Activity of antimicrobial combinations against KPC-2-producing *Klebsiella pneumoniae* in a rat model and time-kill assay

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Abstract

This study evaluated efficacy of tigecycline (TIG), polymyxin B (PMB) and meropenem (MER) in 80 rats challenged with KPC-producing *K. pneumoniae* infection. Time-kill assay was performed with same strain. Triple therapy and PMB+TIG were synergistic, promoted 100% survival and negative peritoneal cultures, while MER+TIG showed lower survival, higher cultures positivity than other regimens (P=0.018) and was antagonistic. *In vivo* and *in vitro* studies showed that combined regimens, except MER+TIG, were more effective than monotherapies for this KPC-producing strain.
Infections due to *Klebsiella pneumoniae* carbapenemase (KPC)-producing Enterobacteriaceae are associated with therapeutic failure and increased mortality (1-5). It has been suggested that antibiotic combination might be a better alternative when compared to monotherapy for treatment of KPC-producing isolates (4-9); however, further investigation on this therapeutic strategy is required (10, 11).

In the present study, we evaluated efficacy of regimes for a KPC-KP strain in an experimental model of systemic infection and in a time-kill assay (TKA).

A KPC-KP strain, coded RM-1209, was isolated from a patient’s blood and identified by Vitek® 2 (BioMérieux, Craponne, France) in 2012. Antibiotics minimal inhibitory concentration (MIC) were determined by agar dilution and interpreted according to CLSI and EUCAST for TIG (12, 13). The strain presented MIC >32mg/L, 1mg/L, and 0.5mg/L for meropenem (MER), tigecycline (TIG), and polymyxin B (PMB) respectively. Detection of *bla*KPC gene was performed with BigDye v1.1 Sequencing Kits (Applied Biosystems, Foster City, CA, USA) and KPC-2 was confirmed at databases queried by NCBI BLAST (6).

The study was approved by the ethical committee, according to the Protocol for the Protection and Welfare of Animals (European Union). Animal model has been previously described,(13). In brief, male and female immunocompetent Wistar rats weighing in between 190–300 g were randomized into each treatment group. Absence of immunosuppression demanded administration of highly concentrated inoculums with 1.5 × 10^10 colony forming units per milliliter (CFU/mL). This solution, when diluted 1:20 presents an absorbance of 0.546 at 625nm wavelength on spectrophotometry (corresponding to tube 50 of nephelometric scale). Seven groups of ten rats were treated with the following regimen: MER; TIG; PMB; MER+PMB; MER+TIG; PMB+TIG or PMB+MER+TIG. Ten rats were untreated (control group). Sample size (80 rats) was calculated using the formula \( n = \frac{z^2PQ}{d^2}\) based on the expected proportion of deaths (50%) and 8% standard error, with a
Animals were injected with 0.7 ml intraperitoneal aliquot of $1.5 \times 10^{10}$ CFU/mL KPC-KP inoculum in log-growth state (14). After infection, rats received antimicrobials intraperitoneally: TIG (Tygacil®) at 7 mg/kg/12 h (15-16), PMB (Polymyxin B) at 2 mg/kg/12 h (17, 18), and MER (Meronem®) at 50 mg/kg/8 h (19), or remained untreated. No pharmacokinetic (PK) data were obtained. Length of survival was observed for 24 h and euthanasia was performed on rats surviving at 24 h. Blood samples were collected by aseptic cardiac puncture and peritoneal fluid samples were obtained through direct observation after incision with aseptic technic. Blood samples were incubated in broth, and peritoneal fluid was incubated on MacConkey agar plates for quantitative cultures with 1 microliter loop.

In vitro TKA was performed by inoculating $5 \times 10^6$ CFU/mL of the clinical strain into 10 mL of fresh cation-adjusted Mueller–Hinton broth (Oxoid, Basingstoke, UK) and incubating at 35°C. MER at 4 mg/L, TIG at 1 mg/L, and PMB at 0.25 mg/L were tested alone and in the same combinations performed in vivo. Aliquots were removed at 1, 6, 12, and 24 h after inoculation. Samples were serially diluted ($10^{-1}$ to $10^{-8}$) and plated in duplicate on blood agar plates for colony count. Antimicrobial carry-over was controlled by streaking the transferred aliquot over the agar plate and observing possible inhibition of growth at the site of the initial streak. Potential in vitro MER hydrolysis was not assessed.

Time-kill curves were constructed by plotting mean colony counts versus time. The results were interpreted after 24 h incubation.

Survival curves were constructed and Gehan-Breslow-Wilcoxon test was performed. Mann Whitney U and Kruskal-Wallis tests evaluated differences in blood, peritoneal cultures and mortality between groups. Alpha-adjustment was performed with Dunn’s multiple comparison tests. The SPSS 16 (IBM, Armonk, NY, USA) software was used for statistical analysis and Prism (Graphpad, La Jolla, CA) for graph construction. A $P$-value $<0.05$ was
considered statistically significant.

