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Synergy of polymyxin B, tigecycline and meropenem against carbapenem-resistant *Enterobacter cloacae* complex isolates

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ABSTRACT

Here, we evaluated the combinations of antibiotics polymyxin B (PMB), tigecycline (TGC) and meropenem (MEM) by *time-kill curves* (TKC) against carbapenem-resistant *Enterobacter cloacae* isolates. Combination of PMB/TGC and PMB/MEM showed promising results in sub-inhibitory concentration of PMB indicating the possibility of reducing the dose of PMB used in the treatment.

Enterobacter cloacae is an important nosocomial pathogen associated with severe infections and outbreaks mainly due to intrinsic resistance to beta-lactamics included cephalosporins. In the last decade this microorganism has become the second most prevalent *Enterobacteriaceae* carrying carbapenemases worldwide (Davin-Regli and Pagès, 2015). This fact associated to the lack of new antimicrobials with activity against these microorganisms led to the use of last resort antimicrobials such as polymyxins (Lee et al., 2016; Pitout and Laupland, 2008). Polymyxin B (PMB) and colistin re-emerged in clinical practice even with pharmacokinetic/pharmacodynamics limitations and the increased risk of a severe renal dysfunction development. Several studies have shown that these drugs would be more effective as part of a combined therapy with other antimicrobial agents such as meropenem (MEM) and tigecycline (TGC) due to the major concern of the development of resistance (Elias et al., 2010; Qureshi et al., 2012; Sharma et al., 2017).

Here, we selected three clinical isolates of *Enterobacter cloacae* according to their susceptibility profile and resistance mechanism and evaluated the *in vitro* activity of PMB alone and in combinations with MEM and/or TGC. The characteristic of isolates are following: NDM-producer, OXA-370 producer and non-carbapenemase-producer (NCP).

The isolates were identified by automated biochemical methods and the presence of carbapenemase genes was evaluated by a real-time PCR multiplex high resolution melting (HRM) with primers specific for bla_{NDM}, bla_{KPC} bla_{GES}, $bla_{OXA\text{-}48\text{-}like}$, bla_{IMP} and bla_{VIM} (Monteiro et al., 2012) plus sequencing.

Broth microdilution method was used to determine the minimum inhibitory concentration (MIC) of the isolates and the results were interpreted according to the breakpoints of the CLSI for MEM and TGC; and colistin breakpoint of the EUCAST was used for PMB (Clinical and Laboratory Standards Institute, 2014; European Committee on Antimicrobial Susceptibility Testing, 2013). All tests were performed in duplicate in cation-adjusted Mueller-Hinton broth.

Time-kill curves (TKC) were used to evaluate *in vitro* antimicrobial activity against the isolates (National Committee for Clinical Laboratory Standards, 1999). Sub-inhibitory concentration, 1×MIC and 2×MIC of PMB and combinations with MEM and TGC were tested. Since MICs indicated resistance to TGC and MEM, breakpoints concentrations according to CLSI for TGC (1 mg/L) and MEM (1 mg/L) were used. These concentrations were chosen based on the dose regimen usually prescribed to patients.

For TKC, initial inoculum used of each concentration tested contained between 10^5 and 10^6 CFU/ml in early to mid-logarithmic growth. Serial microdilution bacterial plate counts were made in 96

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well sterile plates at the initial time of inoculation and at 2, 4, 6, 8, 12 and 24 hours. The TKC were performed in duplicate.

Bactericidal activity was defined by a ≥3 log CFU/ml decrease in colony count compared to initial inoculum. Synergism was defined by a ≥2 log CFU/ml decrease in colony count of the combined antimicrobials compared to colony count of the most active antimicrobial alone in 24 h (National Committee for Clinical Laboratory Standards, 1999).

All isolates were susceptible for PMB (MICs 1 mg/L for NDM, 0.25 mg/L for OXA-370 and 0.5 mg/L for NCP). PMB alone presented bactericidal activity in 1×MIC and 2×MIC concentrations to OXA-370 and NDM isolates (Table A). Bactericidal activity of PMB alone in the NCP isolate was not observed at any concentration (Fig. A.1).

PMB plus TGC was the most promising combination and showed synergistic activity for the OXA-370 and NDM isolates in PMB sub-inhibitory concentration (Table A). PMB plus MEM combination showed synergism in sub-inhibitory PMB concentration to NDM isolate (Table A). Additionally, MEM plus 2×MIC of PMB was able to reduce the inoculum of this isolate to a non-detectable growth (from the second hour), which indicates that this combination would avoid regrowth (Fig. A.2).

The triple combination (PMB, TGC and MEM) was not effective against the carbapenemase-producing isolates; however, showed bactericidal effect and synergism for the NCP. It is possible to speculate that this synergistic effect was independent of PMB as the combinations of PMB/MEM and PMB/TGC did not present a significant reduction of colony counts in 24 hours (Fig. A.3). Therefore, we consider the possibility that the MEM/TGC association was responsible for the synergism presented by the triple combination (data not evaluated in our study).

