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DANIELLE ROSANI SHINOHARA

Avaliação *in vitro* da polimixina B associada à vancomicina contra isolados de *Acinetobacter baumannii* produtores de carbapenemases

Maringá
2018

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Dissertação apresentada ao Programa de Pós-Graduação em Ciências da Saúde do Centro de Ciências da Saúde da Universidade Estadual de Maringá, como requisito parcial para obtenção do título de Mestre em Ciências da Saúde

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“Conheça todas as teorias, domine todas
as técnicas, mas ao tocar uma alma
humana, seja apenas outra alma
humana”

(Carl Jung)

Avaliação *in vitro* da polimixina B associada à vancomicina contra isolados de *Acinetobacter baumannii* produtores de carbapenemases

RESUMO

No presente estudo *in vitro*, avaliou-se o efeito da combinação de polimixina B e vancomicina contra seis isolados clínicos de *Acinetobacter baumannii* pertencentes a seis clones diferentes, sendo três sensíveis e três resistentes à polimixina B. A resistência à polimixina B foi investigada também pela pesquisa do gene mcr-1 e pelo teste de frequência de mutantes. A Concentração Inibitória Mínima (MIC) de vancomicina foi avaliada por microdiluição em caldo. O efeito sinérgico da combinação polimixina B-vancomicina foi determinado pelos métodos de *checkerboard, time-kill*, disco difusão e *Etest*. No ensaio de *checkerboard*, todos os isolados de *A. baumannii* resistentes à polimixina B apresentaram efeito sinérgico (Índice de Fração Inibitória- FICI $\leq$ 0,5), houve também uma redução das MICs de polimixina B em todos os isolados testados com uma alteração da categoria de resistente para sensível em dois isolados resistentes à polimixina B. No ensaio de *time-kill*, observou-se atividade bactericida em dois isolados resistentes e um sensível à polimixina B (redução de 3 $\log_{10}$ na contagem de viáveis em 24h). Nenhum antagonismo foi detectado pelos métodos testados. Surpreendentemente, o ensaio de time-kill demonstrou que a combinação polimixina B-vancomicina apresentou efeito bactericida após 24h contra isolados com alta taxa de mutantes para polimixina B, sugerindo que essa combinação pode bloquear a hipermutação de alguns isolados. Todos os isolados resistentes à polimixina B também mostraram sinergismo no teste de disco difusão e diminuição da MIC de vancomicina pelo método *Etest*. Os resultados sugerem que a combinação polimixina B-vancomicina tem potencial terapêutico contra infecções causadas por *A. baumannii* resistente à polimixina B. Estudos clínicos complementares devem ser realizados para o uso dessa combinação na prática médica.

Palavras-chave: *Acinetobacter baumannii*; polimixina B; vancomicina; combinação antimicrobiana

In-vitro evaluation of polymyxin B associated with vancomycin against isolates of *Acinetobacter baumannii* producing carbapenemases

ABSTRACT

In the present *in-vitro* study, the effect of combination of polymyxin B and vancomycin was assessed against six *Acinetobacter baumannii* clinical isolates belonging to six different clusters (three polymyxin B-susceptible and three polymyxin B-resistant). Additionally, polymyxin B-resistance was investigated with *mcr-1* gene amplification and mutant frequency assay. Vancomycin minimum inhibitory concentration (MIC) was assessed by broth microdilution. The synergistic effect of the polymyxin B-vancomycin combination was determined with the checkerboard, time-kill, disk-diffusion and Etest assays. In the checkerboard assay, all polymyxin B-resistant isolates showed a synergistic effect (FICI $\leq$ 0.5). A reduction in polymyxin B-MICs was observed in all isolates tested, with two polymyxin B-resistant isolates changing category from resistant to susceptible. In the time-kill assay, bactericidal activity was observed in two polymyxin B -resistant and one polymyxin B -susceptible isolate (3 $\log_{10}$ reduction in colony count after 24h). No antagonism was detected by the checkerboard, time-kill, disk-diffusion or Etest assay. Unexpectedly, the time-kill assay demonstrated that the polymyxin B-vancomycin combination had a bactericidal effect at 24h against isolates with a high mutant rate for polymyxin B, suggesting that this combination may block the hypermutation of some isolates. All polymyxin B-resistant isolates also showed synergism in the disk-diffusion test, and a significant decrease in vancomycin MICs in the Etest assay. The findings suggest that the polymyxin B-vancomycin combination has a potential therapeutic effect, especially against infections caused by polymyxin B-resistant *A. baumannii*. Additional clinical studies to evaluate the use of this combination in medical practice are warranted.

Keywords: *Acinetobacter baumannii*; polymyxin B; vancomycin; antimicrobial combination

LISTA DE ABREVIATURAS

CA-MHB	*Cation Adjusted Mueller Hinton Broth*- Caldo Mueller Hinton Cátion Ajustado
CLSI	*Clinical and Laboratory Standards Institute*
COL	*Colistin*- Colistina
ERIC	*Enterobacterial Repetitive Intergenic Consensus*
FICI	*Fractional Inhibitory Concentration Index*- Índice de Fração Inibitória
GN	*Gram Negative*- Gram-Negativo
HUM	Hospital Universitário de Maringá
MDR	*Multidrug Resistant*- Multidroga Resistente
MF	*Mutational Frequency*- Frequência Mutacional
MHA	*Mueller Hinton Agar*- Ágar Mueller Hinton
MHB	*Mueller Hinton Broth* – Caldo Mueller Hinton
MIC	*Minimum Inhibitory Concentration*- Concentração Inibitória Mínima
MRSA	*Methicillin-Resistant Staphylococcus aureus- S. Aureus* Resistente à Meticilina
OXA	*Oxacillinase*- Oxacilinases
PBP	Proteína Ligadora de Penicilina
PCR	*Polymyerase Chain Reaction*- Reação em Cadeia da Polimerase
PMB	*Polymyxin B*- Polimixina B
TKA	*Time-Kill Assay*- Ensaio de Curva de Morte
UTI	Unidade de Terapia Intensiva
VAN	*Vancomycin*- Vancomicina
XDR	*Extreme Drug Resistant*- Extensivamente Resistente

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CAPÍTULO I

INTRODUÇÃO

O gênero *Acinetobacter* é constituido por bactérias Gram negativas cocobacilares, pertencentes à família *Moraxellaceae,* não fermentadoras da glicose, catalase positiva, oxidase negativa e aeróbias estritas (BERGOGNE-BEREZIN, TOWNER, 1996). Com alta prevalência na natureza é facilmente encontrado no solo, água e animais, também é frequente comensal da pele e orofaringe em seres humanos (TOWNER, 2009).

