



Antibiotic Resistance Profiles and Genotypes of *Acinetobacter baumannii* Isolates and *In Vitro* Interactions of Various Antibiotics in Combination with Tigecycline and Colistin

Acinetobacter baumannii İzolatlarının Antibiyotik Direnç Profili ve Genotipleri ile Tigesiklin ve Kolistinin Çeşitli Antibiyotiklerle Kombinasyonlarının *In Vitro* Etkileşimleri

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ABSTRACT

Objectives: The aim of this study was to determine the antibiotic resistance profile, clonal relation and efficacy of antibiotic combinations in nosocomial multidrug resistant (MDR) *Acinetobacter baumannii*.

Materials and Methods: Antibiotic susceptibilities of 84 MDR *A. baumannii* against tigecycline (TGC), colistin (CL), amikacin (AK), ciprofloxacin (CIP), meropenem (MR), moxifloxacin (MXF), rifampicin (RF) were determined by microdilution method. Clonal relationship was investigated by genotyping using AP-PCR and antibiotyping. Interactions of antibiotic combinations were tested against clonally unrelated strains by the checkerboard (CB) method. The efficacy of the best combinations was also assessed on a selected isolate by the time-kill (TK) method.

Results: CIP, RF, MXF, MR, AK resistance was found as 90.47%; 47.62%; 22.62%; 58.33%; 50% respectively; however; CL and TGC were not ascertained. The isolates were distinguished as 25 different antibiotypes and 15 varied molecular patterns. The best synergistic effect was detected in combinations of CL with RF (100%) and MR (100%), in combinations of TGC with RF (53%) against clonally unrelated 15 MDR *A. baumannii* isolates by the CB method. While CL-RF and CL-MR showed synergy by TK method like CB, on the other hand TGC-RF indicated additive interactions by TK.

Conclusion: In this study, both synergy tests showed that CL in combination with RF would be a good option in MDR *A. baumannii*.

Key words: *Acinetobacter*, AP-PCR, checkerboard, time-kill, tigecycline, colistin

ÖZ

Amaç: Bu çalışmada nozokomiyal çoklu ilaç dirençli (ÇİD) *Acinetobacter baumannii* izolatlarının antibiyotik direnç profilinin belirlenmesi, moleküler düzeyde tiplendirmelerinin yapılması ve dirençli izolatlarda antibiyotik kombinasyonlarının aktivitesinin araştırılması amaçlandı.

Gereç ve Yöntemler: Seksen dört ÇİD *A. baumannii* izolatına karşı tigesiklin (TGC), kolistin (CL), amikasin (AK), siprofloksasin (CIP), meropenem (MR), moksifloksasin (MXF) ve rifampisin (RF) minimum inhibitör konsantrasyon (MİK) değerleri sıvı mikrodilüsyon yöntemi ile belirlendi. Epidemiyolojik ilişki, AP-PZR ve antibiyotiplendirme ile saptandı. Klonal ilişkisiz kökenlere antibiyotik kombinasyonlarının etkinliği dama tahtası (CB) yöntemi ile belirlendi. Dama tahtası yöntemi sonucunda, en etkin gözlenen kombinasyonun etkinliği, seçilmiş bir kökene karşı zamana bağlı öldürme eğrisi (TK) yöntemi ile de araştırıldı.

Bulgular: CIP, RF, MXF, MR, AK'nin direnç oranları sırasıyla; %90.47; %47.62; %22.62; %58.33; %50 olarak belirlendi. TGC ve CL direnci görülmedi. Antibiyotik direnç profillerine göre 25 antibiyotip grubu belirlenirken, 15 farklı patern ayırt edildi. Klonal ilişkisiz 15 ÇİD *A. baumannii* izolatında CB yöntemiyle en iyi sinerjistik etki CL-RF (%100), CL-MR (%100) ve TGC-RF (%53) kombinasyonlarında gözlemlendi. Seçilmiş bir kökenden TK yöntemiyle CL-RF ve CL-MR ile sinerji gözlemlendi, TGC-RF ile ise aditif etki saptandı.