In our study, we found synergistic effect among antibiotics even in isolates that presented resistance to MEM and TGC. An important point to consider, regarding antimicrobials combinations assays, is the antimicrobial concentrations used in the tests. In this study, we used breakpoints of CLSI for TGC and MEM whereas some studies used antimicrobial concentrations much higher (which may not be achievable *in vivo*) (Laishram et al., 2015; Sharma et al., 2017). Therefore, the results obtained in our study are more useful to the clinical practice as results obtained at concentrations higher than the breakpoints may not be of value for clinical purposes.

We have different results for the isolates as also found by Lin et al. whom demonstrated different antibacterial activity among isolates that had very similar resistance profile (Lim et al., 2015). Cai et al. findings also corroborated with the fact that the antibacterial activity is strain specific. They used TKC and hollow-fiber infection model to evaluate PMB activity alone and in combinations against extensively drug-resistant *E. cloacae*. Regrowth beyond 24 hours occur in one out of two isolates tested, with PMB/TGC combination, in the hollow-fiber infection model (Cai et al., 2016). Considering regrowth, both studies mentioned above (Cai et al., 2016; Lim et al., 2015) also found regrowth of isolates in PMB monotherapy, the same results we have found. Therefore, the results of these studies reinforce the fact that it is more difficult occur resistant population selection when a combination of antibiotics is used. Despite this, it should be mentioned that Paul et al. (2018) in a recent study did not find superiority of the combination therapy of meropenem with colistin to treat carbapenem-resistant *Enterobacteriaceae* infections, but as the authors themselves justified, the number of patients included in this group was low and that the results of the study are more conclusive for *Acinetobacter* infections. Moreover, the most studies use the colistin in the therapeutic regimens and polymyxin B has pharmacological characteristics that demonstrate superiority to colistin and that direct administration at the infection site could be advantageous (Perez and Bonomo, 2018).

Thus, given the results presented, we considered that synergic effects found in sub-inhibitory PMB concentration combined with TGC or MEM to carbapenemase-producer isolates is a very promising finding as it demonstrates the *in vitro* bactericidal activity of the combination even at low concentrations of polymyxin (Elias et al., 2010).

Conflicts of interest

The authors declare have no conflicts of interest.

Ethical approval

Not applicable.

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Informed consent

Not applicable.

Appendix A

Table A
Log_{10} ΔUFC/mL obtained in time-kill assay to *Enterobacter cloacae* isolates for polymyxin B (PMB), meropenem (MEM) and tigecyclin (TGC) in monotherapy and in combinations.

Enterobacter cloacae isolates (MIC)	Log ΔCFU[a] with antibiotics[b] in monotherapy and in combinations																
	MEM 1mg/L	TGC 1mg/L	PMB 0.125 mg/L	PMB 0.25 mg/L	PMB 0.5 mg/L	PMB 1mg/L	PMB 2mg/L	PMB 0.125 mg/L		PMB 0.25 mg/L		PMB 0.5mg/L		PMB 1mg/L		PMB 2mg/L		PMB + MEM + TGC[c]
								MEM 1mg/L	TGC 1mg/L	MEM 1mg/L	TGC 1mg/L	MEM 1mg/L	TGC 1mg/L	MEM 1mg/L	TGC 1mg/L	MEM 1mg/L	TGC 1mg/L	
NDM (PMB 1mg/L MEM 8mg/L TGC 8 mg/L)	3.22	2.86	N/T	N/T	-1.31	-4.78	-4.78	N/T		N/T		**-4.48**	**-3.44**	-4.78	-4.78	-4.78	-3.64	-1.11
OXA-370 (PMB 0.25mg/L MEM 2mg/L TCG 8mg/L)	2.22	2.22	-0.69	-3.08	-4.30	N/T	N/T	-1.58	**-3.16**	-2.94	-2.87	-4.78	-1.48	N/T		N/T		-1.29
NCP (PMB 0.5 mg/L MEM 4mg/L TGC 4mg/L)	0.22	0.22	N/T	3.22	0.22	0.22	N/T	N/T		0.22	0.22	0.22	-1.09	0.22	-0.37	N/T		**-3.58**

N/T Not tested.
[a] Calculated by subtracting the final colony count from the initial inoculum in each concentration tested. Synergism was calculated by subtracting the final colony count in the most active antibiotic in monotherapy from the final colony count in the combination and is define by a ≥2 log_{10} units decrease (highlighted in bold type).
[b] Sub-inhibitory concentration, 1×MIC and 2×MIC for PMB and breakpoints (1mg/L) for TGC and MEM were tested alone and in combinations. All isolates was susceptible for PMB and resistant for TGC and MEM.
[c] Triple combination was tested with MIC concentrations for PMB (1 mg/L for NDM, 0.25 mg/L for OXA-370, 0.5 mg/L for NCP) and breakpoint (1mg/L) for MEM and TCG.