Acinetobacter baumannii é a principal espécie do gênero possuindo alta prevalência nas infecções hospitalares (JOSHI, LITAKE, 2013). Esse microrganismo tem sido associado a surtos no ambiente hospitalar e, isso se deve especialmente à sua resistência à dessecação, podendo sobreviver em superfícies inanimadas por longos períodos. (DOI et al., 2015; WEBER et al., 2015).

A. baumannii é um agente causador de infecções sanguíneas, urinárias, feridas, pneumonia associada à ventilação mecânica e meningites. Infecções adquiridas na comunidade como pneumonias agudas; também estão relacionadas a *A. baumannii* em indivíduos que apresentam fatores de risco (alcoolismo crônico ou doenças bronco-pulmonares), evoluindo com frequência para choque séptico com taxas de mortalidade entre 40 e 60% (PELEG et al., 2008; ANTUNES et al., 2014).

A prevalência mundial de *A. baumannii* multirresistente (MDR) e com ampla resistência (XDR) tem aumentado e gerado grande preocupação (BARIN e al., 2013; O'HARA et al., 2013). Há vários mecanismos de resistência descritos em *A. baumannii* como a hiperexpressão de bombas de efluxo, a alteração das proteínas ligadoras de penicilina (PBPs), mudança da permeabilidade da membranda externa e a produção de enzimas merecendo destaque a produção de β-lactamases (POURNARAS et al., 2008).

As β-lactamases da classe D de Ambler, oxacilinases (OXA), estão entre as principais carbapenemases detectadas em bactérias do gênero *Acinetobacter* (PELEG et al., 2008). Essas enzimas possuem alta ação hidrolítica contra oxacilina e cloxacilina,conferem resistência a ampicilina e cefalotina, e são fracamente inibidas por ácido clavulânico (POIREL et al., 2010). Alguns autores têm reportado que a OXA-23 juntamente com a OXA-51 (oxacilinase

intrínseca) são as oxacilinases mais frequentes em *A. baumannii* (BALI et al., 2013; MOHAJERI et al., 2013; HOU et al., 2015).

O tratamento de infecções causadas por *A. baumannii* é um grande desafio considerando a facilidade deste microrganismo em adquirir resistência a vários agentes antimicrobianos de uso clínico, impossibilitando sua utilização (ALYAMANY et al. 2015). A correta identificação laboratorial de *A. baumannii* e a terapia empírica adequada tem grande influência na sobrevivência dos pacientes. Viehman et al. sugeriram que a rápida administração de múltiplos agentes antimicrobianos pode ser melhor do que a monoterapia (VIEHMAN et al., 2014). Esta alternativa de associar antimicrobianos é interessante e particularmente para *A. baumannii* se faz necessária pela falta de opções terapêuticas nos dias atuais, assim a investigação da combinação de fármacos visa obter uma terapia mais adequada, no entanto essa associação pode resultar em efeito sinérgico, indiferente ou até mesmo antagônico (BERGEN et al., 2012).

Métodos de associação de antimicrobianos realizados *in vitro* tem demonstrado o potencial das combinações de antimicrobianos para o uso clínico, no entanto, para avaliar melhor seu desempenho, são necessárias pesquisas adicionais *in vivo*, tendo em vista que características farmacocinéticas e farmacodinâmicas podem se mostrar diferentes no paciente (TUON et al., 2015). Há desafios associados à realização de testes de sinergismo, uma vez que exigem habilidades específicas, um trabalho árduo e um longo período de tempo de execução. Por outro lado, testes *in vitro* têm direcionado ações para a descoberta de novas alternativas de tratamento uma vez que as opções terapêuticas para tratar infecções causadas por *A. baumannii* resistente aos carbapenêmicos são escassas e a maioria das possibilidades é baseada na combinação de fármacos (SOPIRALA et al., 2010).

O método *Checkerboard* descrito por Eliopoulos e Moellering em 1991, é baseado no protocolo de microdiluição do *Clinical and Laboratory Standads Institute* (CLSI), no qual são determinadas as concentrações inibitórias mínimas (MIC), do inglês *minimum inhibitory concentration*, de dois fármacos isoladamente e em associação (ELIOPOULOS, MOELLERING, 1991). As vantagens deste método são a possibilidade de testar combinações de antimicrobianos de concentrações diferentes em um mesmo experimento e a utilização de um volume pequeno de cada fármaco testado. Como limitação, o método determina a atividade inibitória e não a atividade bactericida da combinação fármacos (LORIAN, 2005).

Time Kill Assay (TKA) é um método de referência para avaliação de sinergismo, sua padronização foi proposta por Klepser *et al.* em 1998, na qual culturas bacterianas são expostas a concentrações de antimicrobianos sozinhos e em combinação. A técnica demanda muito tempo e é difícil de ser realizada, no entanto, o método avalia a associação entre os agentes antimicrobianos ao longo do tempo (0, 6, 12 e 24h após a incubação), além de demonstrar a atividade bactericida da combinação antimicrobiana (KLEPSER et al., 1998).

O método de disco difusão avalia a associação de antimicrobianos que se difundem em placas de ágar inoculadas com microrganismo. O método foi proposto por Pillar et al., que inseriram alíquotas de dois antimicrobianos distintos em um disco (PILLAR et al., 2009) e por Sakoulas et al., que realizaram a sobreposição de dois discos, cada um preparado com um antimicrobiano diferente (SAKOULAS, 2016). Como vantagens tem a facilidade de execução, rapidez no resultado e baixo custo, sua desvantagem é de ser apenas qualitativo (BASTOS et al., 2007).

