Physiology and Pharmacology 28 (2024) 314-323
Experimental Research Article

Effect of RND-efflux pumps inhibitor on the synergy of different antibiotics combinations against carbapenem-resistant Pseudomonas aeruginosa

Mahsa Vahdatipur Dizaj[1], Reza Ghotaslou[1], Mina Yekani[2,3], Seyyed Reza Moaddab [5], Behrooz Naghili[1], Edris Nabizadeh[1,4], Mohammad Yousef Memar[1*]

1. Infectious and Tropical Diseases Research Center, Tabriz University of Medical Sciences, Tabriz, Iran
2. Department of Microbiology, Faculty of Medicine, Kashan University of Medical Sciences, Kashan, Iran
3. Student Research Committee, Kashan University of Medical Sciences, Kashan, Iran
4. Student Research Committee, Tabriz University of Medical Sciences, Tabriz, Iran
5. Drug Applied Research Center, Tabriz University of Medical Sciences, Tabriz, Iran

ABSTRACT

Introduction: The high-level antimicrobial resistance, particularly carbapenem resistance, in *Pseudomonas* aeruginosa is a global health challenge. The combination of antibiotics and synergy effects is beneficial in control of drug-resistant *P. aeruginosa.* The synergic interaction of antimicrobial agents is af-fected by the mechanisms of antimicrobial resistance. The aim of the current study was to evaluate the effect of efflux pump inhibition on the synergy of antibiotics against carbapenem-resistant *P. aeruginosa.*

Methods: The antibiotics' minimum inhibitory concentration (MIC) was determined by the microbroth dilu-tion method. The synergy effect of antibiotics was determined using the checkerboard assay with-out and with Resistance-Nodulation- Division (RND) efflux pump inhibitor phenylalanine-arginine beta-naphthylamide (PAβN).

Results: The highest levels of synergistic effects were found between cefepime/tobramycin and meropenem/tobramycin combinations in 35.3% of isolates. After adding PAβN, the most frequent synergistic effects were observed between the meropenem/ciprofloxacin and cefepime/ciprofloxacin combinations, found in 64.7% of isolates. The adding PAβN led to an increase in the synergy of all combinations except tobramycin/colistin. The highest effect of PAβN on the synergy effects of antibiotics combination was observed in meropenem/ciprofloxacin, cefepime/ciprofloxacin, and ciprofloxacin/colistin (an increase of 41.2%).

Conclusion: RND efflux pump inhibition has a noticeable effect on the results of synergy tests of some antimi-crobial agent combinations. Given the drug- and strain-dependent effects of PAβN on synergy re-sults, the effects of efflux pump inhibitors should be studied on different combinations of drugs and a large population of bacterial strains.

Keywords:
Synergy
Efflux pumps
Inhibitors
Pseudomonas aeruginosa

* Corresponding author: Mohammad Yousef Memar, memarm@tbzmed.ac.ir
Received 13 July 2023; Revised from 29 January 2024; Accepted 31 January 2024

Citation: Vahdatipur Dizaj M, Ghotaslou R, Yekani Mina, Moaddab S.R., Naghili B, Nabizadeh E, Memar M.Y. Effect of RND-efflux pumps inhibitor on the synergy of different antibiotics combinations against carbapenem-resistant Pseudomonas aeruginosa. Physiology and Pharmacology 2024; 28: 314-323. http://dx.doi.org/10.61186/phypha.28.3.314