Sonuç: Bu çalışmada her iki sinerji testi de ÇİD *A. baumannii* izolatlarına karşı CL ile RF kombinasyonunun tedavide iyi bir seçim olacağını işaret etmiştir.

Anahtar kelimeler: *Acinetobacter*, AP-PZR, dama tahtası, zamana bağlı öldürme eğrisi, tigesiklin, kolistin

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INTRODUCTION

Acinetobacter baumannii is an opportunistic pathogen and causes hospital acquired infections such as bacteremia, septicemia, and ventilator associated pneumonia.^{1,2,3} Before the 1970s, *A. baumannii* was susceptible to most traditional antibiotics such as broad spectrum beta-lactams, cephalosporins and tetracyclines. In recent years *Acinetobacter* related infections have been eradicated with difficulty in general by using single antimicrobial agent because of the ability of the bacteria to develop resistance. Especially multidrug resistant (MDR) *A. baumannii* has been continuously reported as nosocomial pathogen which causes significant morbidity and mortality in critically ill patients.^{4,5}

Carbapenems are considered the drugs of choice for the treatment of serious infections caused by MDR *A. baumannii*. However carbapenem-resistant *A. baumannii* has been reported worldwide.⁶ As a result of this increasing carbapenem resistance, alternative antibiotic classes have become part of treatment.¹ Of these alternative classes, polymyxin and tigecycline (TGC) remain the most active treatments *in vitro* against MDR *A. baumannii* but resistance against these antibiotics is also reported.^{4,6,7,8} Combination therapy is often used in the treatment of MDR *A. baumannii* infections to prevent the emergence of resistance and obtain a synergistic effect.^{1,3,8}

The aim of this study was to determine the antimicrobial resistance profile, clonal relation and efficacy of antimicrobial combinations on nosocomial MDR *A. baumannii*.

EXPERIMENTAL

Microorganisms

Identifications and antibiotic susceptibilities of *Acinetobacter* spp. isolates were elicited by Phoenix TM 100 (BD, United States) at the Clinical Microbiology Laboratory of İzmir Katip Çelebi University Atatürk Training and Research Hospital, between 2009-2010, and 84 MDR *Acinetobacter* spp. isolates were selected for the study. *Escherichia coli* ATCC 25922 and *Pseudomonas aeruginosa* ATCC 27853 were used as control strains.

Determination of minimum inhibitory concentrations and antibiotypes

Minimum inhibitory concentrations (MICs) of TGC (Pfizer, İstanbul, Turkey), colistin (CL) (Sigma-Aldrich, USA), amikacin (AK) (Eczacıbaşı, İstanbul, Turkey), ciprofloxacin (CIP) (Koçak, İstanbul, Turkey), meropenem (MR) (Astra Zeneca, İstanbul, Turkey), moxifloxacin (MXF) (Bayer, İstanbul, Turkey) and rifampicin (RF) (Koçak, İstanbul, Turkey) antibiotics against the isolates were indicated by broth microdilution method according to "Clinical and Laboratory Standards Institute (CLSI)".⁹ Because, CLSI has not suggested available breakpoints, the United States Food and Drug Administration (2005) susceptibility breakpoints for TGC, and the recommendations of Principe et al.⁷ for MXF and RF were utilized. By considering MIC values, the isolates were classified into different antibiotype groups.

Molecular typing

Epidemiologic relations were investigated genetically with M13 universal primers by Arbitrarily-primed polymerase chain reaction (AP-PCR).^{10,11} To obtain crude DNA extracts, freshly cultured bacterial colony suspensions in sterile distilled water were heated for 10 min at 95°C, cooled on ice, and centrifuged at 12.000 g. For totally 50 µL volume PCR mix, 2 mM primers, 200 µM dNTP's together with 1 U of *Taq* polymerase (Fermentas) were used. M13 primers [5'-GTA AAA CGA CGG CCA GTG AA-3' (forward amplification primer) and 5'-GGA AAC AGC TAT GAC CAT GA-3' (reverse amplification primer)] were purchased from Fermentas. The PCR conditions were as follows: 2 cycles of 94°C for 5 min, 40°C for 5 min, 72°C for 5 min followed by 40 cycles of 94°C for 1 min, 40°C for 1 min, and 72°C for 2 min. PCR end products were examined by a ultraviolet transilluminator after electrophoresis for 60 min on 1.5 percent (wt/vol) agarose gels. The genotypes were interpreted according to the band patterns of DNA marker.