A combinação de antimicrobianos pelo método epsilométrico (*Etest*) foi proposto inicialmente por White et al. em 1996, que realizaram a formação cruzada com fitas de *Etest* criando um ângulo de 90° na intersecção (WHITE et al., 1996). Depois, outros autores proporam o método utilizando a sobreposição de fitas (MANNO et al., 2003; PANKEY, ASHCRAFT, 2005) e também a inserção de fitas sobre o ágar suplementado com um segundo antimicrobiano (GORDON et al., 2009; SOPIRALA et al., 2010). Esses métodos têm vantagens de serem simples, rápidos e quantitativos e, sua desvantagem está no custo mais elevado quando comparado a outros métodos como disco difusão (LANG, GARCÍA, 2004; RELLER et al., 2009).

A falta de perspectivas concretas de novas drogas serem lançadas comercialmente como opção terapêutica para o controle de infecções, faz com que antibacterianos utilizados no passado sejam resgatados, como é o caso das polimixinas (CHEN, KAYE, 2011). As polimixinas foram descobertas na década de 1940, tendo seu uso reduzido a partir de 1970 devido à nefrotoxicidade e neurotoxicidade. No entanto, o rápido aumento na resistência aos demais antibióticos gerou o seu ressurgimento na prática clínica (VELKOV et al., 2013).

Existem cinco tipos de polimixinas (A, B, C, D e E) que diferem na sua estrutura polipeptídica, apenas duas são utilizadas clinicamente, B e E, tendo em vista a maior toxicidade das demais (FALAGAS et al., 2006). A diferença essencial entre a PMB e a polimixina E (colistina) é a sua capacidade de ligação proteica. A PMB mantém

concentrações maiores de droga livre em todos os tecidos enquanto a colistina (COL), para obter efeito antimicrobiano eficaz, necessita de concentrações extremamente neurotóxicas às células dos mamíferos (CRAIG, KUNIM, 1973).

O mecanismo de ação das polimixinas consiste em atuar nas membranas externa e citoplasmática com ação semelhante a detergentes catiônicos. As polimixinas ligam-se ao lipopolissacarídeo das bactérias, deslocando por competitividade os íons cálcio e magnésio, o que gera a desestabilização e ruptura da membrana, levando à morte bacteriana (HANCOCK, 1997).

As polimixinas são agentes terapêuticos frequentemente utilizados como último recurso para tratar infecções causadas por *A. baumannii* multirresistente, entretanto, amostras de *A. baumannii* resistentes às polimixinas têm sido detectadas e estão relacionadas à terapia prévia e monoterapia com esses fármacos (WEI et al., 2015). A resistência às polimixinas deve-se principalmente à modificação da 4'-fosfoetanolamina do lípido A no lipopolissacarídeo. Essa alteração pode ocorrrer devido a mutações nos sistemas bacterianos pmrAB e PhoPQ e o regulador mgrB ou a aquisição do gene mcr-1(GAO et al., 2016).

O gene mcr-1 tem alta capacidade de transferência horizontal (LIU et al., 2017), tem se disseminado em vários países, já encontrado em pelo menos 5 espécies em amostras clínicas: *Escherichia coli*, *Salmonella enterica*, *Klebsiela pneumoniae*, *Enterobacter aerogenes* e *Enterobacter cloacae* (GAO et al., 2016), e experimentalmente (testes *in vitro* de conjugação bacteriana) em *Pseudomonas aeruginosa* e *A. baumannii* (LIU et al., 2017).

Além da pesquisa dos genes de resistência às polimixinas, é importante a realização da pesquisa da frequência de mutantes, uma vez que realiza um corte transversal da população bacteriana, em um determinado momento, estimando a quantidade de mutantes a partir de uma alta concentração de bactérias- inóculo $\geq 10^{10}$ UFC/ml (MARTINEZ, BAQUERO, 2000). Mutantes resistentes podem crescer na presença de elevadas concentrações de antimicrobianos (OSBURNE et al., 2011), levando a aumentos da MIC e possível falha no tratamento (ZHANEL et al., 2006).

A PMB tem sido associada com outros fármacos que mesmo não conseguindo penetrar na célula Gram negativa, uma vez rompida a membrana externa, a entrada desses antibióticos é facilitada, propiciando a ação de antimicrobianos até então inativos como é o caso da vancomicina-VAN (KAYE et al., 2016). A VAN pertence à classe dos glicopeptídeos e age

inibindo a síntese do peptideoglicano da parede da célula bacteriana. É amplamente utilizada no tratamento de infecções causadas por *Staphylococcus aureus* resistentes a oxacilina (MRSA) e para infecções graves por microrganismos Gram positivos em pacientes que apresentam reações de hipersensibilidade às penicilinas (MOHR et al., 2007; PETROSILLO et al., 2010). Embora a VAN não tenha efeito contra bactérias Gram negativas, as propriedades da PMB podem permitir que VAN atinja o espaço periplasmático e tenha ação contra esses microrganismos (GORDON et al., 2010).

Alguns estudos avaliaram a combinação de antimicrobianos COL e VAN contra isolados de *A. baumannii* resistentes a múltiplos fármacos (BAE, 2016; GORDON, 2010; PETROSILLO, 2010; YANG, 2016), no entanto, não encontramos estudos na literatura que avaliem a atividade antimicrobiana da combinação de PMB e VAN contra isolados de *A. baumannii* MDR.

JUSTIFICATIVA

Nos últimos anos, o *Acinetobacter baumannii* tem sido o principal bacilo Gram negativo associado a infecções hospitalares no Hospital Universitário de Maringá – HUM. Em 2004 foi endêmico na unidade de terapia intensiva (UTI) adulto e tem se mantido desde então como o microrganismo mais frequente entre os isolados bacterianos desta unidade.

