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Synergistic effect between nisin and polymyxin B against pandrug-resistant and extensively drug-resistant *Acinetobacter baumannii* ☆

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ABSTRACT

Acinetobacter baumannii is an opportunistic pathogen predominantly associated with nosocomial infections. The World Health Organization's data on antibiotic-resistant 'priority pathogens' reports carbapenem-resistant *A. baumannii* as a pathogen which is in critical need of research and development of new antimicrobials. Emerging resistance against polymyxins, last-resort drugs for carbapenem-resistant *A. baumannii*, increases the need for new therapeutic approaches such as synergistic combinations. Nisin, an antibacterial peptide produced by the Gram-positive bacteria *L. lactis*, is a US Food and Drug Administration approved food preservative with bactericidal action predominantly against other Gram-positive bacteria. A 2008 study reported that topical nisin was effective against staphylococcal mastitis in humans. Additionally, nisin has shown activity against Gram-negative bacteria in combination with antimicrobials such as polymyxin B. A recent in vitro study reported that nisin and polymyxin B exhibited synergistic activity against one isolate each of *A. baumannii, Acinetobacter lwoffii* and *Acinetobacter calcoaceticus* using time-kill assay and checkerboard technique. We evaluated the synergistic potential of nisin and polymyxin B against 15 unique clinical *A. baumannii* isolates using time-kill assay. Three of eight (38%) extensively drug-resistant and six of seven (86%) pandrug-resistant *A. baumannii* isolates showed synergy with one or more combinations of nisin and polymyxin B. The synergy seen with the use of lower concentrations of polymyxin B may help in reducing the dose-dependent side effects. Additional studies involving pharmacokinetics and pharmacodynamics of nisin are required to explore clinical possibilities.

1. Introduction

Carbapenem-resistant *Acinetobacter baumannii* have emerged as a serious health concern over the past decade [1,2]. Polymyxins are considered the last resort drugs for *A. baumannii* infections that are resistant to all other antimicrobial classes. Despite the dose-related nephron and neurotoxicity, therapeutic use of polymyxins has increased due to lack of other effective antimicrobials [3]. The mechanisms of antimicrobial resistance in *A. baumannii* include beta-lactamases and changes in penicillin binding proteins (beta-lactam resistance), carbapenemases, and alterations in porin channels (carbapenem resistance) [1], efflux pumps (beta-lactam, tetracycline, aminoglycoside, and quinolone resistance), mutations in DNA gyrase enzyme (quinolone resistance), and aminoglycoside-modifying enzymes. Resistance to polymyxins is likely due to modifications in the lipopolysaccharide membrane of *A. baumannii* [4]. Additionally, resistance against antimicrobial combinations including ceftazidime-avibactam, piperacillin-tazobactam, and ampicillin-sulbactam further reduces possible clinical interventions [5]. *A. baumannii* non-susceptible to ≥1 antimicrobial agent in all but ≤2 classes were classified as extensively drug-resistant (XDR), and strains non-susceptible to all drugs in all antimicrobial classes used for treatment of *A. baumannii* were classified as pandrug-resistant (PDR) for this study [6].

Bacteriocins are ribosomally synthesized antimicrobial peptides produced by bacteria as part of their defense system. They are further classified based on presence or absence of post-translational modification. Class I bacteriocins which undergo post-translational modification include lantibiotics [7]. Nisin, produced by Gram-positive bacteria such as *Lactococcus lactis* and some *Streptococcus* spp., is the most extensively studied and used lantibiotic. Nisin, from *L. lactis*, is used as food preservative and has been listed in the generally recognized as safe category by the US Food and Drug Administration (FDA) [8]. Nisin exerts its

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Table 1
Minimum inhibitory concentrations (MICs; μg/mL) determined by broth microdilution for *Acinetobacter baumannii* isolates.

