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Original Research Article

Synergistic efficacy of meropenem, ciprofloxacin and colistin antibiotics against planktonic and biofilm forms of *Myroides odoratimimus* bacterial isolates

Ayşe Hümeyra Taşkın Kafa *, Mürşit Hasbek

Sivas Cumhuriyet University, Faculty of Medicine, Department of Medical Microbiology, 58140, Sivas, Turkey

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ABSTRACT

Purpose: In this study, it was aimed to investigate the combined synergistic efficacy of colistin (CT), meropenem (MEM), and ciprofloxacin (CIP) antibiotics on planktonic and biofilm forms in *Myroides odoratimimus* strains isolated from various clinical specimens.

Methods: Antibiotic susceptibility was determined by the Kirby-Bauer disk diffusion method. In addition, minimum inhibitory concentrations (MIC) of CIP, MEM, and CT were studied using the standardized broth microdilution method. In vitro synergistic activity of antibiotics against *M. odoratimimus* planktonic bacteria strains was studied by the Micro Broth Checkerboard method. The microtiter plate (MtP) method was used to determine the effectiveness of antibiotics on *M. odoratimimus* biofilm formation.

Results: A zone of inhibition was not observed against other antibiotics used except amikacin and linezolid in all strains. While CT/MEM and CT/CIP combinations have a synergistic effect on all strains, the combination CIP/MEM has an additive effect. According to the biofilm inhibition results, all three antibiotics inhibited biofilm formation. However, the efficacy of MEM (60.3–76.5%) and CIP (60.2–77.8%) was approximately two times higher than that of CT (25.4–34.5%). In addition, the effectiveness of combinations of antibiotics on biofilm formation was examined and the percentage of inhibition was 30.8% when CT was used alone, while the biofilm inhibition rates of CT/MEM and CT/CIP were 92.4% and 91.7%, respectively. MEM/CIP combination was inhibited biofilm formation by 75.7%.

Conclusions: This study is the first report showing the efficacy of CT, MEM and CIP antibiotics, which are frequently used in clinical practice, in combination on *M. odoratimimus* planktonic and biofilm forms. The findings of our study are particularly guiding for combined antibiotic treatment options in immunosuppressed patients admitted to an intensive care unit (ICU). The CT/MEM combination is currently used frequently. In addition, these results are important in terms of supporting in vitro that CT/CIP and MEM/CIP combinations can also be used as a treatment option in *M. odoratimimus* related infections.

1. Introduction

Myroides spp. are gram-negative, non-fermentative, aerobic, non-motile bacillus bacteria. Due to the flexirubin contained in their structure, they make yellow pigments in the media. They have a characteristic fruity (strawberry-like) odor in the media [1,2].

Two different strains of these bacteria are not found in the human flora and have been isolated from clinical sources: *M. odoratus* ve *M. odoratimimus* [2]. These bacteria are rarely isolated from clinical samples. Many strains of this bacteria have multidrug resistance and different virulence characteristics [3].

Although *Myroides* bacterial strains have low pathogenicity, they threaten the patient's life by making opportunistic infections, especially in immunocompromised individuals. It has been reported that these bacteria cause urinary tract infection, endocarditis, and ventriculitis cases [2–4].

Since *Myroides* spp. have high resistance to antibiotics and form a biofilm layer, their treatment is getting more difficult day by day. The biofilm structure is a collection of sessile microorganisms embedded in a matrix consisting of a polymeric substance called exopolysaccharide (eps) and tightly attached to each [5].

The significant feature of biofilm structures is that they have increased resistance to antimicrobials, immune systems, and chemicals

* Corresponding author.
E-mail addresses: ahtaskin@cumhuriyet.edu.tr (A.H. Taşkın Kafa), mhasbek@hotmail.com (M. Hasbek).

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compared to the planktonic forms of bacteria. In addition, it is so hard to elimination a formed biofilm structure. When the formed biofilm structure dries, it is very tolerant of being removed and eliminated from its environment [6].

In many studies published in the literature in recent years, it has been stated that the formation of biofilm together with antibiotic resistance, especially in chronic wounds, causes a delay in healing time [6,7].

In parallel with the increase in microorganisms with multi-drug resistance, the need for new treatment options has increased. The studies aiming to use antibiotics in combination or increase the effectiveness of existing drugs have gained momentum.