A falta de perspectivas concretas do lançamento comercial de novas drogas como opção terapêutica para o controle de infecções faz com que antibacterianos utilizados no passado sejam resgatados, como é o caso da PMB. O uso de PMB na forma de monoterapia não tem sido indicado devido ao risco de desenvolver resistência. Assim, a combinação de fármacos tem o objetivo de ampliar o espectro antimicrobiano, minimizar o surgimento de resistência e diminuir a toxicidade. Diante da alarmante resistência do *A. baumannii* aos antimicrobianos, surge a necessidade de avaliar o uso combinado de PMB com outros fármacos que pode contribuir com a equipe médica, fornecendo uma possível alternativa de terapia combinada.

OBJETIVOS

GERAL

Avaliar *in vitro* a atividade antimicrobiana da combinação de polimixina B e vancomicina contra isolados clínicos de *Acinetobacter baumannii*.

ESPECÍFICOS

- Determinar para todos os isolados a MIC de vancomicina pela técnica de microdiluição em caldo;

- Avaliar a associação entre a polimixina B e vancomicina pelo método de *Checkerboard, Time-kill, Etest* e disco difusão;

- Para todos os métodos de sinergismo, avaliar a presença de sinergismo, indiferença ou antagonismo entre os fármacos nos isolados testados.

- Para os isolados resistentes a polimixina B, investigar a taxa de mutantes e a presença do gene mcr-1.

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CAPÍTULO II

"COULD CARBAPENEM-RESISTANT AND POLYMYXIN-RESISTANT *Acinetobacter baumannii* BE TREATED WITH POLYMYXIN B AND VANCOMYCIN COMBINED?"

Could carbapenem-resistant and polymyxin-resistant *Acinetobacter baumannii* be treated with polymyxin B and vancomycin combined?

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Abstract

In the present *in-vitro* study, the effect of combination of polymyxin B (PMB) and vancomycin (VAN) was assessed against six *Acinetobacter baumannii* clinical isolates belonging to six different clusters (three PMB-susceptible and three PMB-resistant). Additionally, PMB-resistance was investigated with *mcr-1* gene amplification and mutant frequency assay. VAN minimum inhibitory concentration (MIC) was assessed by broth microdilution. The synergistic effect of the PMB-VAN combination was determined with the checkerboard, time-kill, disk-diffusion and Etest assays. In the checkerboard assay, all PMB-resistant isolates showed a synergistic effect (FICI ≤ 0.5). A reduction in PMB-MICs was observed in all isolates tested, with two PMB-resistant isolates changing category from resistant to susceptible. In the time-kill assay, bactericidal activity was observed in two PMB-resistant and one PMB-susceptible isolate (3 $\log_{10}$ reduction in colony count after 24h incubation). No antagonism was detected by the checkerboard, time-kill, disk-diffusion or Etest assay. Unexpectedly, the time-kill assay demonstrated that the PMB-VAN combination had a bactericidal effect at 24h against isolates with a high mutant rate for PMB, suggesting that this combination may block the hypermutation of some isolates. All PMB-resistant isolates also showed synergism in the disk-diffusion test, and a significant decrease in VAN MICs in the Etest assay. The findings suggest that the PMB-VAN combination has a potential therapeutic effect, especially against infections caused by PMB-resistant *A. baumannii*. Additional studies to evaluate the use of this combination in medical practice are warranted.

Keywords: *Acinetobacter baumannii*; polymyxin B; vancomycin; antimicrobial combination

1. Introduction

Acinetobacter baumannii is a challenging Gram-negative (GN) bacterial pathogen that causes hospital-acquired infections. Due to its ability to develop several resistance mechanisms, the incidence of multidrug-resistant (MDR) *A. baumannii* isolates has increased in hospitals worldwide [1].

Because infections caused by MDR *A. baumannii* have high mortality rates, the use of antimicrobial combinations is recommended for adequate empirical coverage [2]. Although the antimicrobials polymyxin B (PMB) and polymyxin E (Colistin) are often the last resort to treat infections caused by MDR *A. baumannii*, isolates resistant to these antimicrobial agents have been identified especially in prior therapy or monotherapy cases treated with these drugs [3].

Therapies using antimicrobial combinations, including polymyxins, have shown promising results as they disrupt the outer membrane of GN bacilli, facilitating the penetration of other antimicrobials into the cell [4]. Vancomycin (VAN) is indicated for infections caused by Gram positive bacteria, and acts by forming a complex with the C-terminal D-alanine residues present in peptidoglycan precursors, interrupting cell wall synthesis. Because of its large size and complex structure, VAN does not penetrate the outer membrane of GN bacteria [5], but the membrane-disrupting properties of polymyxins could allow VAN to reach its periplasmic target and act against GN bacteria [6].

Although PMB and Colistin (COL) often have comparable action, the administered form of COL (COL-methanesulfonate) has a variable rate of conversion to COL, and to attain its required plasma concentration, 4-5 times more COL-

methanesulfonate than PMB must be administered, since the latter reaches the active concentration more rapidly and reliably in the serum [7].

Some studies have evaluated the COL-VAN antimicrobial combination against multidrug-resistant *A. baumannii*. However, to the best of our knowledge no studies evaluating the antimicrobial activity of the PMB-VAN combination against multidrug-resistant *A. baumannii* isolates have been conducted so far. This study assessed the *in-vitro* efficacy of PMB plus VAN against OXA-23-producing PMB-resistant and PMB-sensitive isolates of *A. baumannii* with different clonal origins.

2. Material and methods

2.1. Bacterial samples

The study included six isolates, all carbapenem-resistant *Acinetobacter baumannii* [minimum inhibitory concentration (MIC) ≥ 64 mg/ml], three PMB-resistant (MIC ≥ 4μg/ml) and three PMB-susceptible (MIC≤ 0.5mg/ml) (Table 1). The pathogens were identified was performed using a Phoenix® - BD Automated Microbiology System (Becton Dickinson & Co., Sparks, MD, USA). The isolates used in this study were obtained from clinical specimens: four isolates from tracheal aspirate and two from catheter tips. The susceptibility of the pathogens to PMB was determined by the E test, broth microdilution and agar dilution assays. All isolates conteined $bla_{\text{OXA-51}}$ and $bla_{\text{OXA-23}}$ genes by the polymerase chain reaction (PCR) test, and were classified into six different clusters based on the enterobacterial repetitive intergenic consensus-PCR (ERIC-PCR). These isolates were selected from samples previously analyzed by Menegucci et al. [8], and were stored in Mueller–Hinton broth (MHB) (Difco Laboratories, Sparks, MD) with 30% glycerol (Merck, Darmstadt, Germany) at -20°C until the day of use.

