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ORIGINAL ARTICLE

In vitro synergistic activity of tigecycline and colistin against XDR-*Acinetobacter baumannii*

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The emergence of extensive drug-resistant (XDR) *Acinetobacter baumannii* limits the therapeutic options and leads to high mortality in intensive care units. Combined antibiotic therapy is frequently recommended for the treatment of these infections. Colistin (CO) and tigecycline (TIG), alone or in combination with other antimicrobials, are the most commonly used antibiotics in the treatment of these resistant infections. In this study, the *in vitro* synergistic activity of TIG and CO were tested for 25 XDR-*A. baumannii* strains isolated from ventilator-associated pneumonia by the Etest method. Resistance to CO was not detected, whereas 8% of the strains were resistant to TIG. The TIG–CO combination was more synergistic than TIG–rifampin and CO–rifampin according to the fractional inhibitory concentration index. No antagonism was detected between the drugs in the study. There was no strong correlation between the activity of the combinations with reference to strains or genotypes. Our results suggest that the combined use of TIG and CO may be useful for the treatment of XDR-*A. baumannii* infections.
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INTRODUCTION

The emergence of extensive drug-resistant (XDR) *Acinetobacter baumannii* strains in many geographical regions of the world and the high morbidity and mortality associated with infections caused by these strains, especially in intensive care units (ICU), is a major challenge for clinicians.[1,2] The high resistance rates of *A. baumannii* strains to commonly used antibiotics, including carbapenems, limit the therapeutic options. Although colistin (CO) seems effective *in vitro* against XDR-*A. baumannii* strains, its effectiveness for treatment of ventilator-associated pneumonia (VAP) has been questioned because of its inadequate penetration into pulmonary parenchyma. Therefore, CO was given in combination with other antibiotics, mostly with carbapenem or rifampin.[1,3] Other antibiotic combinations are also used in the treatment of XDR-*A. baumannii* infections such as a combination of netilmycin and cefoperazone-sulbactam, or meropenem and sulbactam.[4,5]

Tigecycline (TIG) is a new glycylcycline antibiotic derived from minocycline and is active *in vitro* against most Gram-positive and Gram-negative aerobes in addition to anaerobes and atypical organisms.[6] Along with CO, TIG seems to be the most effective antibiotic *in vitro* against *A. baumannii* isolates, including multi-drug-resistant strains.[2,7] It may be an alternative agent in the treatment of infections caused by XDR-*A. baumannii* strains. However, the increased MIC levels of TIG among MDR-*A. baumannii* strains should be taken into consideration in clinical usage.[8] There are no data about the combined usage or *in vitro* interaction of TIG with other antibiotics.

The aim of this study was to evaluate the *in vitro* synergistic activity of TIG and CO with each other and with rifampin individually in XDR-*A. baummanii* strains isolated from ICU patients with VAP.

MATERIALS AND METHODS

Isolates were collected from consecutive endotracheal aspirate cultures from six ICUs between June and November 2008. *A. baumannii* strains were isolated on quantitative endotracheal aspirate cultures, identified as the primary agent of VAP by clinical evaluation, and tested for antimicrobial susceptibility by the disk diffusion method. For quantitative endotracheal aspirate, specimens were serially diluted in sterile 0.9% saline solution with concentrations of 10^{-1}, 10^{-3} and 10^{-5}; the diagnostic thresholds were studied at 10^{-5} CFU ml^{-1}. The XDR strains were stored at $-80\,^{\circ}$C until this study was performed. In total, 25 *A. baumannii* strains from VAP were isolated in the study period. Those isolates that were resistant to the antibiotics routinely tested, including carbapenems, aminoglycosides, quinolones, piperacillin/tazobactam, cefepime, cefoperazone/sulbactam and ceftazidime, but susceptible to TIG and/or CO were considered as XDR-*A. baumannii*.[2] Strains were identified using a BBL Crystal Enteric/Nonfermenter ID Kit (Becton Dickinson, Sparks, MD, USA).