It was aimed to investigate in this study, the synergistic efficacy of colistin, meropenem, and ciprofloxacin antibiotics in combination against planktonic and biofilm forms of *M. odoratimimus* strains isolated from various clinical samples.

2. Materials and methods

2.1. Bacterial isolates

M. odoratimimus bacterial isolates were isolated from clinical samples of intensive care patients. The clinical isolates were identified by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF) and Bruker IVD MALDI Biotyper 2.3 (Bruker Daltonik GmbH, Bremen, Germany) automated systems, with a score >2.

2.2. Antimicrobial susceptibility test

Antibiotic susceptibility of the isolates was tested on Mueller-Hinton agar (Bio-Rad, Hercules, CA, USA), using the standard Kirby-Bauer disk diffusion method, using cefotaxime, trimethoprim-sulfamethoxazole, imipenem, amoxicillin, amikacin, ampicillin, meropenem, aztreonam, ceftazidime/clavulanic acid, colistin, linezolid, gentamicin, and levofloxacin antibiotics (Bio-Rad), firstly. In addition, the minimum inhibitory concentration (MIC) of ciprofloxacin, meropenem, and colistin antibiotics were studied by the standardized broth microdilution method. Since there are no standardized inhibition zone diameters, and MIC values determined in European Committee on Antimicrobial Susceptibility Testing (EUCAST) for *Myroides* spp., zone diameters and MIC results were recorded without evaluating such as "*susceptible/resistant*". The MIC results were used to determine the required antibiotic concentration for synergy tests. *Pseudomonas aeruginosa* ATCC 27853 bacteria were used as a quality control strain.

2.3. Checkerboard assay

The in vitro synergistic activity of colistin, ciprofloxacin, and meropenem antibiotics against *M. odoratimimus* bacterial strains was studied using the Micro Broth Checkerboard method [8]. The range values of the tested colistin (CT), ciprofloxacin (CIP), and meropenem (MEM) antibiotic concentrations were set at 4–64 μg/ml, 16–256 μg/ml, and 64–1024 μg/ml, respectively.

The combined antibiotic concentrations such as "CIP/MEM, CT/MEM, CT/CIP" were prepared in cation-supplemented Mueller-Hinton Broth. Then, bacteria were inoculated into the wells with a final bacterial concentration of 5×10^5 bacteria/ml in the well. The microplates were incubated at 37 °C for 24 h and then evaluated.

The fractional inhibitory concentration index (FICi) values were calculated according to the MIC values of both antimicrobial agents alone and in combination as follows:

$$\sum \text{FICi}: \quad \text{FICA} + \text{FICB} = \frac{(MIC \quad Antibiotic \quad combination)}{(MIC \quad Alone \quad A)} + \frac{(MIC \quad Antibiotic \quad combination)}{(MIC \quad Alone \quad B)}$$

FICi was interpreted as follows: Synergistic effect: FICi $\leq$ 0.5, additive effect: 0.5 < FICi $\leq$ 1, indifferent effect: 1 < FICi < 2, antagonist effect: FICi $\geq$ 4 [9].

2.4. Effect of antibiotics against biofilm formation

Microtiter plate (MtP) method was used to determine the efficacy of antibiotics against biofilm formation of *M. odoratimimus* bacteria [10]. To determine the minimum biofilm inhibitor concentration (MBIC) values, antibiotics were prepared at MIC and sub-MIC (MIC/2, MIC/4, MIC/8, MIC/16, MIC/32) concentrations with Tryptic Soy Broth medium containing 2% glucose. In addition, the effects of combinations of antibiotics at sub-MIC (CT 32/MEM 512, CT 32/CIP 64, and MEM 512/CIP 64) concentrations against biofilm formation were investigated. 100 μL of antibiotic solution and 100 μL of bacterial suspension adjusted to 0.5 McFarland (10^8 CFU/ml) absorbance value were added to the wells of 96-well U-bottom microplates. For the positive control, only 200 μL of bacterial suspension was added to the wells. The microplates were incubated at 37 °C for 48 h. At the end of the time, all the wells were washed 3 times with PBS and the planktonic bacteria were removed. Then, the biofilm layer in the wells was fixed with 95% methanol for 15 min. After fixation, 0.1% crystal violet was added to the wells which dried at room temperature and stained for 30 min. At the end of the time, the wells were washed three times with PBS to remove excess dye and left to dry. Afterward, 33% acetic acid solution was added to the wells to dissolve the stain in the wells. After 20 min, biofilm inhibition was evaluated by spectrophotometer at 570 nm wavelength. The percent biofilm inhibition was calculated according to the formula below [11]. All experiments were repeated three times.

$$\%\text{Inhibition} = 100 - \left(\frac{\text{OD570 sample}}{\text{OD570 control}} \times 100\right).$$

3. Results

In this study, some antibiotics against five *M. odoratimimus* clinical isolates were tested by Kirby-Bauer disk diffusion method. No zone of inhibition was observed in all bacterial strains against antibiotics other than amikacin and linezolid antibiotics (Table 1). MIC and MBC values of CIP, MEM, and CT antibiotics against bacterial strains are shown in Table 2.