Introduction

Pseudomonas aeruginosa, a ubiquitous opportunistic bacterium, is commonly associated with a broad range of life-threatening infections including respiratory tract infections, ventilator-associated infections, wound and soft tissue infections, sepsis, and urinary tract infections (UTIs) (Khalili et al., 2019). This bacterium is a common cause of nosocomial infections, being principally problematic in burn patients, cystic fibrosis patients, and patients in intensive care units (ICUs) (Bayat et al., 2023). Some of *P. aeruginosa* have been described as non-susceptible to most classes of antibiotics, including aminoglycosides, β-lactams, quinolones, and tetracyclines (Memar et al., 2016). Treatment options for *P. aeruginosa* infections are now imperfect and not cost-effective due to high levels of resistance to antibiotic therapy (Ugwuanyi et al., 2021). Carbapenems are the drug of choice in the antimicrobial therapy of multi-drug resistant (MDR) *P. aeruginosa*. However, the increased resistance to carbapenems has limited their efficiency (Britt et al., 2018). Combination therapy is one of the important options to treat infections caused by drug-resistant *P. aeruginosa*. Combination antimicrobial therapy is commonly used to achieve synergic effects, decrease toxicity, and reduce the risk of developing resistant strains during antibiotic therapy. The resistance to antimicrobial drugs can influence the choice and potential of antibiotics for achieving synergy effects in combination therapy (Ghorbani et al., 2017). Both pharmacological interactions between antibiotics (e.g., synergy) and the mechanisms of antibiotic effect and resistance are suggested to impact the outcome of combination therapy (Yekani et al., 2023). The expelling activity of bacterial efflux pumps decreases antimicrobial agents' levels lower than their lethal concentrations, leading to decreased susceptibility to antibiotics. To date, several resistance-nodulation-division (RND) family efflux pumps have been described in *P. aeruginosa* strains that can pump out several structurally unrelated drugs (Yoneda et al., 2005). Understanding microbial resistance mechanisms is essential to provide the method of antimicrobial therapy and the design of new antimicrobial drugs, as well as for the control of MDR strains in community and health care centers. Considering the common use of combination therapy in the treatment of infections caused by *P. aeruginosa*, the effects of efflux pumps on the synergistic effects between different antibiotics are important. The aim of this *in vitro* study was to determine the effects of RND efflux pump inhibition on the synergistic effects of different antibiotics on carbapenem-resistant *P. aeruginosa*.

Materials and Methods

Bacterial isolates

Primary, 100 hundred non-duplicated *P. aeruginosa* isolates were obtained from clinical specimens and identified using the colony morphology, gram staining and standard microbiological tests. These tests including catalase, oxidase, oxidative-fermentative (OF), Simmons citrate, motility, pigment production, growth at 42°C and 4°C and growth on Cetrimide agar. This study was conducted at the Infectious and Tropical Diseases Research Center, Tabriz University of Medical Sciences, Tabriz, Iran, during 2021-2022. *Pseudomonas aeruginosa* ATCC 27853 and *Escherichia coli* ATCC 25922 were used for quality control of microbiological tests. This study was approved by the Ethics Committee of Tabriz University of Medical Sciences (IR.TBZMED.VCR.REC.1400.286).

Antibiotic susceptibility testing

The screening of carbapenem-resistant isolates was performed using the disk diffusion method according to the CLSI guidelines (Weinstein et al., 2020). the disk diffusion method was performed to determine the antibiotic susceptibility pattern using disks of amikacin (30µg), aztreonam (30µg), ceftazidime (30µg), cefepime (30µg), ciprofloxacin (5µg), levofloxacin (5µg), meropenem (10µg), and piperacillin-tazobactam (100/10µg). *Pseudomonas aeruginosa* ATCC 27853 was used as the positive control of antimicrobial susceptibility testing. Modified carbapenem inactivation method (mCIM) was used for the detection of carbapenemase-producing isolates. Briefly, tested isolates were inoculated on sheep blood agar (SBA). After 24 hours, a 10-µL loopful of each isolate was inoculated in a tube containing 2 mL tryptic soy broth (TSB). A meropenem disk (10µg) was added to each tube, and the tubes were incubated for 4 h at 35°C. The meropenem disks were removed from the tube with loops and then placed on plates of Mueller-Hinton inoculated with *Escherichia coli* ATCC 25922. Carbapenemase production was considered positive if the zone of growth inhibition was 6 to 15 mm or determined 16 to 18 mm with satellite colonies. An inhi-

bition zone of ≥19 was considered carbapenemase-negative and considered indeterminate if the inhibition zone of 16 to 18 mm was observed without satellite colonies (Uechi et al., 2017). Efflux-pumps mediated carbapenem resistance was detected using the phenotypic assay of efflux pumps by minimum inhibitory concentration (MIC) of meropenem in the absence and presence of phenylalanine-arginine beta -naphthylamide (PAβN) as previously described (Khalili et al., 2021).