Efficacy of the antibiotic combinations

Chequerboard (CB) method: Activity of TGC and CL combination, and combinations of these antibiotics individually with AK, CIP, MR, MXF, RF against clonally unrelated 15 MDR *A. baumannii* isolates were tested. *In vitro* interactions by fractional inhibitory concentration index (FICI) of each agent was calculated as a ratio of MIC when used in combination and MIC when used alone. For each antibiotic, seven concentrations (8X MIC, 4X MIC, 2X MIC, MIC, MIC/2, MIC/4 ve MIC/8) were investigated. FICI were interpreted as synergistic, indifference, additivity and antagonism. FICI was interpreted as follows: synergy FICI≤0.5, additivity/indifference 0.5<FICI≤4 and antagonism FICI>4.0.^{7,12}

Time-kill (TK) method: Effective combinations found by the CB method were also approved by TK method against one of the test isolates. TK studies were performed in flasks containing Mueller Hinton Broth (Merck, Darmstadt, Germany) at 37°C. Samples were removed at 0th, 6th, and 24th h of incubation from the test and growth-control cultures and appropriately diluted and inoculated onto Mueller-Hinton Agar (Merck, Darmstadt, Germany) plates. After incubation at 37°C for 24-48 h, bacterial colonies were counted. All TK studies were performed twice. Synergy or antagonism was defined as an increase or decrease of at least 100-fold compared to the effect of the most active agent singly and an increase of 100 times less than additive interaction.¹²

RESULTS

While 25 different antibiotypes were observed according to antibiotic resistance profiles of 84 MDR *Acinetobacter* spp. isolates, 15 different patterns were distinguished by AP-PCR with M13 primers. Thirty six (43%) isolates showed similar genetic pattern and 22 of these isolates were found in the same resistance pattern according to antibiotype groups and the band patterns of 50 bp DNA marker (Table 1) (Figure 1).

Resistance rates in 84 MDR *Acinetobacter* spp. isolates against CIP, RF, MXF, MR, AK were found as 90.47%; 47.62%; 22.62%; 58.33%; 50% respectively. CL and TGC resistance were not found.

CL in combination with MR and RF demonstrated higher levels of synergy than the other antibiotics according to the CB method. As shown in Table 2, the best synergistic effect was detected in the CL combinations for CL-RF (100%), CL-MR (100%), in the TGC combinations for TGC-RF (53%) combinations. The lowest synergy was seen in the CL combinations for CL-AK (47%) and seen in the TGC combinations for TGC-CIP (20%). Generally, the combinations with TGC demonstrated a higher rate of additive interaction in compared with the CL combinations. Antagonistic

interaction was observed between TGC-CL (20%), TGC-AK (6%), TGC-MXF (6%) and CL-CIP (6%) (Table 2).

TGC-RF combination (0.015-2 µg/mL) indicated synergy according to the CB method, but it indicated additive interactions by the TK method. Besides, CL-RF (0.06-0.25 µg/mL) and CL-MR (0.03-0.12 µg/mL) combinations showed a synergistic effect when considering both CB and TK methods. TGC-CL combination (0.03-0.5 µg/mL) was found as additive according to the CB method, but that combination demonstrated synergistic effect at 3th and 6th hours and an additive effect at 24 h as to the TK method. The synergistic effect of CL-RF (0.06-0.25 µg/mL) combination by the TK method was demonstrated in the Figure 2.