XDR isolates (*n*=8)	NISIN	PMB	AK	GM	TO	DOR	IMI	MER	P/T	CPM	CTX	CIP
279	100	2 S	>64 R	>16 R	>16 R	>8 R	64 R	>8 R	>128 R	32 R	>32 R	>8 R
283	50	1 S	>64 R	>16 R	>16 R	>8 R	64 R	>8 R	>128 R	32 R	>32 R	>8 R
290	100	1 S	>64 R	>16 R	>16 R	>8 R	64 R	>8 R	>128 R	>32 R	>32 R	>8 R
291	50	1 S	>64 R	>16 R	>16 R	>8 R	64 R	>8 R	>128 R	32 R	>32 R	>8 R
294	50	1 S	>64 R	16 R	4 S	>8 R	64 R	>8 R	>128 R	>32 R	>32 R	>8 R
295	100	1 S	4 S	4 S	1 S	>8 R	64 R	>8 R	>128 R	>32 R	>32 R	>8 R
297	100	1 S	32 I	>16 R	16 R	>8 R	64 R	>8 R	>128 R	>32 R	>32 R	>8 R
298	50	1 S	>64 R	>16 R	>16 R	>8 R	64 R	>8 R	>128 R	>32 R	>32 R	>8 R
PDR isolates (*n*=7)												
288	50	>128 R	>64 R	>16 R	>16 R	>8 R	32 R	>8 R	>128 R	>32 R	>32 R	>8 R
303	100	128 R	>64 R	>16 R	>16 R	>8 R	64 R	>8 R	>128 R	>32 R	>32 R	>8 R
304	50	16 R	32 I	>16 R	>16 R	>8 R	32 R	>8 R	>128 R	>32 R	>32 R	>8 R
307	100	16 R	>64 R	>16 R	>16 R	>8 R	16 R	>8 R	>128 R	32 R	>32 R	>8 R
308	100	8 R	>64 R	>16 R	>16 R	>8 R	16 R	>8 R	>128 R	32 R	>32 R	>8 R
309	100	4R	>64 R	>16 R	>16 R	>8 R	>64 R	>8 R	>128 R	>32 R	>32 R	>8 R
310	100	4 R	>64 R	>16 R	>16 R	>8 R	>64 R	>8 R	>128 R	>32 R	>32 R	>8 R

Nisin and polymyxin B (PMB) minimum inhibitory concentrations (MICs) determined in present study were performed in triplicate, using the mean MIC value to determine concentrations used in the time-kill study. All other drugs were previously tested at the CDC. AK, amikacin; CIP, ciprofloxacin; CPM, cefepime; CTX, ceftriaxone; DOR, doripenem; GM, gentamicin; I, intermediate; IMI, imipenem; MER, meropenem; P/T, piperacillin-tazobactam; PDR, pandrug-resistant; R, resistant; S, susceptible; TO, tobramycin; XDR, extensively drug-resistant.

