits of this novel method may be shorter time to results and theoretically increased translatability to the in vivo clinical scenario compared with the standard GDS cross method. First, there is no need to wait for MIC results to proceed with the synergy test, and both can be performed simultaneously which shortens the experiment by 24 h. Second, crossing at C_{ss} allows the technique to more closely mimic the in vivo condition in terms of achievable concentrations. Furthermore, the Css values are available in the current literature for common antibiotics and can be adapted for different dosing regimens and for renally impaired patients.

Some limitations of this study should be mentioned. First, some authors have cautioned regarding the possible disagreement between in vitro and in vivo synergy assessments [17,18]. Second, the real in vivo C_{ss} may be potentially higher or lower depending on patient-specific pharmacokinetics and dosing regimen applied. However, the selected C_{ss} represent an average of in vivo concentrations based on standard dosing regimens and normal kidney function; and may be closer than some exceedingly high MICs. Furthermore, the data described reflect only two dual antibiotic combinations in a small sample of *P. aeruginosa* isolates and remain insufficient to support selection of antibiotic combinations; although we evaluated a phenotypically diverse population in which MICs varied widely, similar larger studies that include additional antibiotic combinations and genotypic information are needed.

Despite the limitations and the challenges with synergy studies, including GDS and TKA methods), this novel method may be performed routinely and that may be closer to the scenario of treated patients in terms of antimicrobial concentrations.

5. Conclusion

Our results suggest that using pre-determined average C_{ss} achieved with standard antibiotic regimens for crossing GDSs may allow for rapid synergy assessments feasible for routine practice in *P. aeruginosa* isolates. Further comparisons of this novel technique with TKA using other drug combinations are needed.

Acknowledgements

We thank Sara Giovagnoli, Michelle Insignares, Alissa Padgett, Kimelyn Greenwood, Elizabeth Cyr, Jennifer Tabor-Rennie, Debora Santini and Janice Cunningham from the Center for Anti-Infective Research and Development, Hartford Hospital, Hartford, CT, USA for their assistance with the conduct of the study. We acknowledge the support of ERS and SEPAR - LTRF 2017.

Competing Interests

D.N, is a consultant, grant recipient and/or on the speaker's bureau for Achaogen, Actavis, Bayer, Cepheid, Merck, Pfizer, Macrolide Pharmaceuticals, Melinta, Tetraphase, The Medicines Company, Shionogi.

Ethical Approval

Not required.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.ijantimicag.2019.03.006.

References

[1] Vincent JL. Nosocomial infections in adult intensive-care units. Lancet 2003;361(9374):2068–77.

[2] Bollenbach T. Antimicrobial interactions: mechanisms and implications for drug discovery and resistance evolution. Curr Opin Microbiol 2015;27:1–9.

[3] Pillai SK, Moellering RC, Eliopoulos GM. In: Lorian V, editor. Antimicrobial combinations: Antibiotics in Laboratory Medicine. Lippincott Williams and Wilkins; 2004. p. 365–440.

[4] 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.

[5] 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(8):1914–18.

[6] Kalil AC, Metersky ML, Klompas M, Muscedere J, Sweeney DA, Palmer LB, et al. Management of adults with hospital-acquired and ventilator-associated pneumonia: 2016 Clinical Practice Guidelines by the Infectious Diseases Society of America and the American Thoracic Society. Clin Infect Dis 2016;63(5):e61–111.

[7] Crandon JL, Ariano RE, Zelenitsky SA, Nicasio AM, Kuti JL, Nicolau DP. Optimization of meropenem dosage in the critically ill population based on renal function. Intensive Care Med 2011;37(4):632–8.

[8] Conil JM, Georges B, Ruiz S, Rival T, Seguin T, Cougot P, et al. Tobramycin disposition in ICU patients receiving a once daily regimen: population approach and dosage simulations. Br J Clin Pharmacol 2011;71(1):61–71.

[9] Drusano GL, Preston SL, Fowler C, Corrado M, Weisinger B, Kahn J. Relationship between fluoroquinolone area under the curve: minimum inhibitory concentration ratio and the probability of eradication of the infecting pathogen, in patients with nosocomial pneumonia. J Infect Dis 2004;189(9):1590–1597.

[10] Wong G, Briscoe S, Adnan S, McWhinney B, Ungerer J, Lipman J, et al. Protein binding of beta-lactam antibiotics in critically ill patients: can we successfully predict unbound concentrations? Antimicrob Agents Chemother 2013;57(12):6165–70.

[11] Boselli E, Breilh D, Djabarouti S, Guillaume C, Rimmele T, Gordien JB, et al. Reliability of mini-bronchoalveolar lavage for the measurement of epithelial lining fluid concentrations of tobramycin in critically ill patients. Intensive Care Med 2007;33(9):1519–23.

[12] Odds FC. Synergy, antagonism, and what the chequerboard puts between them. J Antimicrob Chemother 2003;52:1.

[13] Monogue ML, Stainton SM, Baummer-Carr A, Shepard AK, Nugent JF, Kuti JL, et al. Pharmacokinetics and tissue penetration of ceftolozane-tazobactam in diabetic patients with lower limb infections and healthy adult volunteers. Antimicrob Agents Chemother 2017;61(12).

[14] Doern CD. When does 2 plus 2 equal 5? A review of antimicrobial synergy testing. J Clin Microbiol 2014;52(12):4124–8.

[15] Balke B, Hogardt M, Schmoldt S, Hoy L, Weissbrodt H, Haussler S. Evaluation of the E test for the assessment of synergy of antibiotic combinations against multiresistant Pseudomonas aeruginosa isolates from cystic fibrosis patients. Eur J Clin Microbiol Infect Dis 2006;25(1):25–30.

[16] Isenberg HD, Alperstein P, France K. In vitro activity of ciprofloxacin, levofloxacin, and trovafloxacin, alone and in combination with beta-lactams, against clinical isolates of Pseudomonas aeruginosa, Stenotrophomonas maltophilia, and Burkholderia cepacia. Diagn Microbiol Infect Di 1999;33(2):81–86.

[17] Monogue ML, Abbo LM, Rosa R, Camargo JF, Martinez O, Bonomo RA, et al. In vitro discordance with in vivo activity: humanized exposures of ceftazidime-avibactam, aztreonam, and tigecycline alone and in combination against new delhi metallo-beta-lactamase-producing Klebsiella pneumoniae in a murine lung infection model. Antimicrob Agents Chemother 2017;61(7).

[18] Stainton SM, Monogue ML, Nicolau DP. In vitro-in vivo discordance with humanized piperacillin-tazobactam exposures against piperacillin-tazobactam-resistant/pan-beta-lactam-susceptible Klebsiella pneumoniae strains. Antimicrob Agents Chemother 2017;61(7).