Table 1. The distribution of *A. baumannii* isolates according to antibiotypes and genotypes

Antibiotypes	Genotypes														
	P _A	P _B	P _C	P _D	P _E	P _F	P _M	P _N	P _O	P _P	P _Q	P _S	P _X	P _Y	P _Z
A _A	-	-	-	-	-	1	-	-	-	-	-	-	1	-	-
A _B	-	-	-	1	-	1	-	-	-	-	-	-	-	-	-
A _C	3	-	-	2	1	-	-	-	-	-	-	-	-	-	-
A _D	-	1	-	-	-	-	-	-	-	2	-	-	-	-	-
A _E	22	-	-	-	-	3	-	-	1	-	-	-	-	-	-
A _F	1	1	-	-	-	1	-	-	-	-	-	-	-	-	-
A _G	-	1	-	-	-	-	-	-	-	-	-	-	-	-	-
A _H	-	-	-	-	-	-	-	-	1	-	1	2	-	-	1
A _I	-	-	-	1	-	-	1	-	-	-	-	-	-	-	-
A _J	-	-	1	-	-	-	-	-	-	-	-	-	-	-	-
A _K	-	1	-	-	-	-	1	1	-	-	-	-	-	-	-
A _L	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-
A _M	-	-	-	-	-	-	-	-	1	-	-	-	-	-	-
A _N	6	-	-	2	-	-	-	-	-	-	-	1	-	-	-
A _O	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-
A _P	-	2	-	-	-	-	-	-	-	-	-	-	-	-	-
A _Q	-	-	-	1	-	-	-	-	-	-	-	-	-	-	-
A _R	-	-	-	2	-	1	-	-	-	-	-	-	-	-	1
A _S	-	1	-	-	-	-	-	-	-	-	-	-	-	-	-
A _T	1	1	-	-	-	-	-	-	-	-	-	-	-	-	-
A _X	-	1	-	-	-	-	-	-	-	-	-	-	-	-	-
A _U	-	1	-	-	-	-	-	-	-	-	-	-	-	1	-
A _V	-	-	-	-	-	-	1	1	-	-	-	-	-	1	-
A _Y	-	-	-	-	-	-	-	1	-	-	-	-	-	-	-
A _Z	-	-	-	-	-	-	-	-	1	-	-	-	-	-	-
Total	35	10	1	9	1	7	3	3	4	2	1	3	1	2	2

∴ There is no isolate, A: Antibiotypes, P: Genotypes

DISCUSSION

Antibiotic susceptibility testing and molecular typing are necessary for the monitoring and treatment of infections caused by *Acinetobacter* species that become resistant against many antibiotics easily through more than one mechanism. AP-PCR is a quite common PCR-based genotyping method currently because it is easily applicable, is easily distinguishable and provides quick results. It is reported in some studies that the use of M13 universal primers in the AP-PCR generates more distinct band patterns, gives good results and has quite good distinguishing ability is quite good.^{13,14} In this study 15 different band patterns were detected according to AP-PCR method by using M13 universal primers. Almost 8-15 bands were obtained after the gel electrophoresis of the PCR products. The number of the bands was found sufficient to determine clonally associated strains. To see the reproducibility of the results, the

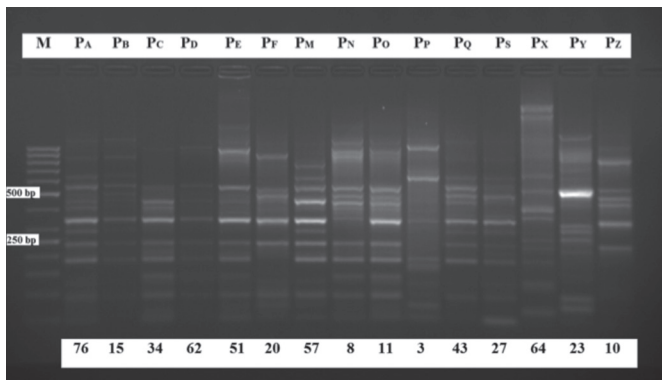


Figure 1. Genotypes of *A. baumannii* isolates to 15 different band patterns obtained by arbitrarily primed-polymerase chain reaction with M-13 primers

M: 50 bp DNA marker

Table 2. *In vitro* effects of antibiotic combinations against 15 *A. baumannii* isolates according to the checkerboard method