The combined effect of CIP, MEM, and CT antibiotics against the tested bacterial strains is shown in Table 2. Although CT/MEM and CT/CIP combinations showed a synergistic effect against all bacterial strains, additive effects were detected in the CIP/MEM combination.

The results of the combined efficacy of antibiotics are shown in the isobologram in Fig. 1.

When the effect of CIP, MEM, and CT antibiotics against the biofilm formations formed by *M. odoratimimus* isolates was tested, the efficacy of MEM (% 60.3–76.5) and CIP (% 60.2–77.8) antibiotics were found to be approximately twice that of the CT (% 25.4–34.5) antibiotic. In addition, the effect of combinations of antibiotics against biofilm formation was tested and the percentage of inhibition was found to be 30.8% when CT antibiotic was used alone, and biofilm inhibition percentages of CT/MEM and CT/CIP antibiotic combinations were 92.4% and 91.7%, respectively. MEM/CIP antibiotic combination inhibited biofilm formation by 75.7% (Table 3).

4. Discussion

Inappropriate and uncontrolled use of antibiotics causes antimicrobial resistance to reach alarming levels all over the world. As a result of this situation, current treatment options are inadequate, and the number of atypical multi-drug resistant microorganisms is increasing day by day. Because of the entering the MALDI-TOF automated system into clinical microbiology laboratories and molecular identification methods such as

Table 1
Inhibition zones of antimicrobial agents tested by Kirby-Bauer disk diffusion method on Mueller-Hinton agar of *M. odoratimimus* strains.

Antibiotics	*M. odoratimimus-1*	*M. odoratimimus-2*	*M. odoratimimus-3*	*M. odoratimimus-4*	*M. odoratimimus-5*
Cefotaxime	*	*	*	*	*
Trimethoprim-Sulfamethoxazole	*	*	*	*	*
Imipenem	*	*	*	*	*
Amoxicillin	*	*	*	*	*
Amikacin	14 mm	14 mm	15 mm	17 mm	13 mm
Ampicillin	*	*	*	*	*
Meropenem	*	*	*	*	*
Aztreonam	*	*	*	*	*
Ceftazidime/Clavulanic Acid	*	*	*	*	*
Colistin	*	*	*	*	*
Linezolid	15 mm	14 mm	15 mm	15 mm	15 mm
Gentamicin	*	*	*	*	*
Levofloxacin	*	*	*	*	*
Ciprofloxacin	*	*	*	*	*

*No inhibition diameter was observed.

Table 2
Efficacy of antibiotic combinations on *M. odoratimimus* planktonic isolates.

Bacteria isolates	Cip (μg/ml)		Mem (μg/ml)		Ct (μg/ml)		Cip/Mem (μg/ml)	Cip/Mem FIC index/Activity	Ct/Mem (μg/ml)	Ct/Mem FIC index/Activity	Ct/Cip (μg/ml)	Ct/Cip FIC index/Activity
	MIC_A	MBC	MIC_A	MBC	MIC_A	MBC	MIC_C		MIC_C		MIC_C	
M. odoratimimus-1	128	256	1024	1024<	64	128	64/512	1/Add	16/256	0.5/Syn	16/32	0.5/Syn
M. odoratimimus-2	128	128	1024	1024<	64	128	64/512	1/Add	16/256	0.5/Syn	16/32	0.5/Syn
M. odoratimimus-3	128	128	1024	1024<	64	128	32/512	0.75/Add	16/128	0.375/Syn	16/32	0.5/Syn
M. odoratimimus-4	128	256	1024	1024<	64	128	64/512	1/Add	16/256	0.5/Syn	16/32	0.5/Syn
M. odoratimimus-5	128	128	1024	1024<	64	128	32/512	0.75/Add	16/128	0.375/Syn	16/32	0.5/Syn

FICi was interpreted as follows: Synergistic (Syn) effect: FICi ≤ 0.5, additive (Add) effect: 0.5 < FICi ≤ 1, indifferent effect: 1 < FICi < 2, antagonist effect: FICi ≥ 4 [9].