2.2. Vancomycin MICs

After two subcultures, 0.5 McFarland standard was prepared using a nephelometer (PhoenixSpec™ nephelometer; Becton Dickinson). VAN MICs against each isolate were determined using the broth microdilution method as described in the CLSI M07-A10; Approved Standard [9].

2. 3. Synergy studies

The *in-vitro* activity of the PMB-VAN combination was evaluated using four different methods, as described below.

2.3.1. Checkerboard assay

PMB and VAN alone and in combination were evaluated by the checkerboard assay in 96-well microtiter plates (Inlab, São Paulo, Brazil) [10]. Briefly, the inoculum of each bacterial isolate was prepared in cation-adjusted Mueller-Hinton broth (CA-MHB) at a 0.5 McFarland standard and added to the wells with a final concentration of 5×10^5 CFU/ml. The test was conducted in duplicate and in cases of discordant values, the experiment was repeated. After incubation at 37°C for 16 to 20h, the modal MICs for the antibiotics alone and in combination were determined for each individual isolate. The activity of the drug combination was classified according to the fractional inhibitory concentration index (FICI), which was calculated by adding the MIC of each individual drug when used in combination, divided by the MIC of the drug used alone. The the effect of the antimicrobial combination therapy was classified according to the following categories: synergism, FICI $\leq$ 0.5; indifference, 0.5 < FICI < 4; and antagonism, FICI $\geq$ 4.

2.3.2. Time-kill assay

In the time-kill assay, tubes containing CA-MHB and concentrations of PMB and VAN alone and in combination (0.5 × MIC) were inoculated with 10^6 CFU/ml of each isolate and incubated with continuous shaking at 37°C. Samples were collected at 0, 6, 12, and 24 h, serially diluted in saline, plated on Mueller-Hinton Agar (MHA), and counted after 24 h of incubation. Time-kill curves were constructed by plotting mean colony counts ($\log_{10}$ CFU/ml) versus time. The drug combination was considered synergistic when a reduction in colony count of $2\ \log_{10}$ was achieved after 24 h (bacteriostatic activity $\geq 2\ \log_{10}$ and $<3\ \log_{10}$; and bactericidal activity $\geq 3\ \log_{10}$) in comparison with the more active single agent. The drug combination was classified as indifferent when $<2\ \log_{10}$ increase or decrease in colony counts was observed, while it was considered antagonistic when $> 2\ \log_{10}$ increase in colony counts was reached after 24 h, in comparison with the more active single agent [11].

2.3.3. Disk-diffusion and Etest assays

Disk-diffusion and Etest assays were based on the Etest assay described by Gordon et al. [6]. PMB was incorporated into agar plates (0.5 × MIC). Briefly, plates were inoculated in three directions with inoculum equivalent to a 0.5 McFarland turbidity standard, and a VAN disk (30μg/ml) and a VAN Etest were applied on the surface of agar plates with PMB and incubated at 37°C for 18-20h. VAN MICs (Etest) in the presence of PMB were compared toVAN MICs in the absence of PMB. If the VAN MIC was reduced within a 1-fold dilution, the result was interpreted as indifferent; if it was reduced by a 2-fold dilution, the result was considered additive; if it was reduced by a >3-fold dilution, the result was considered synergistic [12]. The synergistic effect in the disk-diffusion was defined when the inhibition zone of the VAN disk in the presence of PMB increased at least 5mm in relation to the inhibition zone of the VAN disk without PMB.

2.4. Additional PMB-resistance analyses

2.4.1. *mcr-1* gene amplification

The *mcr-1* gene was detected using the PCR method. Bacterial DNA was extracted with the boiling method. The primers in this study (forward primer, 5'-CGGTCAGTCCGTTTGTTC-3', and reverse primer, 5'-CTTGGTCGGTCTGTAGGG-3') were previously described by Liu *et al*. [13] and used at a concentration of 10 pmol/µL in the PCR mixture. The following cycling parameters were employed for amplification: initial DNA denaturation at 95 °C for 5 min, followed by 30 cycles at 95°C for 1 min, annealing at 56°C for 45 s and extension at 72 °C for 1 min, followed by a single, final, elongation step at 72 ºC for 5 min. Amplified products were analyzed on gel contained 1.5% agarose, stained with ethidium bromide. After running for 50min at a constant voltage (100 V), the gel was visualized and photographed against ultraviolet light under a transilluminator L-PIX HE (Loccus Biotecnologia ®, São Paulo, Brazil).

2.4.2. Mutant frequency

Mutant frequency (MF) was determined as previously described by Zhanel et al. [14], with some modifications. The bacterial concentration of 10^{10} CFU/ml of the samples was quantified by serial dilutions and 20 µl of each dilution was plated on MHA-plates (Becton Dickinson) with and without the antimicrobials, in triplicate, in order to count mutant colonies. PMB concentrations tested ranged from 1 to 8 times the PMB MIC of each isolate. The MFs for PMB were calculated by multiplying the number of mutant colonies by 50 to convert to CFU/ml, and the result was divided by the initial bacterial inoculum.

3. Results and Discussion

Infections caused by *A. baumannii* are difficult to treat. Identification of synergism between antimicrobials is important because of the few treatment options available [12]. Although several studies have demonstrated the synergistic effect of the COL-VAN combination against *A. baumannii* [6,15,16], our data add significant information, demonstrating PMB-VAN synergism in 50% of the isolates tested, regardless of the method used (Table 1).

