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In vitro effects of antibiofilm agents and antibiotics on coagulase-negative staphylococci

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ABSTRACT: Coagulase negative staphylococci (CoNS) are important nosocomial pathogens that cause biofilm infections. Biofilm provides advantages for microorganisms to resist antibiotics and host immune systems. Considering the increased antibiotic resistance, alternative treatments are needed to combat biofilm infections. In the present study, the effects of antibiotics including gentamicin (GEN), ciprofloxacin, doxycycline (DOX), rifampicin (RIF) and antibiofilm agents including N-acetylcysteine (NAC), ethylenediaminetetraaceticacid (EDTA), nisin (NIS), farnesol (FAR) on clinical CoNS biofilm and *IS256, icaA* gene expression levels were evaluated. Forty-five CoNS strains were isolated from patients' catheters, at Manisa Celal Bayar University Hospital. The minimum inhibitory concentrations (MICs) of agents were detected by broth microdilution method with European Committee for Antimicrobial Susceptibility Testing (EUCAST) criteria. The combined effects of agents were investigated by checkerboard method. The antibiofilm effects of combinations were investigated by spectrophotometric microplate method. The effects of combinations on *IS256* and *icaA* gene expressions were evaluated by real-time quantitative reverse-transcriptase PCR. Twenty-four isolates (53.3%) were detected as strong biofilm producer. Biofilm production was inhibited in seven isolates in the presence of EDTA+RIF and NIS+DOX while NIS+GEN combination and RIF inhibited biofilm in six isolates. Nine combinations were found to have synergistic effect against isolate #6 which are resistant to four different antibiotics. The expressions of *icaA* and *IS256* were downregulated in the presence of EDTA, NAC+CIP, NAC+GEN, NIS+GEN, FAR+GEN. Antibiofilm agent/antimicrobial combinations could have promising effects for preventing catheter colonization. The further studies on antibiofilm treatment strategies would be beneficial for decreasing morbidity-mortality rates and healthcare costs caused by biofilms.

KEYWORDS: Coagulase negative staphylococci; biofilm production; antibiofilm agents; synergistic effect; gene expression.

1. INTRODUCTION

Staphylococcus species are clinically important microorganisms which are known to cause biofilm releated nosocomial infections by their many different virulence factors. *Staphylococcus aureus*, *Staphylococcus epidermidis, Staphylococcus haemolyticus, Staphylococcus lugdunensis* and *Staphylococcus saprophyticus* are common species lead to life threating infections. Coagulase-negative staphylococci (CoNS) which are the members of microbiota, can live on human skin and mucous membranes. CoNS microorganisms have recently attracted considerable attention as dangerous nosocomial pathogens. The colonization of bacteria on biotic surfaces such as skin and mucous membranes can cause endogenous infections. In particular, CoNS species that may be capable of forming biofilms on abiotic surfaces are among the major microorganism species that causes infections associated with medical devices. The increase in CoNS infections have been associated with the widespread use of indwelling medical material or implanted foreign bodies such as catheters and artificial heart valves in recent years [1,2].

Biofilm structure can be defined as a micro-ecosytems that contains the community of bacteria adhered to a surface and extracellular polymeric substances, exopolysaccharides (EPS), nucleic acids and proteins [3,4]. Biofilm production capacities of pathogen microorganisms is a major clinical problem for treatment, mainly

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due to the intrinsic resistance of biofilm cells to antibiotics [5]. The bacteria that have biofilm forming capacity, exhibit increased tolerance against antibiotics, disinfectants, and host immune mechanisms. The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of antibiotics against biofilm forming bacteria may be up to 100–1000 fold higher than that of planktonic bacteria [6].It is known that the cell surface proteins and polysaccharide production are contributed to biofilm formation that can protect pathogen bacteria by impeding the penetration of antibiotics and the function of phagocytic immune cells. In the biofilm layer, it is easier for microorganism to escape from phagocytic macrophages and also the biofilm forming bacteria may become more resistant to antibiotics due to exopolysaccharide matrix and low metabolic rates [4-7]. Extracellular polysaccharides, adhesins and biofilm-associated proteins, enable bacteria to form biofilm on biotic or abiotic surfaces [8]. Polysaccharide intracelluler adhesins (PIA), is a major virulence factor required for biofilm formation, hemagglutination, and bacterial aggregation [9,10]. The *icaA* gene (intercellular adhesion gene) is responsible for polysaccharide intercellular adhesin poly-N-succinyl β-1-6 glucosamine (PIAPNSG) which plays an significant role for bacterial biofilm composition and intercellular adhesion [8]. The presence and expression of *icaADBC* operon is essential for the production of staphylococcal biofilm which is also dependent on multiple regulatory proteins. It is known that staphylococcal biofilm formation is a variable process influenced by different genetic mechanisms and chromosomal rearrangements, based on the findings of previous studies [9]. *IS256*, which is an insertion sequence, involved in phase variation of biofilm forming CoNS. Many different studies have also shown that *IS256* insertion sequence, influences expression of *ica* genes and also biofilm phenotype of isolates by causing phase variation of the *icaADBC* operon region. Furthermore, it is noteworthy that the *IS256* has coexisted with some antibiotic resistance genes such as *mec* gene, particularly in biofilm forming CoNS isolates [9,11]. It has also been suggested that *IS256* may have a potential marker gene to distinguish between invasive and noninvasive nature of CoNS isolates which have multidrug-resistance profiles [12].