MIC determination

Based on the CLSI guidelines, the MIC determination of meropenem, cefepime, colistin, ciprofloxacin, and tobramycin was performed using the microbroth dilution method in Mueller-Hinton broth (CAMHB). The MIC was considered the lowest concentration of an antibiotic that entirely suppressed the growth of microorganisms, detectable by the naked eye following incubation in ambient air at 35ºC for 16-20 hours (Wikler 2019).

Phenotypic assay of efflux pumps

The synergy testing was performed on efflux-pumps-mediated carbapenem resistant *P. aeruginosa* isolates. The phenotypic method for the expression of efflux pumps involved using the inhibitory effect of PAβN on the MIC of meropenem, ciprofloxacin, colistin, cefepime, and tobramycin. For this, the MIC of antibiotics was assessed by the microbroth dilution method in the absence and presence of PAβN (40μg/mL) according to the previous studies. An at least-twofold reduction in the MIC value in the presence of PAβN was defined as an overexpression of RND-efflux pumps (Khalili et al., 2021; Khalili et al., 2019).

Fraction inhibitory concentration determination

The antimicrobial effects of combinations of meropenem/colistin, meropenem/tobramycin, tobramycin/colistin, meropenem/ciprofloxacin, cefepime/colistin, and cefepime/ tobramycin were determined using the checkerboard method and FIC index determination as described previously (Memar et al., 2021). Briefly, the MIC of each antibiotic was determined using microbroth dilution method according to the CLSI guideline alone as well as in combination at concentration ranges of 1 to 32 times lower than the MIC of each antimicrobial agent (Wikler 2019). The FIC index was determined as follows:

FIC Index

Synergy was determined as an FIC index ≤0.5, additivity FIC index of > 0.5 to ≥ 1; no interaction (indifference) FIC index of >1 to ≤ 4, and antagonism was determined as a FIC index of >4 (Memar et al., 2021). The effects of efflux pump inhibition on the outcome of drug combinations were determined by the FIC index in the presence of PAβN at a concentration of 40 μg/ml.

Statistical analysis

Statistical Package for the Social Sciences (SPSS) software version 18 was used for the results analysis. Comparison of the results among various groups was performed by non-parametric tests. P-values ≤ 0.05 were considered as a statistical significance.

Results

This study was primarily performed on 100 non-duplicated *P. aeruginosa* isolates collected from clinical specimens of patients referred to Tabriz hospitals during 2021-2022. In this study, 41% of patients were female and 59% were male. The average age of the studied patients was 42.9±25.4 years. The highest frequency of specimens was from the Intensive Care Unit (ICU) with a frequency of 41%, followed by burns (19%), surgery (13%), infectious (7%), neurology (6%), urology (5%), trauma and Cardiac Care Units (CCU) (3%), internal (2%) and transplant (1%) wards. The highest frequency specimens studied in the present study were pulmonary infection specimens (35%), wound (28%), blood (17%), urine samples (15%), middle ear and upper respiratory system samples (2%), and peritoneal fluid (1%).

The antibiotic susceptibility patterns of these isolates were studied using disk diffusion method. The frequency of resistance to different antibiotics is presented in Figure 1 and Table 1. All isolates were susceptible to colistin, while high frequencies of resistance were observed to the other antimicrobial agents. About 99% of the studied isolates were resistant to at least three groups of antibacterial agents and considered multi-drug resistant (MDR). It was found that 57% of the isolated microorganisms were resistant to meropenem that were selected for further study.

