The detection of synergy between meropenem and polymyxin B against meropenem-resistant *Acinetobacter baumannii* using Etest® and time-kill assay

George A. Pankey⁎, Deborah S. Ashcraft

Infectious Disease Research, Ochsner Clinic Foundation, New Orleans, LA 70121, USA

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Abstract

Time-kill assay and Etest testing for synergy of meropenem (MER) (1× MIC) plus polymyxin B (1/4, 1/2, and 1× MIC) were performed against 8 genetically unique MER-resistant clinical *Acinetobacter baumannii* isolates. Time-kill assay demonstrated synergy for all isolates, whereas Etest showed synergy in 5 isolates and indifference in 3.

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*Acinetobacter baumannii* resistance to all major classes of antimicrobials (except polymyxins) is occurring worldwide (Giske et al., 2008). These *Acinetobacter* isolates are among the most difficult antimicrobial-resistant Gram-negative bacilli to control and treat (Maragakis and Perl, 2008). Because few antimicrobials are being developed, novel combinations of presently available agents must be investigated. Carbapenems, including meropenem (MER), are commonly used to treat severe infections in critically ill patients. Carbapenems remain the treatment of choice if *Acinetobacter* isolates retain susceptibility to this class of drugs. However, resistant strains are emerging. With limited therapeutic options, clinicians have returned to the use of polymyxin B (PB) or polymyxin E (colistin) for highly resistant *Acinetobacter* infections (Maragakis and Perl, 2008). Antimicrobial susceptibility testing of such *Acinetobacter* has shown equally high susceptibility rates (95–99%) for PB and (98–100%) for colistin (Diez et al., 2004; Dizbay et al., 2008; Gales et al., 2006; Landman et al., 2007; Manikal et al., 2000; Mezzatista et al., 2008; Reis et al., 2003). However, 1 study from Spain found that only 80.9% (93/115) *A. baumannii* isolates were susceptible to colistin (Arroyo et al., 2005). Several in vitro studies, using small numbers of carbapenem-resistant *A. baumannii* isolates, used the combination of a carbapenem and a polymyxin (Pankuch et al., 2008; Wareham and Bean, 2006; Yoon et al., 2004). Therefore, we tested the combination of MER plus various concentrations of PB against MER-resistant *A. baumannii*. Because there is no standard for in vitro synergy testing, we compared 2 synergy methods: time-kill assay (TKA) and our Etest method (Pankey and Ashcraft, 2005).

Standard laboratory powders of MER (AstraZeneca Pharmaceuticals LP, Wilmington, DE) and PB (Sigma-Aldrich, St. Louis, MO) and Etest strips of MER and PB (AB Biodisk, Solna, Sweden) were used. Etest MICs were performed in triplicate. The strip concentration range was 0.002 to 32 μg/mL for MER and 0.064 to 1024 μg/mL for PB.

Eight genetically unique MER-resistant (Etest MICs: 24 to >32 μg/mL) clinical *A. baumannii* isolates were collected during 2005 to 2006. The isolates were collected...
from patients in Kentucky (1), Louisiana (1), Massachusetts (1), New York (3), and Washington (2). Isolates were cultured from the lower respiratory tract (6) and blood (2). In addition to MER resistance, all isolates were resistant to ampicillin, aztreonam, cefazolin, cefepime, cefotaxime, ciprofloxacin, levofloxacin, and piperacillin/tazobactam but were susceptible (≤2 μg/mL) to PB (Etest MICs: all 0.5 μg/mL). Two isolates (A and D) were positive for the carbapenem hydrolyzing oxacillinase (OXA-23). Isolate F tested negative for OXA-23. Determination on other isolates was not performed. Identification was performed using the Vitek® system (bioMérieux, Durham, NC). Rep-polymerase chain reaction assays were used for genotyping. Mueller–Hinton II broth (TKA) and Mueller–Hinton II agar (MHA) plates (Etest MIC and synergy testing) (Becton-Dickinson Microbiology Systems, Sparks, MD) were used.

