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Research Article

In vitro synergism of combinations of colistin with selected antibiotics against colistin-resistant Acinetobacter baumannii

In-vitro-Synergismus von Kombinationen von Colistin mit ausgewählten Antibiotika gegen Colistin-resistente Acinetobacter baumannii

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Abstract

Aim: The in vitro activity of colistin in combination with sulbactam, netilmicin, and vancomycin against colistin-resistant *A. baumannii* strains was investigated. Furthermore, the clonal relationship of the strains was analyzed.
Methods: Clonal relationship was investigated using rep-PCR. To screen for synergysm, the fractional inhibitory concentration index (FICI) was calculated using checkerboard assay. The killing kinetics of the combination of colistin with vancomycin was assessed using time-kill assay.
Results: Three different clones were found among 10 clinical isolates of colistin-resistant *A. baumannii* strains. Thereof, 8 strains were susceptible to netilmicin. Synergistic interaction was detected in 1 strain with the combination of colistin-netilmicin, in 5 strains with colistin-sulbactam, and in 9 strains with colistin-vancomycin. None of combinations had antagonistic activity. Colistin-vancomycin combination resulted in rapid bactericidal activity.
Conclusion: These results show a distinct in vitro synergism between colistin and vancomycin, which might be useful to treat infection with multiple-resistant strains, prevent emergence of resistant strains, and to lower doses for both antibiotics to be used.

Keywords: Acinetobacter baumannii, colistin resistance, fractional inhibitory concentration index (FICI), time-kill assay, synergism, vancomycin, sulbactam, netilmicin

Zusammenfassung

Ziel: Die Kombination von Colistin mit Sulbactam, Netilmicin und Vancomycin wurde in vitro gegen Colistin-resistente *A. baumannii*-Stämme untersucht und die klonale Verwandtschaft der Stämme analysiert.
Methoden: Die klonale Verwandtschaft wurde mittels rep-PCR untersucht. Der Fractional Inhibitory Concentration Index (FICI) Bruch wurde mit dem Checkerboard-Assay berechnet. Die Abtötungskinetik Colistin-Vancomycin-Kombination wurde mit dem Zeit-Kill-Assay ermittelt.
Ergebnisse: Es wurden 3 verschiedene Klone identifiziert. Alle Isolate waren resistent gegen Colistin, 8 Stämme waren empfindlich gegen Netilmicin. Eine synergistische Wechselwirkung wurde für einen Stamm mit der Kombination Colistin-Netilmicin, für 5 Stämme mit der Kombination Colistin-Sulbactam und für 9 Stämme mit der Kombination Colistin-Vancomycin festgestellt. Bei keiner Kombination trat Antagonismus auf. Die Kombination von Colistin mit Vancomycin ergab eine rasche bakterizide Aktivität.
Fazit: Die In-vitro-Ergebnisse zeigen einen Synergismus für die Kombination von Colistin mit Vancomycin. Damit ist die Möglichkeit gegeben, Infektionen durch multipel-resistente Erreger zu behandeln und der

Entstehung resistenter Stämme entgegen zu wirken. Zugleich kann die Dosis für beide Antibiotika gesenkt werden.

Schlüsselwörter: Acinetobacter baumannii, Colistinresistenz, Fractional Inhibitory Concentration Index (FICI), Time-kill Assay, Synergismus, Vancomycin, Sulbactam, Netilmicin

Introduction

Acinetobacter baumannii is a Gram-negative opportunistic pathogen and one of the leading causes of hospital acquired infections such as septicemia, pneumonia, bacteremia, and urinary tract infections. In recent years, treatment of infections caused by *Acinetobacter* spp. has become increasingly difficult due to the spread of multidrug-resistant (MDR) strains. For these strains, colistin, an old polimyxin antibiotic, frequently is the only remaining therapeutic option. Nevertheless, colistin-resistant *A. baumannii* strains have been reported recently [1], [2], [3].