antimicrobial activity by binding to lipid-II, a cell wall precursor. The nisin–lipid II complex creates a change in membrane permeability through pore formation and prevents peptidoglycan synthesis [9]. In vitro studies have reported significant antibacterial activity against Gram-positive bacteria such as methicillin-resistant *Staphylococcus aureus*, vancomycin-resistant *Enterococcus, Streptococcus* spp. and food-borne pathogens such as *Clostridium botulinum* and *Listeria monocytogenes* [10–12]. Growing antimicrobial resistance has resulted in increased interest in nisin and its biomedical application as a possible adjuvant to antibiotics in the field of dentistry, medicine, and veterinary science [13]. Nisin was found to be effective against *Enterococcus faecalis* and *Streptococcus gordonii* inoculated in vitro into human root canals [14]. Furthermore, topical preparations of nisin were effective against *Staphylococcus* spp. mastitis in humans. In a small case–control study of eight women with bilateral staphylococcal mastitis, no clinical signs of mastitis were observed in the nisin group on day 14 (*n*=4), whereas the infection persisted in the placebo group (*n*=4) [15]. Nisin may have significant application in the cattle industry because of its potential in the treatment of bovine mastitis [16,17]. A purified nisin intramammary product for bovine subclinical mastitis from ImmuCell Inc. (Portland, ME, USA) is planned to be submitted to the FDA in 2018 [18]. While nisin has limited activity against Gram-negative bacteria due to its inability to penetrate the lipopolysaccharides in the outer membrane, combination therapies with antibiotics or compounds that weaken the bacterial outer membrane, such as ethylenediaminetetraacetic acid (EDTA), have demonstrated increased effectiveness of nisin against Gram-negative bacteria [19]. Nisin plus ceftriaxone or cefotaxime was found to be synergistic in vitro and in vivo against *Salmonella* [20], while nisin plus polymyxin B (PMB) showed in vitro synergy against one reference strain each of *Pseudomonas aeruginosa, Escherichia coli, Klebsiella pneumoniae*, and *Pseudomonas putida* [21]. Furthermore, nisin in combination with PMB demonstrated in vitro synergistic activity against oneisolate each of *A. baumannii, A. lwoffii,* and *A. calcoaceticus* using time-kill assay and checkerboard technique [22]. Synergistic combinations of drugs with PMB may have the benefit of lowered dosage of PMB, thus possibly decreasing adverse side effects. The purpose of our study was to evaluate combinations of nisin and PMB against 15 unique clinical isolates of *A. baumannii*, including seven PDR (PMB-resistant) and eight XDR (PMB-susceptible).

2. Materials and methods

2.1. Microorganisms, media, and antimicrobial agents

A total of 15 unique clinical *A. baumannii* isolates, seven PDR (PMB-resistant) and eight XDR (PMB-susceptible), were obtained from the Centers for Disease Control and Prevention (CDC) and FDA Antimicrobial Resistance Bank. Minimum inhibitory concentrations (MICs) determined by broth microdilution (Table 1) and molecular testing for resistance mechanisms by analysis of whole-genome sequencing using the ResFinder database (Table 2) was performed by the CDC [23]. For the present study, Mueller–Hinton II Broth (Becton–Dickinson Microbiology, Sparks, MD, USA) was prepared in the laboratory. Trypticase soy agar with 5% sheep blood plates (Becton–Dickinson) was used for subcultures of the isolates and for the colony counts in the time-kill assay. PMB sulfate powder and nisin powder (2.5%) (Millipore Sigma, St. Louis, MO, USA) were used.

2.2. Nisin stock preparation

One gram of nisin (2.5%) was dissolved in 25 mL of 0.05% acetic acid solution, centrifuged at 4000 × *g* for 20 min. The supernatant was then filter sterilized to prepare a 1-mg/mL stock solution. The stock solution was kept frozen at -70°C. Lower concentrations were prepared as needed from the stock [24].

2.3. Antimicrobial susceptibility testing

MICs of nisin and PMB were determined in triplicate (mean value used) by broth microdilution using sterile 96-well polystyrene microplates, following Clinical and Laboratory Standards Institute (CLSI) guidelines [25]. MICs were read as the first clear well with no growth. Quality control was included using *E. coli* ATCC 25922 and *P. aeruginosa* ATCC 27853 for PMB according to CLSI guidelines [26]. Quality control strains for nisin included *E. coli* ATCC 25922 and *S. aureus* ATCC 25923. Nisin MICs for quality control strains were compared with findings from other published studies to ensure consistency and reproducibility of values [11,27]. There are no CLSI interpretive guidelines available for nisin quality control.

Table 2
Molecular mechanisms of resistance for *Acinetobacter baumannii* isolates obtained from the Centers for Disease Control and Prevention (CDC) and US Food and Drug Administration (FDA) Antibiotic Resistance Isolate Bank (testing performed by the CDC).