Based on the results obtained in the mCIM method, 11% of the examined isolates were carbapenemase producers. Meropenem resistance was observed due to increased expression of efflux pumps in 17 (29.82%)

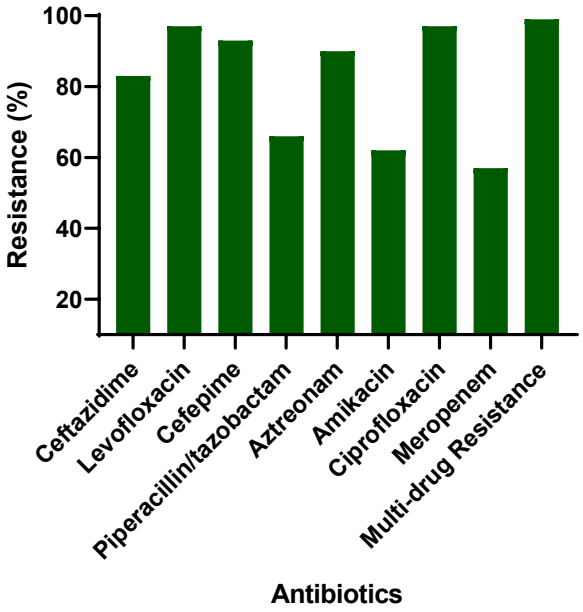

FIGURE 1. The antibiotics susceptibility patterns of bacterial isolates according to the disk diffusion assay.

TABLE 1: The characteristics and origin of RND-efflux pumps mediated carbapenem-resistant isolates

Isolates	Origin		Antibiotic Susceptibility Patterns									
	Ward	Specimens	CEF	COL	LVO	CFM	PTZ	AZM	AMK	CIP	MEM	MDR
1	Burn	Wound	R	S	R	R	R	R	R	R	R	+
2	Burn	Wound	I	S	R	S	S	S	R	R	R	+
3	Burn	Wound	R	S	S	R	S	R	S	S	R	+
4	Burn	Blood	R	S	R	R	R	R	R	R	R	+
5	ICU	Pulmonary	R	S	R	R	R	R	R	R	R	+
6	ICU	Wound	S	S	R	S	S	I	R	R	R	+
7	ICU	Wound	S	S	R	R	R	R	R	R	R	+
8	Infectious	Blood	R	S	R	R	I	S	S	R	R	+
9	Burn	Blood	R	S	R	R	R	R	R	R	R	+
10	Burn	Wound	R	S	R	R	S	R	S	R	R	+
11	ICU	Wound	R	S	R	R	S	R	I	R	R	+
12	ICU	Wound	R	S	R	R	S	R	S	R	R	+
13	Internal	Middle Ear	R	S	R	R	R	R	R	R	R	+
14	ICU	Blood	R	S	R	R	R	R	R	R	R	+
15	Burn	Wound	R	S	R	R	R	R	R	R	R	+
16	ICU	Pulmonary	R	S	R	R	R	R	S	R	R	+
17	Surgery	Wound	R	S	R	R	S	R	I	R	R	+

AMK: amikacin, AZM: aztreonam, CEF: ceftazidime, CFM: cefepime, CIP: ciprofloxacin, COL: colistin, I: intermediate, MDR: multi-drug resistance, LOV, levofloxacin, MEM: meropenem, PTZ: piperacillin-tazobactam, R: resistant, S: susceptible, TOB: tobramycin

isolates. In the current study, the synergistic effects of antibiotics were investigated in isolates that were resistant to meropenem through the expression of RND efflux pumps, which included 17 isolates. The characteristics and origin of these isolates are displayed in Table 1. These 17 isolates were MDR. MIC_{50} and MIC_{90} values for the examined antibiotics are presented in Figure 2.