The widespread use of medical devices such as catheters and artificial heart valves which have suitable surfaces for bacterial biofilm layer has led to a significant increase in the incidence of medical devices associated infections recently. Biofilm-associated infections which are responsible for more than half a million deaths in worldwide, cause devastating complications which prolong disease and result in higher morbidity and mortality, especially in immuno-compromised patients [13]. It is known that there are several challenges for the treatment of infectious diseases, such as increased antibiotic resistance, difficulties about discovery of new compounds and intrinsic resistance caused by biofilm structure [13]. Studies have shown that most of the antibiotics used in treatment are insufficient to eliminate biofilm-associated infections [14]. Given all these difficulties on treatment strategies, it is clear that new antimicrobial and antibiofilm agents are needed for prevention and treatment of biofilm infections. Effects of various molecules used for different therapeutic purposes on the biofilm structure are the subject of various researches in recent years. The main goal of this study was to evaluate the efficacy of some antibiofilm agents and antibiotics against catheter-associated CoNS biofilms and to develop possible antibiotics/antibiofilm agent combinations for combating biofilm associated infections.

2. RESULTS

2.1. Minimum inhibitor concentrations of antibiofilm agents and antimicrobials

It was determined that the MICs of antibiofilm agents were ranged from 16 µg/mL to > 2048 µg/mL according to the results of broth microdilution method. The MICs of antibiofilm agents and the numbers of isolates were shown in Table 1. Within the results of broth microdilution methods, it was determined that the MIC values of four antimicrobial agents ranged from 0.008 µg/mL to >16 µg/mL. The susceptibility profiles of isolates to the four antimicrobial agents studied are shown in Table 2.

2.2. Biofilm quantification

Biofilm quantification for forty-five CoNS was performed by using spectrophotometric microplate method with CV staining [15]. Of 45 CoNS strains isolated from catheters, 24 isolates (53.3%) were found to be strong biofilm producers. Fifteen isolates (33.3%) were detected as moderate biofilm producers while six isolates (13.3%) were detected as weak biofilm producers.

Table 1. The MICs of agents and the numbers of isolates.

MIC (µg/mL)	Agents			
	NAC	EDTA	NIS	FAR
>2048 µg/mL	-	-	2	2
2048 µg/mL	6	2	-	-
1024 µg/mL	33	-	-	15
512 µg/mL	5	7	-	13
256 µg/mL	1	25	2	3
128 µg/mL	-	9	29	4
64 µg/mL	-	2	7	5
32 µg/mL	-	-	5	-
16 µg/mL	-	-	-	3

MIC: Minimum Inhibitory Concentration, NAC: N-acetylcysteine, EDTA: ethylenediaminetetraacetic acid, NIS: Nisin, FAR: Farnesol.

Table 2. The antibiotic susceptibility profiles of CoNS isolates.

	Gentamicin	Ciprofloxacin	Doxycycline	Rifampicin
Resistant (n)	28 (62.2%)	29 (64.4%)	7 (15.6%)	19 (42.2%)
Suspectible (n)	17 (37.8%)	16 (35.6%)	38 (84.4%)	26 (57.8%)
MIC breakpoints*	R: MIC>1 µg/ml S: MIC≤1 µg/ml	R: MIC>1 µg/ml S: MIC≤1 µg/ml	R: MIC>2 µg/ml S: MIC≤1 µg/ml	R: MIC>0.5 µg/ml S: MIC≤0.06 µg/ml
MIC_{50}	8 µg/ml	2 µg/ml	0.06 µg/ml	<0.015 µg/ml
MIC_{90}	16 µg/ml	16 µg/ml	2 µg/ml	>16 µg/ml

R: Resistant, S: Susceptible, *: Breakpoint tables for interpretation of MICs and zone diameters. Version 9.0, 2019. Intermediate susceptible isolates were considered resistant.