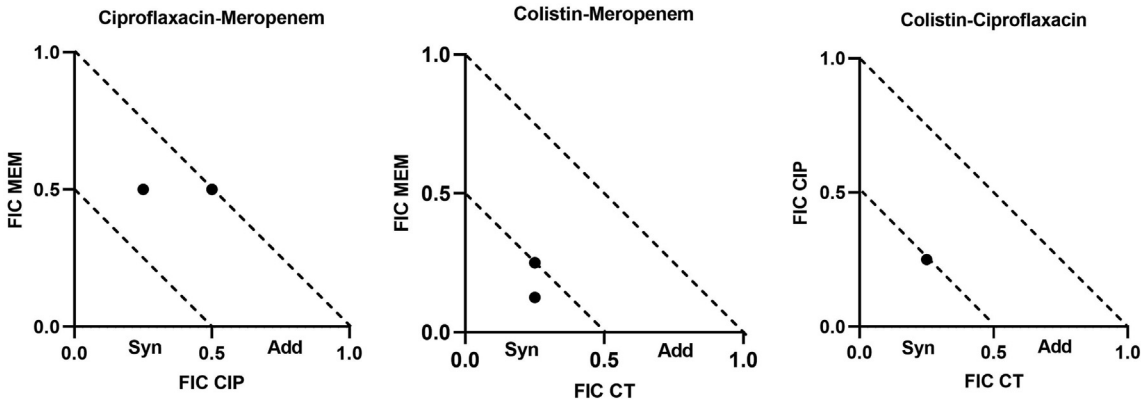

Fig. 1. Isobologram presentation of the effect of antibiotics used against *M. odoratimimus* clinical isolates; Synergistic effect (FICi ≤ 0.5) and additive effect (0.5 < FICi ≤ 1).

16S rRNA sequencing, it is understood that community and hospital-acquired infections caused by atypical pathogens are constantly renewed [3].

M. odoratimimus bacterium is among the atypical microorganisms. It is absent in the human microflora. This bacterium is isolated from environmental sources such as soil and water. It appears as an opportunistic pathogen in immunosuppressed persons using long-term corticosteroid medication [2,12]. *M. odoratimimus* bacteria have developed resistance to many antimicrobial agents, including beta-lactams, monobactams, carbapenems, and aminoglycosides [13].

When the results of the disc diffusion test of the bacterial isolates tested in this study were examined, no antibiotics that formed an inhibition zone other than amikacin and linezolid antibiotics were detected. High MIC values were recorded for CIP, MEM and CT antibiotics, too.

Yang et al. stated that the isolates were resistant to almost all antibiotics, including aminoglycosides and cephalosporins, in their study against twenty-two *M. odoratimimus* bacterial isolates. They reported that the susceptibility of isolates to carbapenems, sulfamethoxazole-trimethoprim, and fluoroquinolone antibiotics decreased [14].

In another study investigating *M. odoratimimus* bacteria isolated from a patient diagnosed with septicemia, it has been reported that the bacterium is sensitive only to piperacillin + tazobactam antibiotics, and it is resistant to all used penicillin and cephalosporin antibiotics, as well as aminoglycosides, quinolones, and carbapenem antibiotics [15].

There is an increase in multi-drug-resistant *M. odoratimimus*, especially in intensive care units patients, due to the presence of a long-term urinary catheter and the large population of immunosuppressive patients. Catheter-associated urinary tract infections caused by hospital-acquired *M. odoratimimus* bacteria have increased in recent years in the world and our country [12,14,16–18].

The combined use of antibacterial drugs has become important in the fight against infectious diseases for a long time. The effect of antibiotic

Table 3
Efficacy of antibiotics on *M. odoratimimus* biofilm formation.