The results of the checkerboard and time-kill assays agreed in 4/6 of the isolates (67%), indicating a lack of direct correlation between tests. This result was expected since these techniques measure different parameters, and should be used complementarily and not comparatively in the detection of synergism [17].

In the checkerboard assay, all PMB-resistant isolates showed synergism (FICI ≤ 0.5) with 2 of them changing categories from resistant to susceptible to PMB. In a study that evaluated three COL-resistant *A. baumannii* isolates, O'Hara et al. [16], also observed a synergistic effect of the COL-VAN combination for all isolates with checkerboard testing, similarly to the present study. None of the isolates showed an antagonistic effect, and all PMB-susceptible isolates showed indifference.The PMB-VAN combination increased bacterial susceptibility to at least one of the antimicrobials tested in combination and four isolates showed reductions of MICs for both antibiotics. The reductions in PMB MICs ranged from 1- to 5-fold for all isolates. Although VAN showed no antimicrobial activity against *A. baumannii* isolates, when combined with PMB, a 4- to 7-fold decrease in the MICs occurred in 4/6 of the isolates (67%).

The time-kill assay indicated a synergistic effect of the PMB-VAN combination in two PMB-resistant isolates (Ac-680 and Ac-Cl) and one PMB-susceptible isolate

189 (Ac-68). For these isolates, the PMB-VAN combination showed a bacteriostatic effect
190 after 6h, and a bactericidal effect after 24h (Fig 1). In relation to isolates that showed
191 indifference (Ac-576, Ac-53 and Ac-S22), despite the ≥ 3 $\log_{10}$ CFU/ml decrease after
192 6h, regrowth occurred between 12 and 24h. In contrast, Yang et al. [15] observed that
193 when the COL-VAN combination was tested against four *A. baumannii* isolates, two
194 COL-susceptible isolates showed a bactericidal effect, with no regrowth. However,
195 although the two COL-resistant isolates initially also showed bactericidal activity,
196 regrowth was observed at 24h.

197 The present study also evaluated synergy using the VAN Etest and a VAN disk
198 in a medium containing PMB (Fig 2). The disk-difussion test showed synergism in all
199 PMB-resistant isolates, while the Etest indicated a significant decrease in MIC values.
200 For all PMB-susceptible isolates, no significant differences between the tests were
201 observed with or without PMB on the MHA plates. Gordon et al. [6] performed synergy
202 testing of the PMB-VAN combination with the Etest method against 40 isolates of
203 MDR *A. baumannii* susceptible to COL. Initial VAN MICs were >256 μg/ml and when
204 the VAN Etest was combined with 0.5 μg/ml of COL supplemented on agar, VAN
205 MICs were reduced in all cases, ranging from 0.016 to 48 μg/ml. Differently from the
206 study conducted by Gordon et al. [6], we used PMB-resistant isolates, PMB instead of
207 COL, and we added a new *in-vitro* synergy test with a VAN disk. Based on these
208 results, we believe that the disk-diffusion and Etest can be reliable methods to evaluate
209 the synergistic effect of PMB-VAN combination in the laboratory, as both showed
210 similar results to the checkerboard test.

211 The PCR demonstrated that all PMB-resistant isolates were negative for the *mcr-*
212 *1* gene. However, caution must be exercised as other mechanisms involved in
213 polymyxin resistance have not been investigated. The mutant frequency investigation

showed unprecedented results. Two isolates (Ac-680 and Ac-Cl) demonstrated high MFs (in concentration of eight times the MIC, MF reached 7.0×10^{-2} and 1.1×10^{-3} for Ac-680 and Ac-Cl, respectively). One isolate (Ac-576) showed confluent growth when 10^{10} cells were applied onto the plate, but no colony was recovered in the two-fold serial dilution. Our time-kill assay results indicated that the PMB-VAN combination showed a bactericidal effect, with no regrowth at 24 h, against the Ac-680 and Ac-Cl isolates, suggesting that this combination has the potential to block the hypermutation of these isolates.

O' Hara et al. [15] and Yang et al. [16] conducted synergy studies using a *Galleria mellonella* model and reported that the COL-VAN combination increased the percentage of *G. mellonella* survival at 96-120 h after infection in comparison to monotherapy with COL. Although COL-methanesulfonate is normally administered to treat human infection, the authors used COL in their study. Therefore, considering the PMB and COL pharmacokinetics, we believe that the data obtained in the present study using PMB could be extrapolated more directly to humans with systemic infections. Studies using COL may not be as reliable because only a small fraction of the COL-methanesulfonate is converted to COL systemically, whereas about 70% of this prodrug is excreted in the urine in humans with normal renal function [18].

To the best of our knowledge, no study has evaluated the increased risk of renal toxicity caused by the PMB-VAN combination, and the data available in the literature on the COL-VAN combination are contradictory. Garnacho-Montero et al. [19] reported that nephrotoxicity was significantly higher in patients treated with COL-VAN, compared to patients treated with COL alone. Kalin et al. [20] found no differences in kidney injury rates in patients using antimicrobials concomitantly or in monotherapy. PMB appears to be less nephrotoxic than COL-methanesulfonate in patients [18],

239 indicating that the PMB-VAN combination may cause less kidney injury than the COL-
240 VAN combination.

241 The present *in-vitro* study on the antimicrobial synergism, of the PMB-VAN
242 combination has some inherent limitations, as *in-vitro* results do not always coincide
243 with *in vivo* results. However, our data clearly showed the potential of the PMB-VAN
244 combination, especially against PMB-resistant isolates (MIC≥ 4 μg/ml). The small
245 number of *A. baumannii* isolates used to evaluate the synergistic effect of the PMB-
246 VAN combination can also be criticized. Nonetheless, the six different bacterial clusters
247 tested can be considered a broad representation of this pathogen.

248 In conclusion, based on the *in-vitro* data obtained in the present study, the PMB-
249 VAN combination clearly showed a synergistic effect, indicating that it may become a
250 treatment option against MDR *A. baumannii*, especially PMB-resistant *A. baumannii*.
251 However, before this combination can be recommended for wide clinical application,
252 with no risk of toxicity, randomized clinical trials are required to identify the lowest
253 practicable doses based on the pharmacokinetics/pharmacodynamics of these drugs.