In Kaplan-Meier survival curves, untreated rats presented 80% mortality, a similar proportion of those treated with TIG and MER monotherapies whose mortality was 60.0% in both groups ($P=0.061$ and $P=0.114$). All animals treated with combinations including PMB survived (PMB+TIG; PMB+TIG+ MER; PMB+MER), whereas a 70.0% survival rate was observed with MER+TIG ($P=0.018$). MER+TIG combination did not produce significantly different survival from PMB monotherapy ($p=0.901$). (Figure 1A and Figure 2).

PMB monotherapy and combinations including PMB significantly sterilized more peritoneal cultures than MER+TIG ($P=0.001$) (Figure 1B). PMB+TIG combination determined the lowest positivity of blood culture, statistically different from control, MER, TIG, and MER+TIG groups ($P<0.001$) (Figure 1C).

PMB+TIG combination and triple therapy were synergistic whereas MER+TIG showed antagonistic effect on TKA (Figure 3).

Evaluating survival and overall cultures sterilization, the better performance occurred with PMB+TIG, followed by triple therapy, PMB+MER, and PMB. Monotherapies did not present the same efficacy observed in the better performing combinations: PMB+TIG or PMB+TIG+MER, corroborating previous studies, which observed lowered mortality with antimicrobial combinations (4-9).

It has been previously described, in retrospective data, that triple therapy might lower mortality in patients infected with KPC-KP, however, MER MIC was not mentioned (4). In the present study, triple therapy was not superior to PMB+TIG, however, this finding should be confirmed using lower MER MIC strain.

Moreover, combination of PMB+MER demonstrated impact on survival and peritoneal cultures, but was not superior to PMB monotherapy for treating bacteremia. Despite the
lower mortality observed with the PMB+MER combination, no synergism was observed with PMB+MER in TKA.

Of interest, we found an antagonistic effect of TIG+MER, which determined worse outcomes than PMB monotherapy, as previously suggested by our group (20). Pournaras et al. 2011 also showed that MER+TIG are not synergic in vitro (21). Previous studies of non-lethal and non-systemic infections were discordant about the antagonistic effects of TIG+MER in vivo (20, 22) and in vitro (24). Further investigation using this combination is required, but we believe that TIG+MER should be used with caution for treatment of infections due to KPC-producing isolates. On the contrary, the activity of TIG+PMB showed favorable results in our study, corroborating previous in vitro (21) and in vivo (23) findings. Tigecycline monotherapy did not perform better than MER alone, potentially due to bacteriostatic effect and inadequate PK to treat blood stream infection, where TIG should be avoided (25).

Strengths of this study are that the in vivo and in vitro experiments have showed similar agreement on findings of antimicrobial activity and sample size with an adequate statistical power to detect relevant differences in clinical and microbiological outcomes. A limitation of the present study was the evaluation of only one strain, as differences among strains can affect study results despite similar MICs (26). We also did not perform a PK evaluation of the antibiotics in the rats, which might contribute to the interpretation of our findings (15, 17, 18, 19).

In summary, combined regimen including PMB resulted in improved outcomes in this experimental study. Triple therapy was not superior to dual combinations with PMB. In vitro antagonism of TIG+MER was correlated with similar outcomes and resulted in worse microbiological findings when compared to MER, TIG and PMB monotherapies.
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Figure 1. Animal model of sepsis of KPC-2-KP and effects of different antibiotic combinations. A) mortality (* $P < 0.05$ in comparison with PMB, MER + TIG, MER, TIG, and control; ** $P < 0.05$ in comparison with control); B) Peritoneal culture (*$P < 0.05$ in comparison with control, MER, TIG, MER+TIG); C) blood culture (**$P < 0.05$ in comparison for control, MER, TIG, MER + TIG).

MER= Meropenem; TIG=Tigecycline; PMB=Polymyxin B.

Figure 2. Survival curves of a rat model of sepsis infected with KPC-2-producing Klebsiella pneumoniae and response to treatment by antibiotic combinations or monotherapies. * $P < 0.05$ from other groups. **$P < 0.05$ from control. Meropenem (MER); tigecycline (TIG); polymyxin B (PMB). The time of x axis is hours.

Figure 3. Time-kill assay of KPC-2-KP using antibiotic combinations. Meropenem (MER); tigecycline (TIG); polymyxin B (PMB). PMB + TIG (A); PMB + MER (B); PMB + MER + TIG (C); MER + TIG (D).

Bactericidal activity was defined as a $\geq 3 \log_{10}$ reduction in the total CFU/mL from the original inoculum at 24 hours;

Bacteriostatic activity was defined as a $< 3 \log_{10}$ reduction in the total CFU/mL from the original inoculum at 24 hours;

Synergism was defined as a difference of $\geq 2 \log_{10}$ in the reduction of the number of CFU/mL between the combination and the most active agent at 24 hours;

Antagonism was defined as $\geq 2 \log_{10}$ increase of the number of CFU/mL between the combination and the most active agent at 24 hours.