A. baumannii Isolates (n=15)	Aminoglycoside	Beta-lactam	Macrolide-Lincosamide-Streptogramin	Phenicols	Sulfonamides
279	aph(3′)-Ic aph(3′)-VIa armA strA strB	ADC-25 OXA-23 OXA-66 TEM-1D	mph(E) msr(E)	catB8	sul1 sul2
283	aph(3′)-Ic aph(3′)-VIa armA strA strB	ADC-25 OXA-23 OXA-66 TEM-1D	mph(E) msr(E)	catB8	sul1
288	armA strA strB	ADC-25 OXA-23 OXA-66	mph(E) msr(E)	catB8	sul1
290	aph(3′)-Ic aph(3′)-VIa armA strA strB	ADC-25 OXA-23 OXA-66 TEM-1D	mph(E) msr(E)	catB8	sul1
291	aph(3′)-Ic aph(3′)-VIa armA strA strB	ADC-25 OXA-23 OXA-66 TEM-1D	mph(E) msr(E)	catB8	sul1
294	aac(3)-IIa aph(3′)-VIa strA strB	OXA-23 OXA-65 TEM-1B			sul2
295	strA strB	ADC-25 OXA-23 OXA-66 TEM-1D			sul2
297	strA strB	ADC-25 OXA-23 OXA-66	mph(E) msr(E)		sul2
298	aph(3′)-Ic armA strA strB	ADC-25 OXA-237 OXA-66 TEM-1D	mph(E) msr(E)	catB8	sul1 sul2
303	aph(3′)-Ic aph(3′)-VIa strA strB	ADC-25 OXA-23 OXA-66	mph(E) msr(E)	catB8	sul1 sul2
304	aac(3)-Ia strA strB	ADC-25 OXA-66 OXA-72			sul2
307	aph(3′)-Ic armA strA strB	ADC-25 OXA-237 OXA-66	mph(E) msr(E)	catB8	sul1 sul2
308	armA strA strB	ADC-25 OXA-71 TEM-1D	mph(E) msr(E)	catB8	sul1
309	aac(3)-Ia armA strA strB	OXA-23 OXA-82	mph(E) msr(E)		sul1
310	aac(3)-Ia armA strA strB	OXA-23 OXA-82	mph(E) msr(E)		sul1

2.4. Synergy testing

In vitro synergy testing was performed using time-kill assay following CLSI guidelines [28]. The inoculum (approximately 5×10^5 cfu/mL; range 2×10^5 to 1×10^6 cfu/mL) was verified after plating in duplicate using a spiral plater and scanner (Spiral Biotech Inc., Norwood, MA, USA). Each isolate was tested with each drug in combination and alone. Three nisin and PMB combinations were tested: (1) both drugs at 1 × MIC; (2) both drugs at ½MIC; and (3) nisin (½MIC) plus PMB (¼MIC). The second and third combinations were evaluated to see whether using lower concentrations of nisin and PMB would still demonstrate synergy. Serial dilutions were performed in 0.85% saline when needed. The spiral plate/scanner can detect bacterial counts as low as 20 cfu/mL. Plates were incubated in ambient air at 35°C for 18–24 h. Colony counts on all isolates were performed at 0 and 24 h. The mean colony count from duplicate samples was used to determine synergy. Synergy was defined as $\geq$2 $\log_{10}$ decrease in the colony count after 24h by

Table 3
Colony counts (colony-forming units (cfu)/mL) of nisin and polymyxin B (PMB) alone and in combination after 24 h determined by time-kill assay for *Acinetobacter baumannii* isolates.