2.3. The effects of antibiotic and antibiofilm agents on bacterial biofilm formation

The investigations on antibiofilm effects of agents were carried out with the isolates, which were strong biofilm producers. Of all CoNS isolates, nine strains that are strong biofilm producer (strain no 3, 7, 9, 10, 11, 12, 31, 37, and 49), resistant to at least three antimicrobial agents, were selected for this study. NIS, NAC, EDTA, and FAR alone inhibited biofilm in five, three, four and three isolates, respectively. The effects of antibiofilm/antibiotic combinations on biofilm formation of strains were shown in Table 3. It was found that the EDTA+RIF and NIS+DOX combinations reduced biofilm formation in seven of nine isolates while NIS+GEN combination caused inhibition of biofilm formation in six isolates. NAC+DOX and NIS+RIF combinations reduced biofilm formation in five isolates.

2.4. The effect of agent combinations on mature biofilm

The effects of combinations on mature biofilms of CoNS strains were also investigated in this study and showed in Table 4. It was detected that the combination of FAR + RIF inhibited mature biofilm formation in six of nine isolates while the NIS+CIP, NIS+DOX and NIS + RIF combinations showed inhibitory effect on mature biofilm in five isolates. In four isolates, the mature biofilm had been inhibited in the presence of NAC+CIP combination and rifampicin alone.

2.5. Combined activity assays

Combination studies were conducted with three isolates (strain no 5, 6, 13) that were found to be resistant to four antibiotics tested by microdilution method. To investigate interactions between the agents, sixteen different combinations were studied for each strain. The agent combinations and interaction types were shown in Table 5.

Table 3. Inhibitory effects of combinations on biofilm production of the isolates.

Agents	Isolate No								
	3	7	9	10	11	12	31	37	40
NAC+GEN	_	↓	_	↓	_	↓	_	_	_
NAC+CIP	_	_	_	↓	_	_	_	_	_
NAC+DOX	_	↓	↓	↓	_	↓	↓	_	_
NAC+RIF	↓	_	_	↓	_	_	↓	_	_
EDTA+GEN	_	↓	↓	↓	_	_	_	_	↓
EDTA+CIP	_	_	_	↓	_	_	_	↓	_
EDTA+DOX	_	_	_	_	_	_	↓	↓	_
EDTA+RIF	↓	↓	_	↓	↓	↓	↓	↓	_
NIS+GEN	↓	↓	↓	↓	_	↓	_	_	↓
NIS+CIP	↓	_	↓	↓	_	_	_	↓	_
NIS+DOX	↓	↓	↓	↓	_	_	↓	↓	↓
NIS+RIF	↓	↓	_	↓	_	_	↓	↓	_
FAR+GEN	↓	↓	↓	↓	_	_	_	_	_
FAR+CIP	↓	_	_	↓	_	_	_	_	_
FAR+DOX	_	_	_	↓	_	_	↓	_	_
FAR+RIF	_	↓	_	↓	_	_	↓	↓	_

NAC: N-acetylcysteine, EDTA: ethylenediaminetetraaceti acid, NIS: Nisin, FAR: Farnesol, GEN: Gentamicin, CIP: Ciprofloxacin, DOX: Doxycycline, RIF: Rifampicin, (↓) : inhibitory effect on biofilm formation, (-) : no inhibition, the data was the mean of three replicates and significant results were added to the table.

Table 4. Inhibitory effects of combinations on mature biofilm of the isolates.

Agents	Isolate Number								
	3	7	9	10	11	12	31	37	40
NAC+GEN	↓	_	_	_	_	_	_	_	↓
NAC+CIP	↓	_	_	_	_	_	↓	↓	↓
NAC+DOX	↓	_	_	_	_	_	↓	_	_
NAC+RIF	_	_	_	_	_	_	↓	↓	↓
EDTA+GEN	_	_	_	_	_	_	↓	_	↓
EDTA+CIP	_	_	_	_	_	_	_	_	_
EDTA+DOX	_	_	_	_	_	_	↓	_	_
EDTA+RIF	_	_	↓	_	_	_	_	↓	↓
NIS+GEN	_	_	↓	_	_	_	_	↓	↓
NIS+CIP	↓	_	↓	_	_	_	↓	↓	↓
NIS+DOX	↓	_	↓	_	_	_	↓	↓	↓
NIS+RIF	↓	_	↓	_	_	_	↓	↓	↓
FAR+GEN	_	_	↓	_	_	_	_	↓	↓
FAR+CIP	_	_	_	_	_	_	_	↓	↓
FAR+DOX	_	_	↓	_	_	_	_	↓	↓
FAR+RIF	_	↓	↓	_	↓	_	↓	↓	↓

NAC: N-acetylcysteine, EDTA: ethylenediaminetetraaceti acid, NIS: Nisin, FAR: Farnesol, GEN: Gentamicin, CIP: Ciprofloxacin, DOX: Doxycycline, RIF: Rifampicin, (↓) : inhibitory effect on biofilm formation, (-) : no inhibition, the data was the mean of three replicates and significant results were added to the table.

Table 5. The checkerboard method results (Combined effects and FICI values).