The *in vitro* antimicrobial susceptibilities for TIG, CO and rifampicin (RIF) were determined by Etest (AB BIODISK, Solna, Sweden). Large (150 mm) agar plates with Mueller–Hinton medium (Becton Dickinson) were inoculated with suspensions of the strains equivalent to 0.5 McFarland standard, and Etest strips of each antibiotic were applied. After incubation for 24 h at 35 °C, MICs were read and interpreted according to the manufacturer's instructions. To prevent misinterpretation of TIG MICs, the agar plates were used within 12 h after preparation. TIG susceptibility or resistance was judged using interpretive criteria as supplied by the manufacturer, Wyeth Research (Wyeth Pharmaceuticals, Collegeville, PA, USA). For simplicity, TIG susceptibility

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criteria for *Enterobacteriaceae* were applied to all isolates (susceptible, MIC $<2\,mg\,l^{-1}$; intermediate, MIC=$4\,mg\,l^{-1}$; resistant, MIC $>8\,mg\,l^{-1}$). The breakpoint for CO was defined as $\leqslant 4\,mg\,l^{-1}$ for susceptible strains based on published recommendations.[9] The breakpoints for rifampin based on the CLSI guidelines for Gram-positive bacteria were $\leqslant 1\,mg\,l^{-1}$ susceptible and $\geqslant 4\,mg\,l^{-1}$ resistant.[10]

Three different antibiotic combinations (TIG–RIF, CO–RIF and TIG–CO) were evaluated for *in vitro* synergistic effect by the Etest method according to the manufacturer's recommendations. The E-test strip of an antibiotic (drug A) was applied to the surface of the Mueller–Hinton agar plates and left for 1 h at room temperature. Subsequently, the strip was removed and another strip (drug B) was applied onto the imprint of strip A. The plates were incubated at 35 °C for 48 h and then the MIC levels of each drug and combination were read. The fractional inhibitory concentration (FIC) index was calculated with the formula:

$$FIC = MICAB/MICA + MICBA/MICB$$

The results of combination tests according to the FIC index were interpreted as follows: synergistic (FIC $\leqslant 0.5$), additive (FIC >0.5 and $\leqslant 1$), indifferent (FIC >1 and $\leqslant 4$) and antagonistic (FIC >4).

Pseudomonas aeruginosa ATCC 27853 and *Escherichia coli* ATCC 25922 were used as quality control strains.

To show a possible clonal relationship between isolates, an arbitrarily primed-PCR method was used. For DNA extraction, bacterial strains were extracted by the boiling method using 1×Tris-EDTA (pH 8.0) buffer. Extracted DNA was stored at −80 °C until use. For PCR, 10×PCR buffer, 100 μM of a primer, 25 mM $MgCl_2$, and 2.5 mM dNTP's together with $5\,U\,\mu l^{-1}$ of *Taq* polymerase were used. The primer used was M13 (5′-GTAAAACGACGGCC AGTGAA-3′). The amplification reaction was performed in an Applied Biosystems (Foster City, CA, USA) thermocycler at 94 °C for 2 min followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at 45 °C for 1 min, and extension at 72 °C for 40 s.[11] Amplified products were analyzed by electrophoresis on 2% agarose gels.[12] The gel was stained with ethidium bromide ($5\,mg\,ml^{-1}$) and photographed under UV light.

RESULTS

All of the XDR-*A. baumannii* isolates were susceptible to CO. Susceptibility to TIG varied: 14 of the 25 isolates were susceptible (56%), 9 were intermediate (36%) and 2 (8%) were resistant to TIG. None of the isolates were susceptible to rifampin (Table 1).

By E-test, the best results were obtained in the TIG–CO combination. Synergistic activity was observed in 18 of 25 (72%) isolates. The CO–RIF combination revealed synergistic activity in 60% of the isolates. Synergistic activity was detected in only three strains with the TIG–RIF combination. None of the combinations revealed antagonism in the study. There was no strong correlation among the individual isolates according to the FIC index of the different antibiotic combinations or susceptibility pattern (Table 2).