Colistin is a cationic antibiotic, which is bactericidal for Gram-negative bacteria, interacting with lipid A causing disarrangement of the outer membrane [4]. Colistin resistance amongst *A. baumanii* can occur by two mechanisms: lipopolysaccharide loss or modification of lipid A [5], [6], [7]. Because of the difficulties in the treatment of MDR *A. baumannii* infections, clinicians have been forced to use combination therapy to achieve synergistic activity. Some studies have demonstrated that colistin combined with rifampin, minocycline, ceftazidime, imipenem, azithromycin can be effective against MDR *A. baumannii* [8], [9], [10].

In the present study, we investigated the in vitro activity of colistin in combination with sulbactam, netilmicin, and vancomycin against clinical colistin-resistant *A. baumannii* strains.

Materials and methods

Bacterial isolates

Ten colistin-resistant *A. baumannii* strains isolated from different patients hospitalized in intensive care units (ICU) were used. Thereof, 5 were isolated from endothracheal aspirates, 2 from bronchoalveolar lavage, and 1 from blood, wound, and pleural fluid each. Identification and antibiotic susceptibility were performed using BD Phoenix Automated Microbiology System (Becton Dickinson, NJ, USA). Antimicrobial susceptibilities against colistin, sulbactam, netilmicin and vancomycin were investigated using microdilution method [11]. The clonal relationship of strains was explored using rep-PCR (DiversiLab, bioMérieux, France).

Checkerboard assay

Antibiotic interactions were determined using checkerboard assay as previously described [12]. The concentrations tested for colistin were 2–1,024 µg/ml; for sulbactam 2–128 µg/ml; for netilmicin 0.25–16 µg/ml, and for vancomycin 8–512 µg/ml. Microbroth dilution plates were inoculated with each *A. baumannii* isolate to yield ~$5x10^5$ CFU/ml in a 200 µl final volume and incubated overnight at 35 °C.

The fractional inhibitory concentration index (FICI) was calculated for each combination using the following formula: $FIC_A+FIC_B=FICI$, where FIC_A=MIC of drug A in combination/MIC of drug A alone, and FIC_B=MIC of drug B in combination/MIC of drug B alone. The FICI was interpreted as follow: synergism = FICI≤0.5; indifference = 0.5<FICI≤4; antagonism = FICI>4 [13].

Time-killing assays

Tubes containing Mueller-Hinton broth (Oxoid, UK) supplemented with the respective antibiotics were inoculated with *A. baumannii* isolates to density of ~$5x10^5$ CFU/ml in a final volume of 5 ml and incubated at 37 °C. The killing kinetics of the colistin vancomycin combination was assessed against each of the isolates using standard time-killing assay and viable bacterial counts. The final concentration of colistin and vancomycin were 1 µg/ml, and 20 µg/ml respectively, to simulate the minimal steady-state concentrations achieved with standard dosing regimens of colistin methanesulfonate (1,000,000 IU of colistin) and continuously infused vancomycin. Aliquots were removed at time 0, 3, 6 and 24 h post incubation, serially diluted in saline for determination of viable counts. Diluted samples (100 µl) were plated on blood agar plates and colonies were counted after overnight incubation. Bactericidal activity was determined as 3 $\log_{10}$ CFU/ml reduction in the colony count relative to the initial inoculum [14].

Results

Clonal relationship

The rep-PCR analysis showed a clonal relationship among the isolates. Six isolates were identified in A clone, 3 isolates in B clone and 1 isolates in C clone (Figure 1).

Antimicrobial susceptibility results

The MIC_{50}, MIC_{90}, and MIC ranges of the isolates are presented in Table 1. According to the CLSI breakpoints [15] for *Acinetobacter* spp., all isolates were resistant to colistin and 8 strains were susceptible to netilmicin.