	Isolate No 5				Isolate No 6				Isolate No 13			
	GEN	**CIP**	**DOX**	**RIF**	**GEN**	**CIP**	**DOX**	**RIF**	**GEN**	**CIP**	**DOX**	**RIF**
NAC	I (2.0)	I (2.0)	S (0.5)	S (0.5)	I (2.0)	A (0.75)	S (0.5)	I (2.0)	I (2.0)	I (2.0)	I (2.0)	I (2.0)
EDTA	I (2.0)	I (2.0)	S (0.5)	S (0.5)	A (1.0)	S (0.5)	S (0.5)	S (0.5)	A (1.0)	I (2.0)	I (2.0)	I (2.0)
NIS	I (2.0)	I (2.0)	A (0.75)	S (0.5)	S (0.5)	S (0.5)	S (0.5)	I (2.0)	I (2.0)	I (2.0)	A (1.0)	I (2.0)
FAR	I (2.0)	A (0.75)	A (0.75)	S (0.5)	S (0.5)	A (0.75)	S (0.5)	I (2.0)	I (2.0)	I (2.0)	I (2.0)	I (2.0)

FICI: Fractional inhibitory concentration index, NAC: N-acetylcysteine, EDTA: Ethylenediaminetetraacetic acid, NIS: Nisin, FAR: Farnesol, GEN: Gentamicin, CIP: Ciprofloxacin, DOX: Doxycycline, RIF: Rifampicin, S: Synergistic, A: Additive, I: Indifferent

2.6. Expression analysis of biofilm associated genes *icaA* and *IS256*

The effects of tested agents on expression levels of *icaA* and *IS256* genes were detected according to analysis of RT-qPCR. The expression rates of *icaA* and *IS256* were downregulated in the presence of NAC+CIP and NAC+GEN combinations. NIS+GEN, FAR+GEN combinations and EDTA alone also caused downregulation in the expression levels of *icaA* and *IS256* combinations. In addition, *IS256* gene was downregulated in the presence of doxycycline, gentamicin, nisin and farnesol alone. It was observed that RIF alone had shown no significant effect on *IS256* gene expression, but upregulated *icaA* gene expression. EDTA+CIP, EDTA+RIF, NIS+DOX, NIS+RIF, FAR+DOX and FAR+RIF combinations were determined as the combinations that caused downregulation of *IS256* gene expression according to the expression analyses.

3. DISCUSSION

Coagulase-negative staphylococci are one of the most common microorganisms which cause nosocomial infections and catheter associated infections. Bacterial biofilm formation is thought to be one of the main virulence mechanisms in CoNS, especially for *S. epidermidis*. Studies on the effects of various drug molecules on bacterial biofilm formation are increasing rapidly to prevent biofilm associated infections. Considering the antibiotic resistance rates and biofilm infections which are increasing day by day, it is understood that the elucidation of bacterial biofilm structures and new antibiofilm treatment strategies are essential for the fight against infectious diseases worldwide. Nowadays, biofilm structure of different microorganism species and antibiofilm activity of various agents are wide research areas which have been studied by the researchers [13,17].

In this study, it was determined that NIS and EDTA were the agents which have the highest antibiofilm effect among the tested antibiofilm agents. Although NAC alone had shown lower antibiofilm effect, it has been observed that NAC/antibiotic combinations can inhibit biofilm formation with a synergistic effect. It was detected that NAC+DOX combination had shown the highest antibiofilm effect compared to NAC+other antibiotic combinations. The antibiofilm effect of NAC has been investigated on different microorganism species previously. In parallel to some studies in literature, our findings suggest that NAC can have potential as an antibiofilm agent especially in combination with antibiotics, for preventing biofilm associated infections [18]. In a research on the effects of NAC on biofilm production of *S. epidermidis*, it was found that there were significant differences between the biofilm production capacities of methicillin-resistant and methicillin-sensitive *S. epidermidis* isolates were reported in the presence of various concentrations of NAC. They reported that NAC could have biofilm inhibitory effect that depend on concentration for these isolates [18].

In a study conducted in 2010, the effect of NAC on the antibiotic susceptibility of different microorganism species such as *Escherichia coli, Klebsiella aerogenes* and *Pseudomonas aeruginosa* was investigated. They found that the MIC values of penicillin and ampicillin decreased in the presence of NAC for *P. aeruginosa* and *K. aerogenes.* The researchers found no change in MIC values of chloramphenicol and tetracycline while they detected that both the MICs of quinolones such as ciprofloxacin, ofloxacin and the MICs of aminoglycosides such as streptomycin, kanamycin and spectinomycin increased with the presence of NAC

[19]. El-Feky et al., studied the effect of CIP and NAC, alone and in combination, on biofilm production on ureteral stent surfaces and on preformed mature bacterial biofilms. *S. aureus, S. epidermidis, E. coli, Klebsiella pneumoniae, P. aeruginosa* and *Proteus vulgaris* strains were tested and it has been noted that combinations of NAC+CIP have high potential inhibitory effect on biofilm of these species [20]. In contrast to these results, it was detected that both NAC alone and in combination with CIP showed lower antibiofilm effect than the other combinations tested in our study. NAC+CIP combination inhibited biofilm production only one isolate while it showed the antibiofilm effect on mature biofilm of four isolates.