Twenty-five strains isolated from patients have been analyzed phylogenetically for identifying the clonal relations following arbitrarily primed-PCR. According to electrophoretic band patterns, two main and in total six groups have been identified (Table 2). There was no correlation between the FIC index of the strains according to genotypes, even though the strains were within the same genotype.

Table 1 MIC and susceptibility rates of extensive drug-resistant *A. baumannii* isolates

	MIC ($mg\,l^{-1}$)			*Susceptibility rate (%)*		
Antibitoic	*Range*	MIC50	*MIC90*	*Susceptible*	*Intermediate*	*Resistant*
Tigecycline	0.38–16	2	4	56	36	8
Rifampin	2–32	32	32	0	0	0
Colistin	0.064–1	0.38	0.75	100	—	—

DISCUSSION

Multidrug-resistant and even XDR-*A. baumannii* isolates are emerging as a cause of numerous large outbreaks and are also endemic organisms in ICUs in Europe, North and South America, and Asia.[1,2] Multidrug resistance in *Acinetobacter* spp. appears to be a particular problem in Turkey. The results of the MYSTIC study, which was performed in nine centers in Turkey from 2000 to 2003, revealed that 49% of the *A. baumannii* isolates were multidrug resistant, and one-third of them (33.3%) were resistant to all antibiotics tested.[13] In our hospital, *A. baumannii* has been placed as the primary nosocomial pathogen since 2006. In a previous study performed in our hospital, 80.5% of *A. baumannii* strains isolated from VAP were determined to be MDR.[8] Some of these strains were resistant to all antibiotics except CO and TIG. As reported in a Taiwanese study, the emergence of XDR or pandrug-resistant *A. baumannii* strains is associated with increasing use of carbapenems and ciprofloxacin.[14]

All of the *A. baumannii* strains in our study were resistant to carbapenems, aminoglycosides, quinolones and cephalosporins. Resistance to CO was not detected. CO is very active against *Acinetobacter* spp. Susceptibility to CO was reported as 97.9–100% in various studies.[15–17] However, poor pharmacokinetic properties and adverse effects such as nephrotoxicity and neurotoxicity limit its wide clinical

Table 2 The results of antibiotic combinations by E-test

No.	*TIG–RIF*		*TIG–CO*		*CO–RIF*		
	FIC	*Activity*	*FIC*	*Activity*	*FIC*	*Activity*	*Genotype*[a]
1	1.6	ID	0.25	S	0.51	ADD	A
2	0.83	ADD	0.18	S	0.1	S	A
3	1.9	ID	1.09	ID	0.11	S	A
4	2.6	ID	1.95	ID	0.13	S	A
5	0.7	ADD	0.18	S	0.18	S	D
6	1.4	ID	0.37	S	0.1	S	B
7	1.4	ID	0.24	S	0.76	ADD	B
8	0.48	S	0.5	S	0.36	S	C
9	1.12	ID	0.85	ADD	0.77	ADD	B
10	0.56	ADD	0.29	S	1.01	ID	D
11	0.16	S	0.42	S	0.34	S	A
12	0.84	ADD	0.27	S	1.06	ID	A
13	0.27	S	0.34	S	0.79	ADD	A
14	1.09	ID	0.37	S	0.77	ADD	E
15	1.04	ID	0.61	ADD	0.34	S	A
16	1.25	ID	0.28	S	0.3	S	B
17	1.06	ID	0.44	S	0.12	S	B
18	0.72	ADD	0.5	S	0.1	S	F
19	1.06	ID	0.56	ADD	0.76	ADD	B
20	0.79	ADD	0.12	S	0.33	S	B
21	1.25	ID	0.38	S	1.25	ID	A
22	0.67	ADD	0.9	ADD	0.25	S	B
23	1.01	ID	0.83	ADD	0.33	S	B
24	1.3	ID	0.2	S	1.32	ID	B
25	1	ID	0.16	S	0.26	S	A