A. baumannii (*n*=15)	Nisin 1 × MIC cfu/mL in 24 h	PMB 1 × MIC cfu/mL in 24 h	Nisin + PMB 1 × MIC cfu/mL in 24 h (Log_{10} change)	Nisin ½MIC cfu/mL in 24 h	PMB ½MIC cfu/mL in 24 h	Nisin + PMB ½MIC cfu/mL in 24 h (Log_{10} change)	PMB ¼MIC cfu/mL in 24 h	Nisin ½MIC + PMB ¼MIC cfu/mL in 24 h (Log_{10} change)
XDR isolates (*n*=8)								
279	2.24×10^4	3.76×10^5	1.56×10^5 (+0.9 IND)	3.86×10^6	4.10×10^5	1.54×10^5 (−0.3 IND)	5.82×10^8	8.02×10^5 (−0.5 IND)
283	4.30×10^6	1.18×10^6	9.66×10^4 (−1.2 IND)	6.75×10^7	3.88×10^8	4.34×10^5 **(−2.2 SYN)**	1.57×10^9	2.00×10^5 **(−2.4 SYN)**
290	7.46×10^5	5.93×10^7	4.85×10^5 (−0.3 IND)	5.18×10^6	1.65×10^8	4.07×10^5 (−1.1 IND)	9.24×10^8	3.74×10^5 (−1.2 IND)
291	5.15×10^6	9.82×10^7	3.07×10^5 (−1.2 IND)	1.72×10^7	3.08×10^8	3.53×10^5 (−1.8 IND)	3.81×10^8	3.88×10^5 (−1.8 IND)
294	2.34×10^6	2.66×10^5	2.95×10^6 (+1.0 IND)	2.14×10^7	2.86×10^7	3.74×10^4 **(−2.9 SYN)**	1.11×10^8	7.23×10^5 (−1.5 IND)
295	5.85×10^5	1.43×10^5	5.98×10^4 (−0.6 IND)	1.37×10^6	5.46×10^5	3.75×10^5 (−0.2 IND)	8.71×10^6	5.30×10^5 (−0.5 IND)
297	1.08×10^6	7.63×10^5	1.14×10^5 (−0.6 IND)	6.51×10^5	1.84×10^6	7.23×10^5 (+0.1 IND)	3.41×10^8	5.11×10^5 (−0.1 IND)
298	2.47×10^6	3.88×10^5	2.00×10^3 **(−2.1 SYN)**	7.29×10^6	1.04×10^4	1.00×10^1 **(−3.0 SYN)**	4.90×10^7	2.58×10^4 **(−2.5 SYN)**
PDR isolates (*n*=7)								
288	6.12×10^5	3.49×10^6	1.0×10^1 **(−4.0 SYN)**	8.63×10^6	6.46×10^7	1.72×10^4 **(−2.7 SYN)**	7.71×10^7	2.80×10^4 **(−2.6 SYN)**
303	4.97×10^5	2.94×10^7	3.93×10^5 (−0.1 IND)	2.07×10^6	2.94×10^7	2.66×10^5 (−1.0 IND)	8.27×10^7	6.95×10^5 (−0.6 IND)
304	2.00×10^3	1.0×10^1	1.0×10^1 (0 IND)	4.03×10^4	6.11×10^4	6.0×10^1 **(−2.8 SYN)**	2.38×10^7	1.0×10^1 **(−3.0 SYN)**
307	2.30×10^5	4.42×10^4	1.99×10^4 (−0.3 IND)	2.58×10^7	6.82×10^6	6.12×10^4 **(2.0 SYN)**	5.37×10^7	2.11×10^5 **(−2.0 SYN)**
308	8.60×10^5	1.06×10^4	2.58×10^3 (−0.9 IND)	9.20×10^6	1.08×10^7	5.80×10^2 **(−4.4 SYN)**	3.53×10^7	7.84×10^4 **(−2.2 SYN)**
309	1.09×10^5	8.40×10^5	1.0×10^1 **(4.0 SYN)**	7.79×10^5	8.81×10^7	3.90×10^3 **(−2.4 SYN)**	8.00×10^7	5.00×10^3 **(−2.2 SYN)**
310	1.09×10^6	1.06×10^8	1.0×10^1 **(−5.0 SYN)**	1.60×10^6	8.43×10^7	1.0×10^1 **(−5.0 SYN)**	8.44×10^7	1.0×10^1 **(−5.0 SYN)**

IND, indifference; MIC, minimum inhibitory concentration; PDR, pandrug-resistant; SYN, synergy; XDR, extensively drug-resistant.

the combination compared to that of the most potent agent alone. Indifference was defined as a <2 log_{10} decrease or increase in the CFU/mL after 24 h, and antagonism, >2 log_{10} increase in CFU/mL after 24h [28].