Researches on the antimicrobial and antibiofilm effects of chemical agents that used in pharmaceutical and cosmetic industries have been conducted on a rapidly increasing scale all over the world. EDTA, NIS and FAR are prominent molecules that have attracted attention with their potential antimicrobial and antibiofilm activities in recent years. According to our results EDTA was one of the molecules which showed high antibiofilm effect among four antibiofilm agents tested. When the effects of EDTA on CoNS biofilms, in combination with different antibiotics were analyzed, we detected that EDTA+RIF combination and EDTA+GEN combination were the best combinations that caused reduction on biofilm formation.

It was reported that EDTA, lactic acid, citric acid and phytic acid molecules, which were used as chelators, could make some microorganisms more susceptible to antimicrobial agents by increasing the permeability of the bacterial cell membrane in another research [20]. With the results of different studies, it has been pointed out that these chemical agents block iron which is required for microbial metabolism and growth, thus being important agents for increasing the effectiveness of antimicrobial agents that are used against resistant Gram-negative bacteria [21]. Birteksöz Tan et al. reported that EDTA is not considered to be an antimicrobial agent alone, but it may show synergistic effect with some antibiotics, other preservatives (benzalkonium chloride, parabens) and cationic surfactants (quaternary ammonium compounds) [22]. Additionally, it has been known that EDTA has a synergistic effect with synthetic preservatives and it makes Gram-negative bacteria more sensitive to antimicrobials by disrupting lipopolysaccharide structure in the outer membrane. In another research on activities of NAC, EDTA, ethanol and lactoferrin against monomicrobial and polymicrobial biofilms, it was found that the combinations of NAC, EDTA with antimicrobials were synergistic against *S. epidermidis* and *Candida albicans* biofilms. They reported that EDTA, NAC and ethanol alone or in combination with some antibiotics can be used in catheter solutions for preventing catheter-related infections, possible resulting in reducing the patient's morbidity and health costs directly [23].

Nisin is an antimicrobial peptide that is produced by *Lactococcus lactis* and it has bactericidal activity against Gram-positive bacteria. Farnesol is a sesquiterpene alcohol produced by many organisms and is also found in essential oils. The antibiofilm effects of NIS and FAR which are widely used as a preservative in food industry, are being studied by different researchers. It is stated that FAR affects the growth of bacteria and fungi and has potential as an antimicrobial agent [24]. It was determined that NIS alone was the most potent agent showed biofilm inhibitory effect on five isolates in the present study. In the combination assays, NIS+GEN and NIS+DOX combinations showed the highest biofilm inhibitory effect compared to other NIS and antibiotics combinations. It was detected that farnesol inhibited biofilm production in three of nine CoNS isolates and showed lower antibiofilm effect than NIS and EDTA. Among the combinations of FAR and antibiotics, FAR+GEN and FAR+RIF were the combinations with the most potential promised biofilm inhibitory effect in our study. In the findings of a research on antimicrobial effects of NIS on *Listeria monocytogenes*, it was reported that bacterial growth was inhibited and cellular deterioration symptoms were observed on the surfaces in contact with nisin, therefore this agent could have a potential as an antimicrobial agent [25]. As a result of different studies examining the effects of farnesol on *S.epidermidis*, high concentrations of farnesol have been reported to have antibiofilm effects and synergistic interactions with antibiotics such as nafcillin and vancomycin [5,26]. The data from an investigation on effects of farnesol on methicillin-resistant and susceptible *S. aureus* strains has shown that farnesol inhibits biofilm formation and damages cell membrane integrity. The researchers also reported that farnesol could have an impact on antibiotic resistance profiles at its high concentrations. It was reported in the literature that the combination of farnesol and gentamicin at different concentrations could have a synergistic effect on bacterial biofilm [27].

Although there are many studies on the effects of different antibiotics on virulence genes, the knowledge on the effects of antibiofilm agents and their combinations with antibiotics on gene expression is very limited. Thanks to various researches on the relationship between antibiotics exposure and gene expressions, it is known that the genes which encode several adhesins and virulence factors in *Staphylococcus* spp. also showed increased levels of expression in the presence of antibiotics at sub-MIC levels [28,29]. Gomes et al. showed that

enhanced *icaA* expression in *S. aureus* strains exposed to rifampicin at sub-MIC as well as RIF in combination with GEN or clindamycin [28]. It was also reported that quinupristin-dalfopristin and erythromycin at sub-MIC levels may stimulate *icaA* expression [29,30]. In another study it was demonstrated that tigecycline may alter the pathogenesis of staphylococcal infections by leading to increased *icaA* expression level. In contrast to this situation, it was reported that ciprofloxacin induced an increase of *icaA* mRNA levels in the same study [31].