Abbreviations: ADD, additive; FIC, fractional inhibitory concentration; ID, indifferent; S, synergistic.
[a]The genotypes related to arbitrarily primed-PCR band patterns have been identified with the gel documentation system from Syngene Corporation (Cambridge, UK) using GeneSnap software.

usage.[18] Although CO was recommended in salvage therapy of nosocomial MDR-*A. baumannii* infections, clinical success is not as high as expected in the treatment of VAP.[18,19]

Among the anti-MDR-*A. baumannii* antibiotics, TIG has received significant attention. Along with CO, TIG appears to be the most potent agent *in vitro* against *A. baumannii*.[1,20] In a study from Greece, all of the MDR-*A. baumannii* strains isolated from ICU patients were found to be susceptible to TIG, including CO-resistant isolates.[7] TIG was found to be active in 86.7–93.3% of *Acinetobacter* spp. in various studies.[6,15,16] However, there are some reports showing high TIG resistance among *A. baumannii* isolates without clonal relationship.[21,22] A previous study in our hospital revealed that 25% of the MDR-*A. baumannii* strains isolated from VAP were resistant to TIG.[8] Furthermore, exposure to subtherapeutic levels of TIG for even short periods of time may promote the rapid emergence of TIG resistance and cause therapeutic failure.[23] An efflux pump mechanism that confers reduced susceptibility to aminoglycosides, fluoroquinolones, erythromycin, chloramphenicol and tetracyclines is the most probable resistance mechanism for TIG in *A. baumannii*. This emphasizes the need for accurate interpretation of resistance.[2] The isolation of *Acinetobacter* resistance to TIG should be interpreted and reported with caution because resistance can be intermediate and method-dependent.[7] Our study was performed using XDR-*A. baumannii* strains isolated from ICUs. In this study, MIC levels were higher than in previous studies.[6,15,16] Although 8% of the isolates were resistant to TIG, increased MIC levels (intermediate) were detected in 36% of the isolates. The increased MIC levels of TIG in these strains are worrisome, as TIG may not be adequate in the treatment in monotherapy.

The treatment options are very limited in infections caused by XDR-*A. baumannii*. Combination antibiotic therapy is a strategy often used in the treatment of MDR-*A. baumannii* infections.[1] This approach attempts to achieve synergy, particularly against MDR strains. The combinations of sulbactam with aminoglycosides, rifampin and azithromycin have shown synergy against imipenem-susceptible strains.[1] CO is frequently used in combination with rifampin, carbapenems or azithromycin. It is speculated that the role of polymyxins in combination with other antibiotics allows the rapid permeabilization of the outer membrane, permitting the entry of other agents into the bacterial cell.[1] As far as we know, there are no data about the combined use or *in vitro* synergistic activity of TIG with other antibiotics. In this study, the *in vitro* synergistic activity of TIG and CO with each other and with rifampin individually was investigated. As rifampin was shown to be synergistic with CO or imipenem both *in vitro* and clinically, we enrolled rifampin as a third agent to try in combination.[1,17,24] The best results were obtained in the TIG–CO combination. Synergistic activity was found in 72% of the isolates with the TIG–CO combination, in 60% with the CO–RIF combination, and in only 12% with the TIG–RIF combination. No antagonism was detected with the three antibiotic combinations in the study. As shown in Table 2, the synergistic activity was specific to the strains, and there was no strong correlation between the combinations regarding strains or genotypes. Therefore, each strain should be tested individually for synergistic activity.

Our results provide an option for treating infections caused by XDR-*A. baumannii*. The synergistic activity observed between TIG and CO *in vitro* should be evaluated with clinical studies. The combined use of TIG and CO may contribute to the solution of problems in the treatment of XDR-*A. baumannii* infections.

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