3. Results

3.1. MIC determination by broth microdilution (Table 1)

Eight *A. baumannii* isolates were XDR and PMB-susceptible (MICs 1-2 μg/mL), and seven isolates were PDR (PMB MICs 4 to >128 μg/mL). The CLSI defines PMB ≥4 μg/ml for *A. baumannii* as resistant [26]. Nisin MICs ranged from 50 to 100 μg/mL. There are no interpretative breakpoints for nisin.

3.2. Time-kill assay (Table 3)

The combination of nisin and PMB, with each agent at a concentration equal to the MIC, showed synergy in one of eight XDR *A. baumannii* isolates and in three of seven PDR isolates. Using the combination with each agent at ½MIC, synergy was seen in three of eight XDR isolates and six of seven PDR isolates. The combination of nisin (½MIC) and PMB (¼MIC) showed synergy in two of eight XDR isolates and six of seven PDR isolates. Isolates not demonstrating synergy were classified as indifferent. No antagonism was seen. Examples of synergy (isolate #310) and indifference (isolate #303) are shown in Figs. 1 and 2, respectively.

4. Discussion

Nisin was FDA approved in 1988 for use as an antimicrobial food preservative to inhibit the growth of *C. botulinum* spores and toxin formation in pasteurized cheese products. The FDA recommends a maximum of 250 parts per million of nisin in the finished product [8]. In 2017, the European Food Safe Authority re-evaluated the toxic potential of nisin and approved an acceptable daily intake of up to 1 mg nisin/kg body weight per day for use in certain food products [29]. The high safety threshold of nisin combined with low reports of resistance [30], despite its extensive use in the food industry, warrants further investigation into its therapeutic potential. Despite implementation of strict antimicrobial stewardship programs, PDR *A. baumannii* isolates continue to emerge. Synergistic combinations of nisin and existing antimicrobial compounds might provide an alternative to overcoming antibiotic resistance.

In our study, the highest amount of synergy was seen in six of seven [86%] of PDR isolates with the combination of nisin and PMB at ½MIC. The underlying mechanism of synergy may be due to the action of PMB on the outer membrane of *A. baumannii* enabling nisin to target the cell membrane. Interestingly, six of the seven PDR isolates demonstrated synergy with one or more of the combinations, indicating that a complete susceptibility to PMB may not be required for synergistic interactions. One PDR isolate (#304) showed indifference with the 1 × MIC combination but synergy with both lower concentrations at 24 h. This isolate gave consistent results after being repeated in triplicate. We have no explanation for this finding.