In the present study we evaluated the expression levels of biofilm associated genes *icaA* and *IS256* in one CoNS strain (*S. epidermidis*) in the presence of the antibiofilm+antibiotic combinations in addition to *in vitro* phenotypic methods. With the analysis of our RT-qPCR data it was detected that the expressions levels of *icaA* and *IS256* were downregulated in the presence of NAC+CIP, NAC+GEN, NIS+GEN, FAR+GEN combinations and EDTA alone. *IS256* gene was also downregulated in the presence of doxycycline, gentamicin, nisin and farnesol alone. It was noteworthy that rifampicin showed no significant effect on *IS256*, but upregulated *icaA* gene expression. According to the results it has thought that the antibiofilm effects of NAC+CIP, NAC+GEN, NIS+GEN, FAR+GEN combinations may be based on the downregulation of *icaA* and *IS256* genes. It is clear that further studies and data are needed to understand the molecular mechanisms of biofilm inhibition.

It is known that catheters are associated with a definitive risk of infections and catheter-related infections contribute to the increasing problem of nosocomial infections in all over the world. The ability of microorganisms to adhere to medical devices including catheters and the production of biofilm is one of the most important feature of their pathogenicity. It is clear that new antimicrobial and antibiofilm agents are needed for prevention and treatment of biofilm-related infections. New strategies such as antiadhesive and colonisation resistant materials would be suitable candidates to avoid colonization and infection. Impregnation of devices including central venous catheters and urinary catheters with various compounds such as antimicrobials, antiseptics or metals alone and/or in combination is another strategy for the prevention of medical device-related infections [32-38]. In addition, catheter lock therapy using various antimicrobials and other agents has been also proposed for the prevention and treatment of catheter-related infections [39-41]. Although the problem of catheter-related infections is still one of the major challenges, the novel strategies aforesaid have shown promising progress according to the data of *in vitro*/*in vivo* experiments and clinical trials. According to the results of our experiments, it is thought that the studied combinations may have potential and may provide for new therapeutic opportunities against biofilm-related and medical device-related infections in accordance with the strategies mentioned above.

4. CONCLUSION

Considering the antibiotic resistance rates worldwide and the challenges on development of new antibiotics, it is of great importance to develop new treatment strategies with therapeutic agents in use. It is obvious that there is an urgent need for the discovery of functional combinations of antibiofilm agents/antibiotics and the development of novel therapeutic strategies in order to combat biofilm infections and antimicrobial resistance. Based on the knowledge in literature and the results of our study, it is thought that some agents that are in use for different therapeutic purposes may have potential to prevent CoNS colonization and catheter related infections, alone and in combination with antibiotics. We hope that further studies in this area will contribute significantly to the understanding of the bacterial biofilm structure and the fight against antimicrobial resistance.

5. MATERIALS AND METHODS

5.1. Bacterial isolates

Forty-five clinical CoNS isolates were isolated from catheters at Bacteriology Laboratory, Department of Medical Microbiology, Manisa Celal Bayar University, were used in this study. Catheter tips were processed by using both semi quantitative method described in by Maki et al. and quantitative sonication method [42]. The semi quantitative technique is also called the roll plate method was performed by rolling the external surface of a catheter tip back and forth on the surface of a Columbia Agar with 5% sheep blood plate (BD GmbH, Germany) at least three times. The inoculated agar plates were incubated at 35°C and 5% CO2 for 72 h. The quantitative sonication technique was performed by placing the catheter in 5 ml of 0.9% NaCl, sonicating it for 1 minute and vortexing it for 15 s, then 0.1 ml of the sonication fluid was cultured on a Columbia Agar plate supplemented with 5% sheep blood and then incubating the plate at 5% CO2 and 35°C

for 72 h [43]. Microorganisms recovered from the plates were identified and counted by standard microbiological methods. Definitions: Catheter tip colonization was defined as a positive semi quantitative tip culture of ≥15 CFU/ml for the roll plate method or ≥100 CFU/catheter segment for the sonication technique, as described elsewhere [42-44]. Identification of the isolates was performed using conventional methods and VITEK-2 device. Forty-five isolates identified as CoNS were stored in brain-heart infusion broth (BHIB) (Merck) with 10% glycerine at –80°C until use. *Staphylococcus aureus* ATCC 29213 was used as quality control strain for antimicrobial susceptibility testings.