254

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Table 1

Synergistic effects of polymyxin B plus vancomycin against six strains of carbapenem-resistant *Acinetobacter baumannii*

Strain	Clonality	MIC (µg/ml)		Checkerboard		Time-kill assay (0.5 × MIC) Log▲ (Log_{10}CFU/ml)	Disk-diffusion▲ (mm)	VAN E-test (MIC-µg/ml)
		PMB	VAN	PMB/VAN (µg/ml)	FICI			
Ac-576	A	16	>256	0.5/16	0.094 (Synergy)	–1.759 (Indifference)	8 (Synergy)	4 (Synergy)
Ac-680	B	16	>256	4/2	0.258 (Synergy)	–3.611 (Synergy/bactericidal activity)	7 (Synergy)	8 (Synergy)
Ac-Cl	C	4	>256	0.5/8	0.156 (Synergy)	–3.329 (Synergy/bactericidal activity)	5 (Synergy)	8 (Synergy)
Ac-53	D	0.5	>256	0.25/4	0.516 (Indifference)	–1.157 (Indifference)	2 (Indifference)	>256 (Indifference)
Ac-68	E	0.5	>256	0.25/256	1.5 (Indifference)	–3.718 (Synergy/bactericidal activity)	1,5 (Indifference)	>256 (Indifference)
Ac-S22	F	0.5	>256	0.06/256	1.12 (Indifference)	–1.415 (Indifference)	0 (Indifference)	>256 (Indifference)

MIC: minimum inhibitory concentration; PMB: polymyxin B; VAN: vancomycin; FICI: fractional inhibitory concentration index; In checkerboard, synergy was defined as FICI≤ 0.5 and indifference as 0.5 < FICI < 4; Log▲ (final inoculum of the combined drugs minus final inoculum of the more active drug in the combination (log_{10} CFU/ml)). In Time-kill assay, synergy was defined as a ≥2 log_{10} decrease in colony count after 24h by the combination when compared with the more active single agent, indifference as a <2 log_{10} increase or decrease in colony count at 24 h by the combination when compared with that by the more active drug alone and bactericidal activity as ≥3 log_{10} decrease in colony count after 24 h; Negative values indicate a decrease in colony count. Disk-diffusion▲ (zone of inhibition (mm) of the VAN disk in the presence of PMB minus the zone of inhibition (mm) of the VAN disk on agar without PMB). In the VAN-Etest, PMB was incorporated into agar (0.5 × MIC).

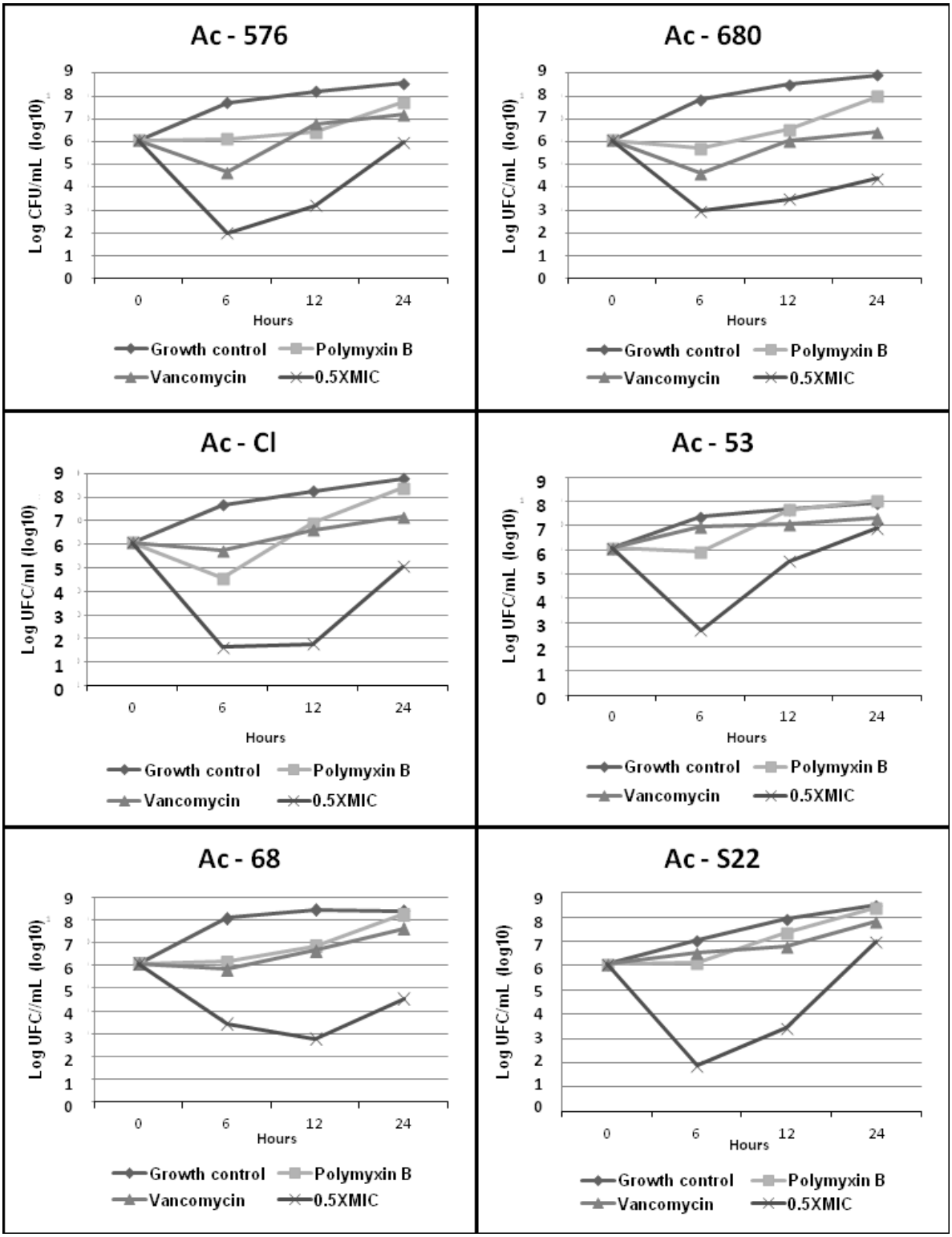

Fig 1: Time-kill curves of isolates using the drugs alone and in combination at 0.5 × MIC against *A. baumannii* isolates. Isolate Ac-576 and Ac-680 (polymyxin B- 8µg/ml), Ac-Cl (polymyxin B- 2 µg/ml), Ac-53, Ac-68 and Ac-S22 (polymyxin B- 0.25µg/ml). For all isolates a vancomycin concentration of 128 µg/ml was used.