Antibiotic combinations	Synergy n (%)	Additive n (%)	Ineffective n (%)	Antagonism n (%)
CL-MR	15 (100)	0	0	0
CL-RF	15 (100)	0	0	0
CL-CIP	9 (60)	1 (6)	4 (27)	1 (6)
CL-MXF	9 (60)	3 (20)	3 (20)	0
CL-AK	7 (47)	8 (53)	0	0
TGC-MR	7 (46)	6 (40)	2 (14)	0
TGC-RF	8 (53)	2 (14)	5 (33)	0
TGC-CIP	3 (20)	3 (20)	9 (60)	0
TGC-MXF	6 (40)	4 (27)	4 (27)	1 (6)
TGC-AK	4 (27)	6 (40)	4 (27)	1 (6)
TGC-CL	7 (46)	1 (6)	4 (27)	3 (20)

CL: Colistin, MR: Meropenem, RF: Rifampicin, CIP: Ciprofloxacin, MXF: Moxifloxacin, AK: Amikacin, TGC: Tigecycline, n: Number of isolate, synergy FICI ≤ 0.5 , additivity/indifference $0.5 < \text{fractional inhibitory concentration index} \leq 4$ and antagonism fractional inhibitory concentration index > 4.0

trials were triplicated. Similar to recent studies,^{10,15} our results showed intense clonal spread of resistant *A. baumannii* strains in the intensive care units (80%), particularly. The isolates in the same or close antibiotic resistance patterns were often observed in the same clonal group according to molecular classification with AP-PCR. It was considered that isolation dates and locations in the hospital of *A. baumannii* isolates resulted in different molecular clones.

The most important issue in the *Acinetobacter* infections is the development of resistance against many kinds of antibiotics including primarily preferred carbapenems, CL and sulbactam.¹⁶ Due to the difficulties in the treatment of hospital-acquired infections, broad spectrum antibiotics are commonly chosen. Thus, causative strains become resistant easily. Consequently, new treatment alternatives have been required. TGC, a promising semisynthetic tetracycline, is considered as first choice of the new drugs for the therapy of carbapenem-resistant *A. baumannii* infections. This broad-spectrum antibiotic is shown to be highly effective against MDR *Acinetobacter* spp. isolates.¹⁶⁻¹⁹ However, several studies have currently reported decreased susceptibility to TGC. Certain *in vivo* and *in vitro* researches also highlighted developed resistance to sub-MIC concentrations of TGC. CL is shown as the only effective antibiotic against MDR *A. baumannii* in many countries that have not used TGC yet. In recent years, CL again has become a current issue for the treatment of infections caused by *Acinetobacter* species resistant to all antibiotics except CL. However, there are some major disadvantages of CL when used alone, because of its pharmacokinetic properties, side effects and fast, easy improvement of resistance. Thus, combined use of antibiotics is recommended to prevent the development of resistance and increase the success in the treatment of MDR *A. baumannii* infections since.^{7,19,20}

Recent studies in Turkey found that imipenem-netilmicin,²¹ RF-ampicillin/sulbactam,²² CL-TGC,²³ CL-vancomycin³ in combination showed synergistic interaction, and they were considered as strong choices in the treatment of infections caused by MDR *A. baumannii*.

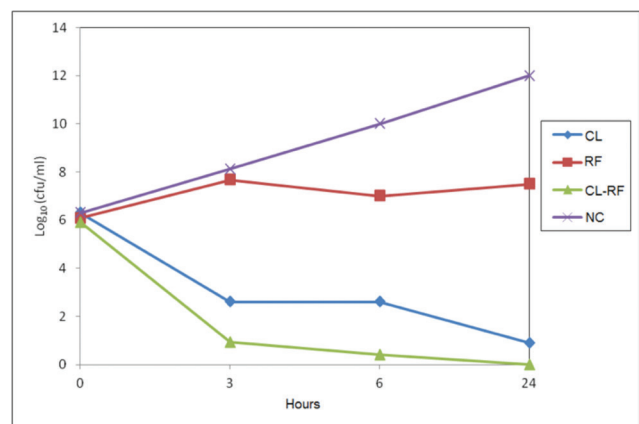


Figure 2. The synergistic effect of CL-RF (0.06-0.25 $\mu\text{g/mL}$) combination against *A. baumannii* in 3, 6, and 24 hours by time-kill method

CL: Colistin, RF: Rifampicin, NC: Negative growth control without antibiotics

In this study, the interactions of TGC and CL combinations with different antibiotics; AK, CIP, MR, MXF, RF and each other were investigated by CB and TK methods. The best synergistic effect was detected in the CL combinations by CB method, for CL-RF (100%), CL-MR (100%), and in the TGC combinations for TGC-RF (53%). Besides, synergy between TGC and CL was found as 46%.