5.2. Antibiofilm agents and antibiotics

N-acetylcysteine (NAC) (Abdi Ibrahim, Turkey), ethylenediaminetetraacetic acid (EDTA) (Sigma), nisin (NIS) (Sigma) and farnesol (FAR) (Sigma) were used as antibiofilm agents. Gentamicin (GEN) (Bayer, Turkey), ciprofloxacin (CIP) (Bayer, Turkey), doxycycline (DOX) (Sigma) and rifampicin (RIF) (Sigma) were antimicrobial agents used in this study. To prepare working stock solutions, antibiofilm and antimicrobial agents were dissolved by using 50% dimethyl sulfoxide (DMSO)/distilled water (v/v) and distilled water alone, respectively.

5.3. Determination of minimum inhibitory concentrations

The minimum inhibitory concentration (MIC) values of agents were determined by broth microdilution method according to European Committee for Antimicrobial Susceptibility Testing (EUCAST) criteria. *Staphylococcus aureus* ATCC 29213 was used as the control strain. The antibacterial activity of DMSO which was used to dissolve agents was also studied alone. All the experiments were performed in triplicate.

For antibiotic/antibiofilm agent's susceptibility testings, forty-five CoNS strains were grown on a Mueller–Hinton Agar (MHA) (Merck) and incubated at 35°C for 24 h. The bacterial suspensions were prepared by using 0.9% sodium chloride and adjusted to 0.5 McFarland turbidity standard. Then bacterial suspensions were diluted in ratio 1/100. Briefly, 50 µL Mueller-Hinton II broth (MHB II) (Merck) was distributed to the wells of sterile 96-well microplates for the broth microdilution method. Then, 50 µL of antimicrobial agents were added to the first wells of the microplates and serial dilutions were prepared by using other wells. Following the addition of bacterial suspensions to the wells, the microplates were incubated at 37 °C for 24 h. After the incubation period, the lowest agent concentration that inhibited the growth of bacteria were determined as MIC values. MIC_{90} and MIC_{50} values were defined as the lowest concentration of the antibiotic at which 90% and 50% of the isolates were inhibited, respectively.

5.4. Biofilm quantification

Bacterial biofilm formation was quantified by a modification of the method used by Stepanovic et al., in this study [15]. Initially, biofilm production capacities of isolates were determined and then the effects of agents on both biofilm formation and mature biofilm of the selected strains were investigated with crystal violet (CV) staining assay. In our experiments, we investigated the anti-biofilm activities of the agents against biofilm production of the isolates by adding the agents to the inoculum before incubation. Incubation of inoculum without agents at 37°C for 24 hours were performed in order to mature biofilm on microplate surface. Bacterial strains were cultured on MHA at 37°C for for 24 h. Bacterial suspensions (20 µL) which were adjusted to 0.5 McFarland turbidity standard were added into the wells of microplates that contained 180 µL Tryptic soy broth (TSB) containing 2.5% glucose. Then the microplates were incubated at 37 °C for 24 hours for biofilm production. Following the incubation period, the wells were aspirated to remove the medium and were washed three times with sterile phospate buffered saline (PBS) (Oxoid) 200 µL per well to remove non-adherent cells. After washing steps, the plates were air dried then the remaining attached microorganisms were fixed with 200 µL of methanol for 15 min. The content of wells was poured off, methanol was discarded, and the wells were air-dried. After drying process, 200 µL of 0.1% CV solution were added to the wells for 5 min, at room temperature. After drying process, 200 µL of 0.1% CV solution was added to the wells for 5 min, at room temperature. Then the CV solution was removed by washing with tap water, and the plates were dried again. 200 µL of of 95% ethanol was added to each well to remove the stain and the plates incubated for 15 min. At the end of 15 min, the contents of the wells were transferred to sterile microplates and spectrophotometric measurements were performed at 570 nm using microplate reader (Varioskan Flash, ThermoScientific, USA). The optical density (O.D.) values of the wells which contained only medium were used as negative controls. The cut-off O.D. (O.D.c) was defined as three standard deviations above the mean O.D. of negative controls. All tests were carried out in triplicate. Statistical analyzes were performed using Graphpad Prism 5.03 (t test) program and the values of $p<0,5$ were considered significant. O.D.≤ O.D.c: no biofilm production, O.D.c < O.D. ≤ (2×O.D.c): weak biofilm producer, (2×O.D.c) < O.D. ≤ (4×O.D.c): moderate biofilm producer and (4×O.D.c) <O.D: strong biofilm producer [15].