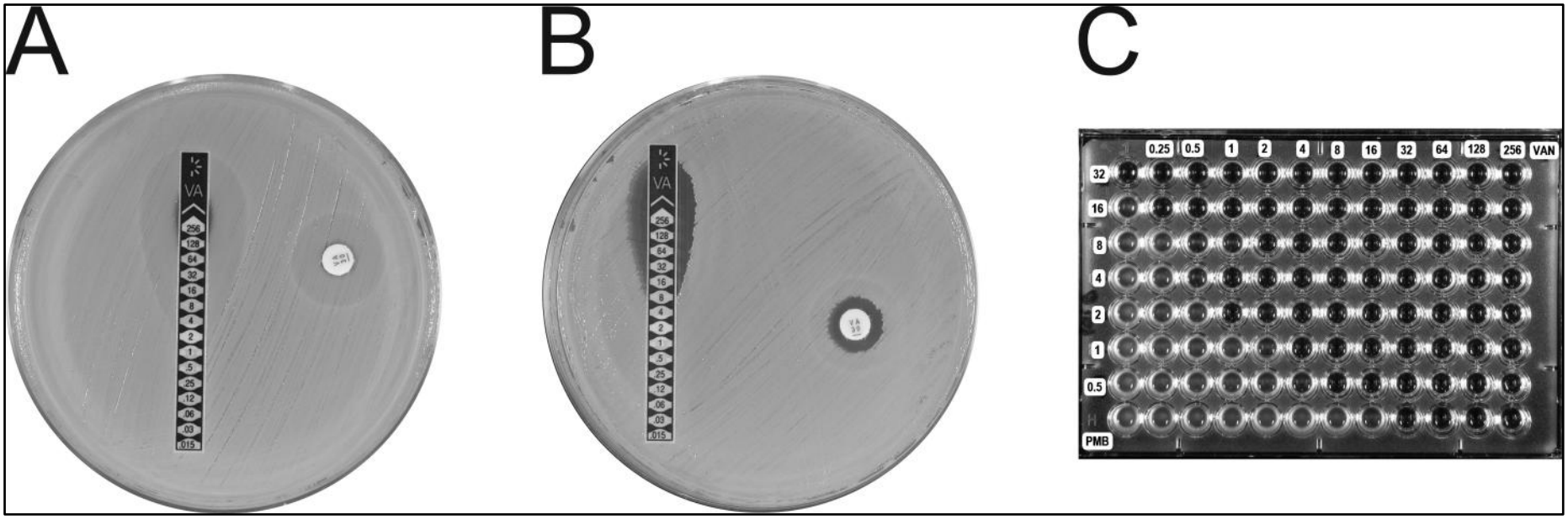

Fig 2. Demonstration of the synergistic action of polymyxin B plus vancomycin against PMB-resistant *A. baumannii*. A: Etest and disk-diffusion assay with agar plates without polymyxin B. B: Etest and disk-diffusion assay with agar plates supplemented with polymyxin B (0.5 × MIC). C: representation of the checkerboard assay.

CAPÍTULO III

CONCLUSÕES

Este estudo avaliando a combinação de polimixina B e vancomicina contra diferentes *clusters* de *Acinetobacter baumannii* produtores de carbapenemase, sendo três *clusters* sensíveis e três resistentes à polimixina B mostrou que:

1) A combinação dos antimicrobianos testados apresentou sinergismo em 50% dos isolados independente da metodologia.
2) A associação dos antimicrobianos teve melhor resultado em isolados resistentes a polimixina B.
3) Nenhum antagonismo foi verificado.
4) O sinergismo verificado pelo método *checkerboard* e pelo método *time-kill* mostrou concordância de 67% nos resultados.
5) Os resultados obtidos pelos métodos *checkerboard*, *Etes*t e Disco Difusão apresentaram 100% de concordância;
6) Os isolados resistentes a polimixina B apresentaram ausência do gene mcr-1, porém 2 de 3 isolados mostraram alta taxa mutacional para polimixina B.
7) Os isolados que mostraram alta taxa de mutantes para polimixina B também apresentaram efeito bactericida na combinação (polimixina B e vancomicina) pelo teste de *time-kill*, sugerindo o potencial dessa combinação para bloquear a hipermutação desse isolados.
8) Maiores investigações são sugeridas para determinar se nossos achados *in vitro* são eficazes na prática clínica.

PERSPECTIVAS FUTURAS

- Avaliar a combinação de polimixina B e vancomicina contra um número maior de isolados de *Acinetobacter baumannii* através dos métodos *checkerboard*, *Etest* e disco difusão a fim de verificar se para todos os casos testados permanece a concordância de 100% entre eles.

- Avaliar essa combinação antimicrobiana por meio de estudos de farmacocinética e farmacodinâmica;

- A partir dos resultados obtidos pelos estudos de farmacocinética e farmacodinâmica, propor estudos *in vivo* a fim de verificar a possibilidade de uso clínico dessa combinação.

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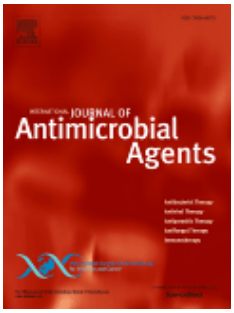

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