Bacteria isolates	Antibiotic concentration	Biofilm inhibition %
M. odoratimimus-1	Ct 32 (MIC/2)	34.5 ± 0.2
M. odoratimimus-2		30.8 ± 0.6
M. odoratimimus-3		25.4 ± 0.8
M. odoratimimus-4		27.9 ± 1.2
M. odoratimimus-5		31.2 ± 2.1
M. odoratimimus-1	Mem 512 (MIC/2)	72.3 ± 0.5
M. odoratimimus-2		76.5 ± 0.8
M. odoratimimus-3		67.8 ± 0.4
M. odoratimimus-4		75.7 ± 3.1
M. odoratimimus-5		60.3 ± 2.2
M. odoratimimus-1	Cip 64 (MIC/2)	76.6 ± 1.4
M. odoratimimus-2		77.8 ± 1.1
M. odoratimimus-3		60.2 ± 0.2
M. odoratimimus-4		68.5 ± 0.4
M. odoratimimus-5		71.4 ± 0.6
M. odoratimimus-2	Ct 32/Mem 512	92.4 ± 0.8
	Ct 32/Cip 64	91.7 ± 1.4
	Mem 512/Cip 64	75.7 ± 3.1

combinations against *M. odoratimimus* bacteria in planktonic form was tested in our study. It has been determined that CT/MEM and CT/CIP antibiotic combinations have synergistic effects and EM/CIP antibiotic combinations have an additive effect against all bacteria. MIC values of CT, MEM, and CIP antibiotics alone were four times higher than the combined efficacy of antibiotics.

M. odoratimimus bacterium has a strong biofilm-forming ability. The high level of biofilm formation in *Myroides* spp. complicates the treatment of infections and also predisposes to recurrent infections [19]. Biofilms are sessile microorganism communities that form on biotic or abiotic surfaces. Microorganisms in the biofilm structure have higher pathogenicity than planktonic microorganisms. Due to the biofilm structure, bacteria have a high resistance to antibiotics and disinfectants. The bacteria can escape the host's immune system and acquire highly virulence characteristics, too.

It is reported that biofilms are responsible for 80% of all microbial infections in the human body [20]. Biofilm-related infections include urinary tract infections, catheter-related infections, dental plaque formation, gingivitis, and cystic fibrosis [21].

The antibiotics with the most effective biofilm inhibition rates in this study were; MEM (60.3–76.5%) and CIP (60.2–77.8%) antibiotics. The inhibition percentage of CT antibiotics (25.4–34.5%) was found to be lower than that of MEM and CIP antibiotics. Although the CT antibiotic alone had low inhibition, the level of inhibition increased approximately 3-fold when used in combination with MEM or CIP antibiotics.

For the clinical isolate "*M. odoratimimus*-2", although the percent inhibition was 30.8% when CT antibiotic was used alone, the biofilm inhibition rates of CT/MEM and CT/CIP antibiotic combinations were 92.4% and 91.7%, respectively. The combination of MEM/CIP antibiotics inhibited biofilm formation by 75.7%.

According to the results of this study, CT/MEM and CT/CIP antibiotic combinations, which have synergistic effects against planktonic *M. odoratimimus* isolates, also showed the highest inhibition effect on biofilm formation. Therefore, the synergistic activity results of the antibiotics were found to be compatible with both planktonic and biofilm forms of the tested isolates.

The literature was reviewed and no in vitro studies were found researching the effect of antibiotic combination or biofilm inhibition against *M. odoratimimus* bacteria. A study reported the combined use of rifampicin and quinolone antibiotics has been reported in the treatment of some urinary tract infections caused by the *M. odoratimimus* bacteria [12]. In another study, positive results were reported from treatment with the meropenem or piperacillin/tazobactam antibiotic combinations [2,22].

5. Conclusion

This study is the first report showing the efficacy of CT, MEM and CIP antibiotics, which are frequently used in clinical practice, in combination on *M. odoratimimus* planktonic and biofilm forms. Choosing the appropriate antimicrobial therapy in the treatment of infections caused by *Myroides* may be difficult due to limited clinical experience. The results obtained in this study are important in terms of guiding the selection of combinational therapies, especially in the antimicrobial treatment process of immunosuppressed patients receiving treatment in the intensive care unit. The combination of CT/MEM antibiotics is one of the combination therapies that are frequently used in clinical services. In addition, these results are important in terms of supporting in vitro that CT/CIP and MEM/CIP combinations can also be used as a treatment option in *M. odoratimimus* related infections.

CRediT authorship contribution statement

Ayşe Hümeyra TAŞKIN KAFA: Conceptualization, Methodology, Formal analysis, Validation, Investigation, Data curation, Writing – original draft. Mürşit HASBEK: Resources, Visualization, Data curation, Writing – review & editing.

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Declaration of competing interest

No conflict of interest was declared by the authors.

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