Investigating the effect of RND-type efflux pumps in resistance to different antibiotics was determined using phenotypic methods. PaβN had no significant effect on

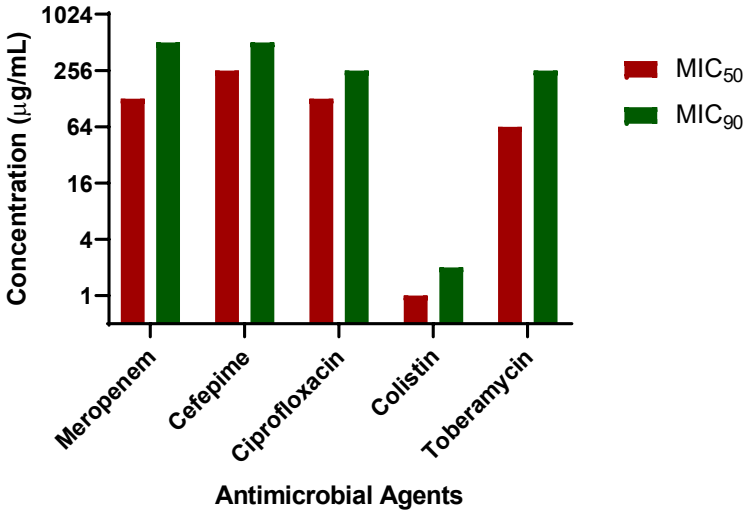

FIGURE 2. MIC50 and MIC90 values for the tested antibiotics for bacterial isolates screened for synergy testing.

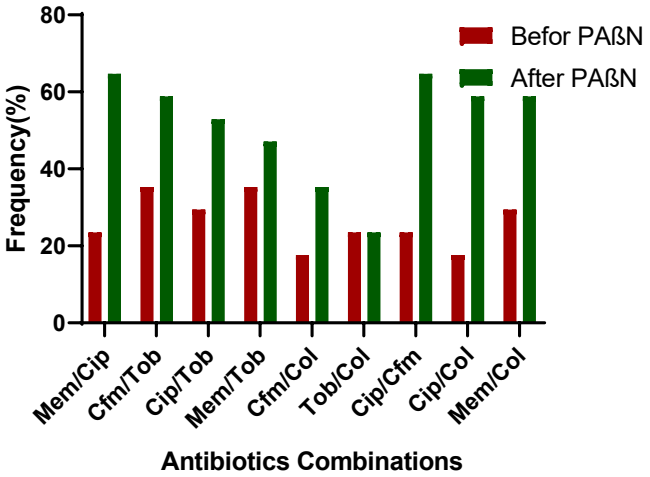

FIGURE 3. Comparison of frequency of synergy results between different antibiotics combinations before and after exposure to PaβN. Cfm: cefepime, Cip: ciprofloxacin, Col: colistin, Mem: meropenem, Tob: tobramycin.

the MIC of colistin. RND efflux pump-dependent resistance to cefepime was observed in 6 isolates (35.3%), tobramycin in 6 isolates (35.3%), and to ciprofloxacin in 9 isolates (52.9%).

Before adding PAβN, the most frequent synergistic effects between cefepime/tobramycin and meropenem/tobramycin combinations in 35.3% of isolates, followed by ciprofloxacin/tobramycin and meropenem/colistin with a frequency of 29.4%, meropenem/ciprofloxacin, tobramycin/colistin, cefepime/ciprofloxacin with a frequency of 23.5% and cefepime/colicin, ciprofloxacin/colicin with a frequency of 17.6%. Antagonist effects were not observed in any of the antibiotic compounds (Table 2 and Table-3).

After adding PAβN, the most frequent synergistic effects were observed between meropenem/ciprofloxacin and cefepime/ciprofloxacin combinations in 64.7% of isolates, followed by cefepime/tobramycin, meropenem/colistin and ciprofloxacin/colistin with a frequency of 58.8%, tobramycin/ciprofloxacin with a frequency of 52.9%, tobramycin/meropenem with a frequency of 47.06%, cefepime/colistin with a frequency of 35.3%, and tobramycin/colistin with a frequency of 23.5%. Antagonist effects were not observed in any of the antibiotic compounds (Table 2 and Table 3).