Time-kill assay tests were performed according to Clinical and Laboratory Standards Institute guidelines (National Committee for Clinical Laboratory Standards, 1999). An approximately 10⁷ colony-forming units (CFU)/mL inoculum was verified after plating in duplicate using a spiral plater and scanner (Spiral Biotech, Bethesda, MD). Each isolate was tested in duplicate against MER and PB alone and in combination at a concentration equal to the mean Etest MIC. A concentration equal to the MIC was used, so that TKA results could be compared directly with the Etest synergy method (which uses 1× MIC for each drug). When the MER Etest MIC was >32 μg/mL, an MER concentration equal to 32 μg/mL was used in the TKA. Polymyxin B was also tested at subinhibitory concentrations of 1/2 and 1/4 MIC in combination with MER (1× MIC). Lower concentrations of MER were not tested because the MER MIC was >32 μg/mL (exceeding the highest concentration on the Etest strip) in 5/8 isolates. Colony counts on all isolates were performed at 0 and 24 h. In addition, intermediate times (at 4, 8, and 12 h) were tested for
isolate G. Performing serial dilutions and plating with a spiral plater, which further dilutes and plates the sample, helped reduce the possibility of antibiotic carryover. The spiral plater/scanner was used to accurately detect bacterial counts as low as 20 CFU/mL. Synergy was defined as a ≥2 log$_{10}$ decrease in colony count after 24 h by the combination compared with the most active single agent. The number of surviving organisms in the presence of the combination had to be ≥2 log$_{10}$ CFU/mL below the starting inoculum (Pillai et al., 2005).

Our Etest synergy method was performed in triplicate, the summation fractional inhibitory concentration (ΣFIC) was calculated for each set of MICs, and the mean ΣFIC was used for comparison to TKA. The inoculum and streaked MHA plates were prepared in the same manner as the Etest MIC. To evaluate the effect of the combinations, we calculated the FIC for each antibiotic in each combination. High off-scale MICs (>32 μg/mL) were converted to the next 2-fold dilution (64 μg/mL). The following formulas were used to calculate the ΣFIC: FIC of MER = MIC of MER in combination/MIC of MER alone; FIC of PB = MIC of PB in combination/MIC of PB alone; ΣFIC = FIC of MER + FIC of PB. Synergy was defined by a ΣFIC of ≤0.5. Antagonism was defined by a ΣFIC of >4. Interactions represented by a ΣFIC of >0.5 but ≤4 were termed indifferent (Pillai et al., 2005).

Time-kill assay synergy was demonstrated with all combinations of PB (1/4, 1/2, and 1× MIC) and MER (1× MIC) against all 8 A. baumannii isolates (Table 1 and Figs. 1–4). An increase in synergistic activity at 24 h was noted in 5/8 isolates (63%) using the 1/4 and 1/2 MIC concentrations of PB with the TKA (Table 1: isolates A, B,
C, G, and H). The combination was bactericidal (≥3 log_{10} decrease in CFU/mL) in 7/8 isolates. For isolate G (tested at additional times), synergistic activity with PB and MER at 1× MIC was apparent by 8 h. Synergy with the lower PB concentrations (1/4 and 1/2 MIC) occurred between 12 and 24 h.

The Etest method showed synergy in 5/8 (63%) isolates. Isolates G and H, which did not show synergy by Etest, had an MER MIC >32 μg/mL. The concentration on the Etest strip may limit the use of the Etest synergy method to isolates with an MIC not exceeding the strip concentration. Subinhibitory concentrations of PB (1/4 and 1/2 MIC) could not be tested with Etest because the narrow PB ellipse is difficult to read if the MIC approaches the lower limit of the strip.

Synergy testing methods are not standardized for reproducibility and interpretation, making a comparison of results from different methods (TKA, checkerboard, and Etest) in different studies extremely difficult. We evaluated 2 methods in our study. Checkerboard is an “inhibitory” method and does not compare well with TKA (a bactericidal method). Although the Etest synergy method may be classified as an inhibitory method, it has been found to be a better predictor of bactericidal activity (Pankey and Ashcraft, 2005). In this previous study, minimal bactericidal concentrations correlated more closely with Etest MICs than broth microdilution MICs (77% versus 5.6% essential agreement). The Etest is able to detect slight hazes of growth and resistant subpopulations, and this growth is included when reading the end point for the Etest MIC. The broth microdilution method is often unable to detect the presence of small amounts of growth.

Recently, in vitro studies have evaluated the ability of MER and colistin to produce synergy against A. baumannii using time-kill experiments (Pankuch et al., 2008). Both of the 2 MER-resistant and colistin-susceptible strains showed synergy at 24 h. Another study, using an Etest agar dilution method with imipenem-resistant and PB-susceptible A. baumannii isolates, showed synergy in 2/5 (40%) (Wareham and Bean, 2006). Synergy was also demonstrated with imipenem and PB in 6/7 imipenem-resistant and PB-susceptible A. baumannii isolates using TKA (Yoon et al., 2004).

In conclusion, this study demonstrated in vitro synergy between MER and PB against 8 MER-resistant A. baumannii strains by TKA (100%) and Etest (63%). The mechanism of the observed in vitro synergy is unknown but may relate to high levels of MER entering the bacterial cell via cell membrane disruption by PB. Regardless, in vitro synergy may or may not translate into in vivo benefit.

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