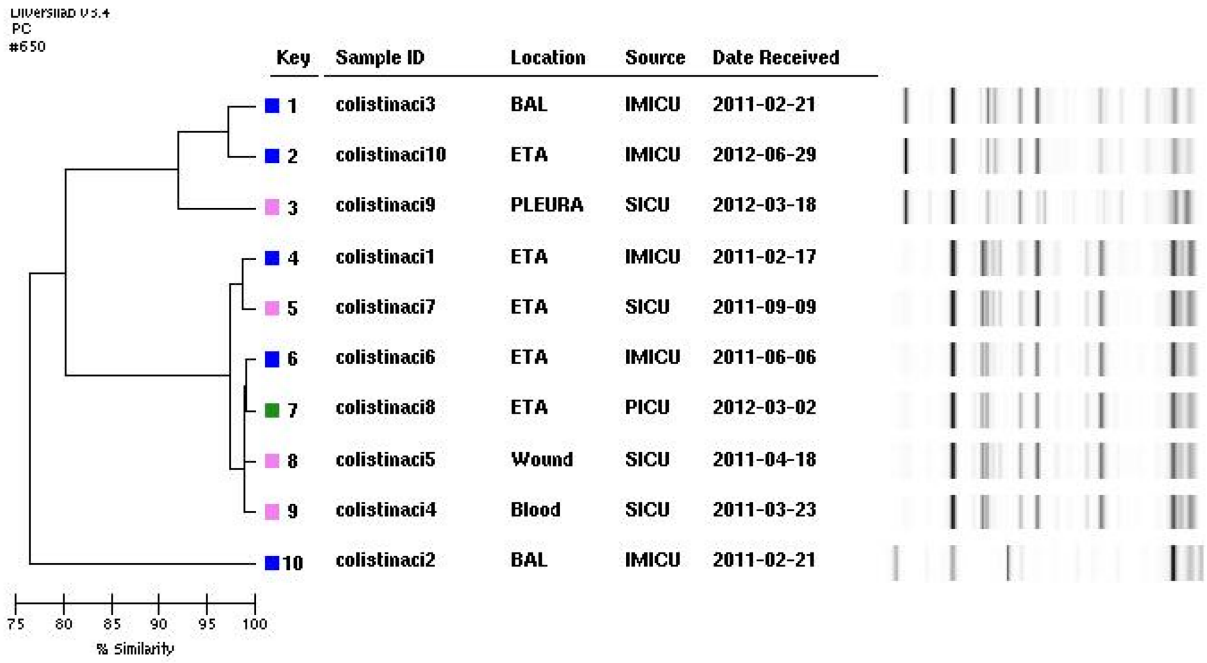

Figure 1: Clonal relationship among clinical isolates

Table 1: MIC values of tested antibiotics

Antimicrobial	MIC_{50} (µg/ml)	MIC_{90} (µg/ml)	MIC range (µg/ml)
Colistin	256	>1,024	64 – >1024
Sulbactam	32	128	8 – >128
Netilmicin	4	>16	1 – >16
Vancomycin	128	256	32 – 256

Table 2: Results of combination effects of the isolates

Isolates	Colistin + Netilmicin		Colistin + Sulbactam		Colistin + Vancomycin	
	FICI	Result	FICI	Result	FICI	Result
1	2	indifference	0.562	indifference	0.126	synergy
2	0.255	synergy	0.265	synergy	0.257	synergy
3	2	indifference	0.507	indifference	0.515	indifference
4	0.562	indifference	0.187	synergy	0.140	synergy
5	0.531	indifference	0.375	synergy	0.046	synergy
6	0.507	indifference	0.281	synergy	0.281	synergy
7	0.532	indifference	0.187	synergy	0.281	synergy
8	1.25	indifference	0.75	indifference	0.124	synergy
9	1.015	indifference	1.031	indifference	0.253	synergy
10	1.25	indifference	1.5	indifference	0.140	synergy

Checkerboard assay

Checkerboard analysis was performed with all antimicrobials in combination with colistin. Synergistic interaction was detected in one strain with the combination of colistin-netilmicin, in 5 strains with colistin-sulbactam, and in 9 strains with colistin-vancomycin (Table 2). None of combinations showed antagonistic activity.

Time-killing assays

Time kill assay was performed for the combination of colistin with vancomycin, since this combination showed the highest synergistic in the checkerboard assay and resulted in rapid bactericidal activity. Reduction of 3 $\log_{10}$ CFU/ml in the colony count was seen after 3 h of incubation for 8 isolates and after 6 h of incubation for 2 isolates. After 24 h of incubation, no regrowth did occur (Table 3).