The addition of the RND-efflux inhibitor was observed to lead to an increase in the synergy of all antibiotic combinations except tobramycin/colistin (Figure 3). The highest effect of PaβN on synergy results of antibiotics combination was observed in meropenem/ciprofloxacin, cefepime/ciprofloxacin, and ciprofloxacin/colistin (Table-2 and Table-3).

TABLE 1: The results of the synergy test in the absence and presence of PaβN in different bacterial isolates

N	Synergy results (without PaβN)									Synergy results (with PaβN)								
	MEM/ CIP	CFM/ TOB	CIP/ TOB	MEM/ TOB	CFM/ COL	TOB/ COL	CIP/ CFM	CIP/ COL	MEM/ COL	MEM/ CIP	CFM/ TOB	CIP/ TOB	MEM/ TOB	CFM/ COL	TOB/ COL	CIP/ CFM	CIP/ COL	MEM/ COL
1	A	A	A	A	A	A	A	A	A	A	A	A	S	A	A	A	A	S
2	A	A	A	S	A	A	I	A	A	A	S	A	S	A	A	I	A	A
3	A	S	A	A	A	A	A	A	S	A	S	S	S	A	A	S	A	S
4	A	A	A	A	A	A	I	S	I	S	A	A	A	S	A	A	S	S
5	A	A	S	A	A	S	A	A	S	S	A	S	A	S	S	S	S	S
6	A	A	A	A	A	A	S	A	A	S	A	S	A	S	A	S	A	S
7	A	A	A	S	S	A	S	A	A	S	S	A	S	S	A	S	S	A
8	S	A	A	A	A	A	S	S	A	S	A	A	A	A	A	S	A	A
9	A	S	A	A	S	A	A	A	S	A	S	A	A	S	A	S	S	S
10	S	S	A	A	A	A	A	A	A	S	S	S	A	A	A	S	A	S
11	A	A	A	A	A	A	A	A	A	A	S	A	S	A	A	A	S	A
12	A	S	S	S	A	S	A	A	I	S	S	S	A	A	S	S	S	A
13	A	S	A	A	A	A	A	S	A	A	S	S	S	A	A	S	S	S
14	S	A	S	S	A	S	I	A	A	S	A	S	A	A	S	I	S	A
15	A	A	S	A	I	S	A	A	S	S	S	S	A	A	S	S	A	S
16	A	S	S	S	A	I	S	A	I	S	S	S	S	A	A	S	S	I
17	S	A	I	S	S	A	I	A	S	S	A	A	S	S	A	A	S	S

A: additive, CFM: cefepime, CIP: ciprofloxacin, COL: colistin, I: indifference, MEM: meropenem, S: synergy, TOB: tobramycin

TABLE 3: The effects of antimicrobial agent combinations in the absence and presence of PaβN

Combinations	Result of combination (without PaβN) (%)				Result of combination ((with PaβN) (%)			
	Synergy	Additive	Indifference	Antagonism	Synergy	Additive	Indifference	Antagonism
MEM/CIP	23.52	76.48	0	0	64.7	35.29	0	0
CFM/TOB	35.29	67.71	0	0	58.82	41.17	0	0
CIP/TOB	29.41	64.71	5.88	0	52.94	47.06	0	0
MEM/TOB	35.29	64.71	0	0	47.06	52.94	0	0
CFM/COL	17.64	76.48	5.88	0	35.29	64.71	0	0
TOB/COL	23.52	70.58	5.88	0	23.52	76.47	0	0
CIP/CFM	23.52	52.94	23.52	0	64.7	23.52	11.76	0
CIP/COL	17.64	82.36	0	0	58.82	41.17	0	0
MEM/COL	29.41	52.94	17.64	0	58.82	35.29	5.88	0

CFM: cefepime, CIP: ciprofloxacin, COL: colistin, MEM: meropenem, TOB: tobramycin