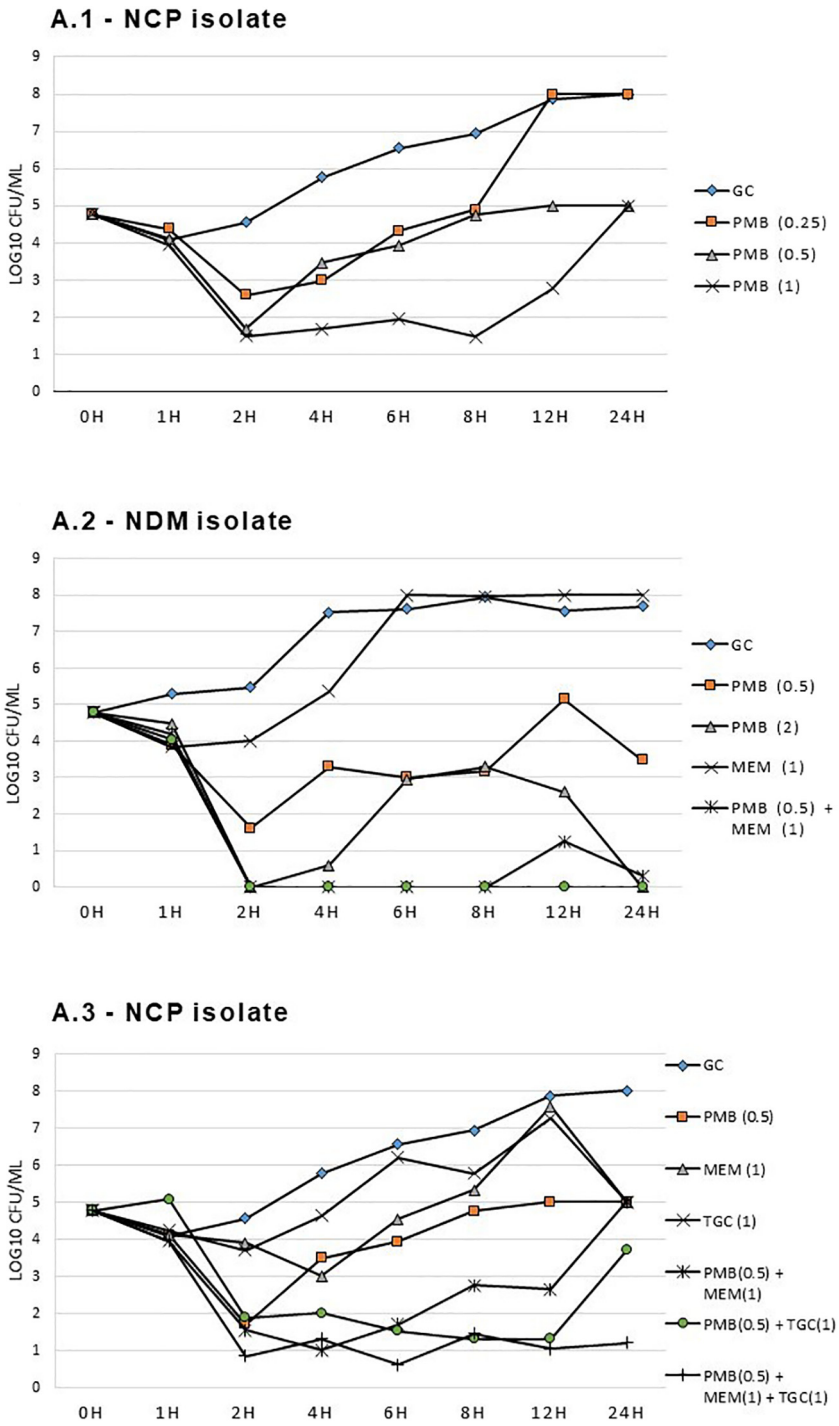

Fig. A. *Time-kill* curves: Initial inoculum used of each concentration tested contained between 10^5 and 10^6 CFU/ml in early to mid-logarithmic growth. Serial microdilution bacterial plate counts were made at the initial time of inoculation and at 2, 4, 6, 8, 12 and 24 hours. Growth control (GC) without antibiotics. **(1)** Activity of PMB in the non-carbapenemase-producer *E. cloacae* isolate with sub-inhibitory concentration (0.25 mg/L), 1×MIC (0.5 mg/L) and 2×MIC (1 mg/L) of polymyxin B (PMB). **(2)** NDM-producer *E. cloacae* isolate with sub-inhibitory (0.5 mg/L) and 2×MIC (2 mg/L) concentrations of PMB in combinations with meropenem (MEM) breakpoint (1 mg/L). PMB plus MEM showed synergism in sub-inhibitory PMB concentration and 2×MIC PMB (2 mg/L) combination reduce the inoculum to a non-detectable growth from the second hour. **(3)** Non-carbapenemase-producer *E. cloacae* isolate with 1×MIC of PMB (0.5 mg/L), breakpoints (1 mg/L) for MEM and tigecycline (TGC) and their combinations. Synergism occurs in triple combination PMB (0.5 mg/L) plus MEM (1 mg/L) plus TGC (1 mg/L).

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