Table 3: Time-killing kinetics of the colistin-vancomycin combination

Isolate no	Clone	3 h	6 h	24 h
1	A	>–3	>–3	>–3
2	C	–2	>–3	>–3
3	B	–2	>–3	>–3
4	A	>–3	>–3	>–3
5	A	>–3	>–3	>–3
6	A	>–3	>–3	>–3
7	A	>–3	>–3	>–3
8	A	>–3	>–3	>–3
9	B	>–3	>–3	>–3
10	B	>–3	>–3	>–3

*$\log_{10}$ colony count lower than that at time zero without antimicrobial agent. –1= Δ1 $\log_{10}$ CFU/ml = 90% killing; –2= Δ2 $\log_{10}$ CFU/ml = 99% killing; –3= Δ3 $\log_{10}$ CFU/ml = 99.9% killing.

Discussion

A. baumannii has emerged as an important nosocomial pathogen, especially in ICUs. This organism develops resistance to a variety of antimicrobial agents like broad-spectrum beta-lactams, aminoglycosides, fluoroquinolones and carbapenems [16]. MDR *A. baumannii* is also endemic in our ICUs and the causative pathogen of critical nosocomial infections with high mortality rate. Carbapenem resistance of *A. baumannii* doubled from 2000 to 2009 in our hospital [17].

The spread of MDR *A. baumannii* has been forcing researchers to find new molecules as little choice for treatment remains. Therefore, the use of quite old antimicrobial drugs is reconsidered again. Colistin is an example of a drug that has good activity against *A. baumannii* and has fallen out of use because of toxicity, but is reemerging now [18], [19]. Nevertheless, there is a risk of emergence of colistin-resistant *A. baumannii* strains during colistin treatment, if monotherapy is administered [20]. Colistin-resistant clinical isolates have already been reported [1], [2], [3]. For this reason, a combination of colistin with other antimicrobial agents could be more rational.

In the present study we investigated synergistic activities of colistin combined with sulbactam and netilmicin, which are in clinical use today, and with vancomycin, which is not routinely used against Gram-negative organisms, but showed proven synergistic activity on *A. baumannii* strains [18]. Kempf et al. [20] demonstrated synergistic activity of colistin combination with sulbactam and found that colistin MIC decreased from 32 µg/ml to 4 µg/ml and the sulbactam MIC decreased from 2 µg/ml to 0.5 µg/ml. Gordon et al. [18] tested 34 clinical isolates having vancomycin MICs of >256 µg/ml. The addition of 0.5 µg/ml of colistin reduced the vancomycin MIC in all cases (range 0.0016 > to 48 µg/ml) and the combination of vancomycin with colistin also resulted in rapid bactericidal activity. The highest synergistic activity was determined in combination of colistin with vancomycin and rapid bactericidal activity was observed in this study. Nephrotoxicity is a reported side effect of both drugs that should be taken into account for clinical use. However, Kalin et al. [19] evaluated concominant antibiotic use, and there was no difference in nephrotoxicity between the patients who received concominant glycopeptide antibiotic and the patients who did not.

In conclusion, our results present a distinct in vitro synergism between colistin and vancomycin. This combination may be useful in the treatment of MDR *A. baumannii* infections. Moreover, it may help to prevent emergence of resistant strains, to lower doses for both antibiotics to be used, and to prevent nephrotoxicity. Further in vivo investigations are needed to prove the use of colistin-vancomycin combination therapy for infections of MDR *A. baumannii*.

Notes

Competing interests

The authors declare that they have no competing interests.

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Please cite as

Percin D, Akyol S, Kalin G. In vitro synergism of combinations of colistin with selected antibiotics against colistin-resistant Acinetobacter baumannii. GMS Hyg Infect Control. 2014;9(2):Doc14. DOI: 10.3205/dgkh000234, URN: urn:nbn:de:0183-dgkh0002345

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Published: *2014-08-19*