Discussion

Despite the progress in health care science and technology and the developing of a broad spectrum of antibiotics, some nosocomial infections caused by drug-resistant bacteria continue to be a most important medical challenge. *P. aeruginosa* is one of the most frequent drug-resistant microorganisms particularly in healthcare centers, which is associated with numerous complications due to high levels of resistance to different classes of antimicrobial drugs (Mobaraki et al., 2018). The source of bacterial isolates has a significant effect on their antibiotic susceptibility patterns. Isolates that have been exposed to antimicrobial agents for a long time usually show high levels of drug resistance. In the present study, most of the isolates were isolated from the ICU and burn departments, which may be the reason for the high resistance to antimicrobial agents. The increasing frequency of MDR *P. aeruginosa* strains is a considerable limitation for antimicrobial treatment. It appears doubtful that any new antibiotics will be introduced in the near future, and clinicians may be increasingly compelled to use older antibiotics to treat bacterial infections, including polymyxins and fosfomycin, regardless of their toxicity and unfordable effects (Mirakhur et al., 2003). Combination therapy is an alternative strategy for treating infections caused by drug-resistant pathogens. Therefore, extensive notice occurs in administrating current antimicrobial drugs in combination to increase antibacterial properties. Combinations of antimicrobial drugs may increase the spectrum and antimicrobial effect, and decrease the emergence of resistant strains and unfavorable toxic effects in patients. Synergy testing evaluates the effectiveness of combining two antibiotics against bacterial isolates (Memar et al., 2021; Tschudin-Sutter et al., 2018). In the current study, similar to previous studies from Iran, a high prevalence of resistance to various classes of antimicrobial agents including β-lactams, quinolones, and aminoglycosides (Japoni et al., 2006; Saderi and Owlia 2015). However, all isolates were not resistant to colistin. In this study, 99% of isolates were resistant to at least one drug in three or more antimicrobial classes and defined as MDR. Other studies have described varying rates of multidrug-resistant (MDR) *P. aeruginosa*, with prevalence ranging from 50% to 100% (Khalili et al., 2019). Several factors may contribute to the different prevalence of MDR *P. aeruginosa* in different studies, such as time and geographic diversity, patient's demographical factors, or access and patterns of antibiotics usage (Memar et al., 2016).

In the present study, overall, the most frequent synergistic effects were found between cefepime/tobramycin and meropenem/tobramycin combinations in 35.3% of isolates. The considerable synergistic effects of carbapenems and cephalosporins in combination with aminoglycosides have been reported for carbapenem-resistant and MDR *P. aeruginosa* strains. This had been reported previously in studies that evaluated the synergistic effects of ceftazidime and imipenem or meropenem in combination with tobramycin against MDR and carbapenem-resistant *P. aeruginosa* isolates (Balke et al., 2006; Campana et al., 2003). In some studies, colistin-carbapenem combinations have been suggested for treating MDR *P. aeruginosa* infections to increase the therapeutic effects and decrease probable colistin resistance. In this study, meropenem/colistin showed a synergic effect with a frequency of 29.4% against carbapenem-resistant

P. aeruginosa. Colistin and meropenem have been reported to show a synergistic interaction against 80% of *P. aeruginosa* including MBLs producing isolates, colistin-resistant and pan-drug-resistant isolates (Montero et al., 2019). The difference in reporting synergic effects provided by the same combination may be due to the effects of the methodology used in the different studies. There is some data as to whether the synergy interaction of antimicrobial agents may be related to the methods of detection and the mechanisms of resistance to drugs or to the clonality of strains, or both (Leite et al., 2016).

Multidrug resistance efflux pumps especially RND-type are capable of providing resistance to structurally unrelated antimicrobial agents (Ghotaslou et al., 2018). In the present study, RND efflux pump-dependent resistance was observed to meropenem, cefepime, tobramycin, and ciprofloxacin which is similar to results reported by some studies from Iran (Khalili et al., 2021). RND efflux pumps play important role in developing MDR *P. aeruginosa* as well as carbapenems-resistant *P. aeruginosa* (Khalili et al., 2021; Khalili et al., 2019). Infections caused by carbapenem-resistant and MDR *P. aeruginosa* strains are particularly concerning for risky ill patients, as these infections can be difficult to treat and can lead to serious complications. Inhibition of RND pumps might help to combat the antibiotic resistance problem. According to the results of the phenotypic method, the resistance to carbapenem was related to the expression of RND type of efflux pumps in 29.82% of isolates. The effect of RND-type efflux pumps on *P. aeruginosa* resistance to carbapenem has been reported by several studies with frequency of 17.3 % to 92.0% (Khuntayaporn et al., 2013; Pan et al., 2016).