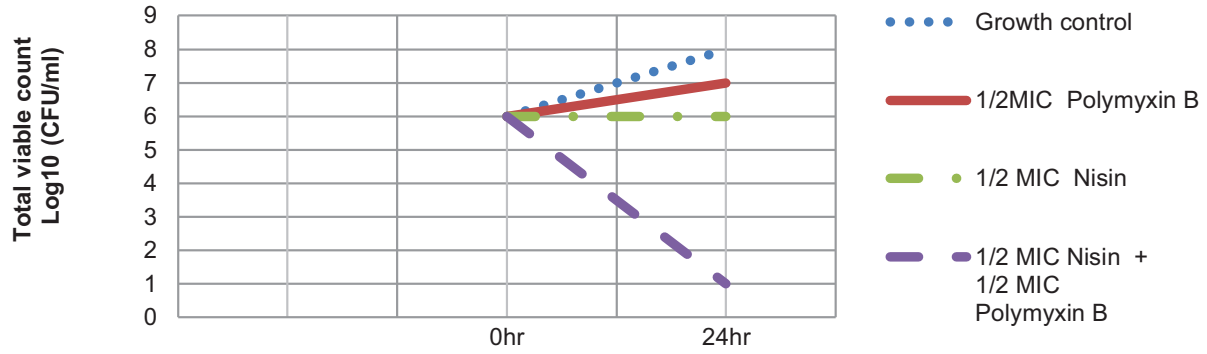

Fig. 1. Time-kill assay with isolate #310 showing synergy with both agents at ½ minimum inhibitory concentration (MIC) (−5.0 $\log_{10}$ change in colony-forming units/mL when compared to most potent agent at 24 h).

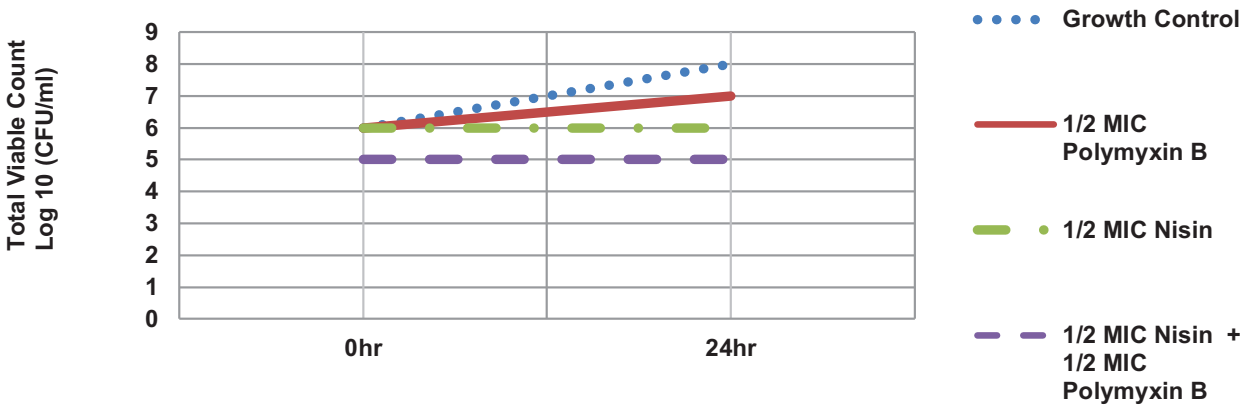

Fig. 2. Time-kill assay with isolate #303 showing indifference with both agents at ½ minimum inhibitory concentration (MIC) (−1.0 $\log_{10}$ change in CFU/mL when compared to most potent agent at 24 h).

This study warrants further evaluation for possible clinical use of nisin and PMB combinations for antibiotic-resistant *A. baumannii* infections. The potential modes of administration and clinical relevance of the combinations were considered. PMB is administered through intramuscular, intravenous, intrathecal, and topical routes. Effective modes of nisin administration are under investigation. Currently, degradation of nisin by enzymatic activity limits the use of oral and intraperitoneal administration [31,32]. However, topical preparations of nisin have been identified as effective against mastitis in humans and cows [15–17]. In addition, nisin exhibits highest solubility in acidic conditions, limiting intravenous use since the physiological pH of human blood is 7.4. Alternatively, bioengineered nisin forms with increased solubility at a neutral pH and increased potency against Gram-negative bacteria further increases the scope of clinical applications [33,34]. Our in vitro results demonstrate that a PMB-nisin preparation may be a possible treatment for PDR *A. baumannii* by inhalation for respiratory infections or as a topical for burns or wound infections. Further studies, including pharmacokinetics and pharmacodynamics of nisin in animal/disease models, may enable better understanding of the extent of synergy and *in vivo* effects of this combination.

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Competing Interests

None.

Ethical Approval

Not required.

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