We also compared the results of CB and TK methods for one of the tested strains. Synergy was determined for the combinations CL with RF and MR by both CB and TK methods. While TGC-RF combination showed synergy by CB, additive interaction was shown by TK method. Percin et al.³ (2014) investigated CL-vancomycin combinations because of good efficacy by TK as well as CB method, and found consistency of both the results.

Interaction of levofloxacin and CIP alone and their combinations with ceftazidime, cefepime, imipenem, piperacillin/tazobactam AK was investigated in a synergy study conducted in 2005, where 5 *Acinetobacter* spp. were evaluated. The highest synergy was seen in beta-lactam-fluoroquinolone and fluoroquinolone-AK combinations.²⁴ While in a study conducted in 2011, Tan et al.⁹ detected rate of synergy by CB method between polymyxin B-RF and TGC-RF as 19%, those between polymyxin B-TGC was 12%. The same rates obtained by TK methods were 56%, 19% and 44% respectively. When all studied strains were evaluated, the observed synergy rate was 40% by TK method and 17% by the checkerboard method. According to these results, researchers have noted that the best observed *in vitro* synergy rate by TK method was between polymyxin B-RF combination. In a study conducted with 31 MDR and polymyxin B-sensitive *A. baumannii* isolates, Lim et al.²⁵ reported that the best level of bactericidal activity at 24th hour is in polymyxin B-RF combination (42%), whereas the TGC-RF combination shows very low levels by TK method. In a study conducted by using TK method, initially antagonism was observed in TGC-polymyxin B combination, then variable action was seen where as no interaction was determined when other antibiotics were combined with TGC.²⁶ In 2013 Lee et al.⁴ investigated by TK method the interaction of CL-RF combination for the first time by changing CL concentrations within *in vitro* pharmacodynamic and pharmacokinetic model in aim to achieve clinical concentrations. They observed that this combination prevents the expression of CL resistant subpopulations in CL- susceptible and -resistant MDR *A. baumannii*. In a study conducted in 2012, it was emphasized that the use of inappropriate antibiotic combination increases CL resistance.²⁷

Principe et al.⁷ for the first time reported that they have noticed the synergy between TGC and CL by TK method in one *A. baumannii* isolate. In two studies conducted by CB method in TGC-CL combination²⁸ and in TGC-sulbactam, TGC-CL combinations²⁹ a synergistic effect was observed.

With regard to the assessment of the interaction of the combination used in our study, only one of the 15 strains tested by CB method has also been evaluated by TK method and similar results were obtained with both methods in this strain.

In 2015 a meta-analysis evaluated several studies. It was noted that CL showed *in vitro* synergy and bactericidal activity with many antibiotics against MDR *A. baumannii* strains, especially RF and carbapenem combinations suppressed CL resistance and synergy was observed at over 50% in CL-resistant strains. Synergy rates according to TK method were higher than CB and E-test methods. However, researchers underlined that *in vivo* studies to support *in vitro* studies are insufficient, because some factors like host immune response, bacterial virulence, infection site and antibiotics concentration can alter the effect of the combination of antibiotics. Thus it was declared that there is a need for randomized clinical trials to support the *in vitro* studies.^{5,6,30}

CONCLUSION

Today, various methods have been developed for investigation of antibiotic combinations; but there is not a standard approach yet. In this study, both synergy tests showed that CL in combination with RF would be a good option in the treatment of MDR *A. baumannii* infections. Although both methods pose some difficulties such as high work-load, the length of time involved and working with the lowest concentration of antibiotic, they are useful when the reliable results are considered. However, those *in vitro* studies should be supported by *in vivo* studies to determine effective new combinations.

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