In this study, we evaluated the effects of RND efflux inhibitor on the synergy effects of several antimicrobial agents on efflux-mediated carbapenem-resistant *P. aeruginosa.* In this respect, the synergistic effects of the antibiotic compounds were identified in the absence and presence of efflux pump inhibitors PAβN. PaβN has a significant effect on the results of synergy tests of different combinations. The addition of the PaβN led to an increase in the synergy of all compounds except tobramycin/colistin. The highest effect of PaβN on synergy effects (increasing of 41.2%) of antibiotics combination was observed in meropenem/ciprofloxacin, cefepime/ciprofloxacin and ciprofloxacin/colistin. Before adding PAβN, the most frequent synergistic effects were detected between cefepime/tobramycin and meropenem/tobramycin combinations, followed by ciprofloxacin/tobramycin and meropenem/colistin, meropenem/ciprofloxacin, tobramycin/colistin, cefepime/ciprofloxacin and cefepime/colicin, ciprofloxacin/colicin. While, in the presence of PAβN, the most frequent synergistic effects were observed between meropenem/ciprofloxacin and cefepime/ciprofloxacin combinations, followed by cefepime/tobramycin, meropenem/colistin and ciprofloxacin/colistin, tobramycin/ciprofloxacin, tobramycin/meropenem, cefepime/colistin and tobramycin/colistin. Studies have reported that inhibiting RND efflux pumps can significantly reduce the intrinsic resistance level and reverse acquired resistance of *P. aeruginosa* to fluoroquinolones, as well as decrease the frequency of highly fluoroquinolone-resistant strains (Lomovskaya et al., 2001). RND efflux pumps have also been suggested to play a significant role in bacterial pathogenesis, suggesting that efflux pump inhibitors could also provide anti-virulence properties. It has been shown that PAβN decreased the *in vivo* virulence of *P. aeruginosa*. The expression of quorum sensing (QS) mediators and of QS-dependent virulence factors was differentially influenced by PaβN due to a strain-dependent mechanism (Rampioni et al., 2017). Unfortunately, PAβN is not safe and has not been approved for human use, hindering future clinical usage and discouraging more studies designed to understand its effects on antimicrobial combination therapy *in vivo* conditions. Actually, toxicity and side effects in humans are the most common limitations of using efflux pump inhibitors clinically. Further studies focusing on efflux pump inhibitors that are effective only on bacteria are needed. However, research on novel efflux pump inhibitors with better pharmacological activities related to PAβN is in progress (Li et al., 2015; Wang et al., 2016).

Conclusion

The finding of the present study exhibits a high frequency of drug-resistant *P. aeruginosa* in health centers, which need appropriate strategies to control the spread of these strains. The *in vitro* synergy results are different from used antimicrobial agents and strain-dependent. RND-efflux pump inhibition showed a noticeable effect on the results of synergy tests of some combinations and indicated that any novel efflux pump inhibitor can be studied not only for its potential to enhance the in-

hibitory effects of antimicrobial agents but also for its effects on the synergy effects between combinations of antibiotics. Given the drug and strain-dependent effects of PAβN on synergy results, the effects of efflux pump inhibitors should be studied on the different combinations of drugs and on the large population of bacterial strains isolated from various clinical specimens.

Acknowledgments

This article was written based on a dataset from a project registered at Tabriz University of Medical Sciences (number 68212). The Vice Chancellor for Infectious and Tropical Diseases Research Center, Tabriz University of Medical Science provided financial support for this research that is greatly acknowledged.

Conflict of interest

The authors reported no potential conflict of interest.
Data availability statement Not applicable

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