5.5. Antibiofilm effects of antibiofilm agents and antibiotics

So as to investigate the antibiofilm effects of antibiotics and other agents, the strains were cultured on MHA at 37°C for 24 h. Each agent (20 µL, final concentration at MIC level) and bacterial suspensions (20 µL) which were adjusted to 0.5 McFarland turbidity, were added into the wells of microplates that contained 160 µL TSB containing 2.5% glucose. The plates were incubated at 37 °C for 24 h. Following the incubation period, the wells were aspirated to remove the medium and washed three times using 200 µL PBS. Upon completion of the washing steps, the microplates were air dried. The bacteria that adhered to surface were fixed with 200 µL of methanol for 15 min. Then the content of wells was poured off, methanol was discarded, and the wells were air-dried. After the drying process, 200 µL of 0.1% CV solution were added to the wells for 5 min, at room temperature. Then the CV solution was removed by washing with tap water and the plates were dried again. 200 µL of of 95% ethanol was added to each well to remove the stain and the plates were incubated for 15 min. At the end of 15 min, the contents of the wells were transferred to steril microplates and spectrophotometric measurements were performed at 570 nm using a microplate reader (Varioskan Flash, ThermoScientific, USA). The optical density (O.D.) values of the wells which had content media with no inoculum were used as negative controls. The cut-off O.D. (O.D.c) was defined as three standard deviations above the mean O.D. of negative controls. All tests were carried out in triplicate. Statistical analyses were performed using t-test (GraphPad program). Certain concentrations of the tested agents were added onto the mature biofilm formed with 24 h incubation to investigate their effects on mature biofilm. The effects on mature biofilm was evaluated by the CV staining method described above.

5.6. Combined activity assays

Interactions between the antibiofilm agents and antibiotics were determined using the checkerboard method with 96-well microplates [16]. Antibiofilm agent/antimicrobial combinations used in checkerboard method were as the follows: NAC+GEN, NAC+CIP, NAC+DOX, NAC+RIF, EDTA+GEN, EDTA+CIP, EDTA+DOX, EDTA+RIF, NIS+GEN, NIS+CIP, NIS+DOX, NIS+RIF, FAR+GEN, FAR+CIP, FAR+DOX, FAR+RIP. The types of interaction between these agents were evaluated based on the fractional inhibitory index (FIX) and fractional inhibitory concentration (FIC) values that were calculated for each combination. The effects of combinations against three CoNS isolates identified as resistant to the four antibiotics were investigated in this study.

Bacterial strains were cultured on MHA at 37°C for 24 h. Bacterial suspensions were prepared with 0.9% NaCl and diluted with 1/100 MHB-II after adjusting to the 0.5 McFarland turbidity standard. Different concentrations of each antibiofilm agents and antibiotics, (2×MIC, MIC, MIC/2, MIC/4 and MIC/8) were studied with the checkerboard method. 50 µL of the four-fold dilutions of tested antibiotics were distributed to wells starting from the first column of the microplate to the fifth column. No antibiotics were added to the last column that was used as growth control. 50 µL of each serial dilution of the antibiofilm agents was added onto the wells which contain antibiotics. Finally, 100 µL of bacterial suspension was added to all the wells. The MIC values of the combination were determined separately in the two antibiotics included in the combination following the incubation period of microplates at 37 ° C for 16-24 hours.

In vitro interactions between the agents were evaluated based on fractional inhibitor concentration (FIC) and fractional inhibitor concentration index (FICI) values for each combination. The effect of the combinations on the sum of obtained FICI values (ΣFIC) was determined to be synergistic, additive, ineffective or antagonistic. The interection types between the agents were determined using the following criteria: FICI ≤ 0.5: synergistic, 0.5 < FICI ≤ 1: additive, 1 < FICI ≤ 4: indifferent, 4 < FICI: antagonistic [17].

5.7. Expression analyses of biofilm associated genes by RT-qPCR

The effects of some antibiofilm agents (N-acetylcysteine, EDTA, nisin, farnesol) and antimicrobials (gentamicin, ciprofloxacin, doxycycline, rifampicin) on the expression levels of *icaA* and *IS256* genes, by using RT-qPCR method, were also investigated in this study. The gene expression studies were performed with a strong biofilm producer CoNS isolate (*S.epidermidis*) identified as possessing the *icaA* and *IS256* genes by conventional PCR [9,10]. The RNA and DNA isolations were performed after *S. epidermidis* strain was exposed to MIC levels of the tested agents for 24 h. Then RT-qPCR experiments were performed in a 96-well plate, by using LightCycler 480 Instrument II (Roche, USA) and LightCycler 480 SYBR Green I Master kit (Roche, USA). The reference gene 16S rRNA was used to normalize data. Gene expression analyses were evaluated by using the expression of the *icaA* and *IS256* genes versus reference gene (16S rRNA), and also comparing the groups which exposed the agents relative to control groups. The PCR cycles were as follows: 5 min at 95 °C for initial

denaturation, followed by 45 cycles of denaturation at 95 °C for 10 seconds, annealing at 43 °C for 10 seconds, elongation at 72 °C for 10 seconds before melting curve analysis. Threshold cycle (C_T) values were calculated using LC480 2 software program. The data was presented as a fold change in gene expression in the presence of tested agents compared to control groups. Delta-delta Ct method was used for the analysis of relative gene expression. All the experiments were performed in triplicate. The statistical analyses were performed using t-test (GraphPad program).

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