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Araştırma Makalesi - Research Article

The Effect of Nisin and Chloramphenicol Combinationon *Staphylococcus aureus* ATCC 6538 Biofilm Structure

Nisin ve Kloramfenikol Kombinasyonunun *Staphylococcus aureus* ATCC 6538 Biyofilmi Üzerindeki Etkileri

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ABSTRACT

Nowadays, due to the rapid spread of antibiotic resistance and the difficulty of discovering new antimicrobial agents, the reuse and combinational strategies of old antibiotics have come to the fore. In this study, we investigated the combinational efficacy of nisin and chloramphenicol against the ATCC 6538 strain of *Staphylococcus aureus*. The MIC values of nisin and chloramphenicol were >64 and 32 µg/mL, respectively. The checkerboard assay was carried out for the inspection of synergism between nisin and chloramphenicol. Furthermore, the crystal violet assay was employed to assess antibiofilm effects. Additionally, the expressions of various virulence genes (*agrA, spa*, *icaA* and *saeR*) were investigated using the colony biofilm assay and qRT-PCR methods. In combination application, MIC values of nisin and chloramphenicol was decreased. In addition, biofilm formation was decreased.It was shown that the expression level of the *agrA* gene decreased compared to the control in the nisin, chloramphenicol and combinational applications. The expression levels of other genes were increased compared to the control. The results showed that the activities of nisin and chloramphenicol combinations had synergistic and antibiofilm activity. This study sheds light on the combinatorial use of older antibiotics.

Keywords- Staphylococcus Aureus, Combination, Antibiotic Resistance, Nisin, Chloramphenicol

ÖZ

Günümüzde antibiyotik direncinin hızla yayılması ve yeni antimikrobiyal ajanların keşfedilmesinin zorluğu nedeniyle eski antibiyotiklerin yeniden kullanımı ve kombinasyon stratejileri ön plana çıkmıştır. Bu çalışmada, *Staphylococcus aureus*'un ATCC 6538 suşuna karşı nisin ve kloramfenikolün kombinasyonel etkinliğini araştırdık. Nisin ve kloramfenikolün MİK değerleri sırasıyla >64 ve 32 µg/mL olarak bulundu. Nisin ve

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kloramfenikol arasındaki sinerjizmin incelenmesi için dama tahtası (Checkerboard) testi yapıldı. Ayrıca, antibiyofilm etkilerini değerlendirmek için kristal viole testi kullanıldı. Koloni biyofilm testi ve qRT-PCR yöntemleri kullanılarak çeşitli virülans genlerinin (*agrA,spa*, *icaA* ve *saeR*) ekspresyonları araştırıldı. Kombinasyonel uygulamada, nisin ve kloramfenikolün MİK değerlerinde azalma gözlendi. Ayrıca, kombinasyonel uygulamada, biyofilm oluşumu azalma gösterdi. Nisin, kloramfenikol ve kombinasyon uygulamalarında *agrA* geninin ekspresyon seviyesinin kontrole göre azaldığı gösterilmiştir. Diğer genlerin ekspresyon seviyeleri ise kontrole göre artış göstermiştir. Sonuçlarımız, nisin ve kloramfenikol kombinasyonlarının aktivitelerinin sinerjistik ve antibiyofilm aktiviteye sahip olduğunu göstermiştir. Bu çalışma, eski antibiyotiklerin kombinasyonel kullanımının önemine ışık tutmaktadır.

Anahtar Kelimeler- Staphylococcus Aureus, Kombinasyon, Antibiyotik Dirençliliği, Nisin, Kloramfenikol

I. INTRODUCTION

Nowadays, one of the most important health problems is infections caused by antibiotic resistance [1,2]. The genetic basis of antibiotic resistance involves mutations and mobile genetic elements. Mutations cause changes in antibiotic targets, lower cell membrane permeability and reduced drug accumulation. Mobile genetic elements give rise to the transfer of antibiotic resistance genes among bacteria. Resistance mechanisms in bacteria have evolved rapidly due to the misuse or overuse of antibiotics. Clinically, one of the rapidly growing antibiotic-resistant bacteria is *Staphylococcus aureus.* It was estimated to be responsible for over 148,000 infections and 7000 deaths in the European Union in 2015 [2]. Furthermore, its biofilm-forming capacity is quite high, and it is shown as a major cause of biofilm-associated infections [3]. Hence, the fight against multidrug-resistant *S. aureus* infections increases the need for new antimicrobial agents and new treatment strategies. One of the approaches used to combat antibiotic resistance is the use of combinational treatment approaches [4–6].It is common to use two antibiotics together to achieve synergistic effects. This way, old antibiotics can be reused to combat antibiotic resistance. Recently, combinations of traditional antibiotics and antimicrobial peptides (AMPs) called next-generation antibiotics have shown promising results to fight infections.

Nisin is an AMP belonging to the bacteriocins group produced from *Lactococcus lactis* [7]. It has an inhibitory effect against Gram-positive bacteria such as *S. aureus* and *Listeria monocytogenes*. It has also gained importance as a food preservative [8]. It creates a pore structure on the bacterial membrane. Moreover, it prevents cell wall synthesis by binding to the lipid II molecule [9]. Chloramphenicol is one of the most active broad-spectrum antibiotics. It inhibits protein synthesis by binding to the 50S subunit of the ribosome. Furthermore, it inhibits peptidyl transferase activity. However, chloramphenicol has serious side effects such as neurotoxicity and hematological disorders [10,11]. Nevertheless, its use is being reconsidered due to the increase in antibiotic resistance.

In this study, we confirmed that *S. aureus* ATCC 6538 is capable of forming strong biofilms under in vitro conditions, and therefore, we selected this strain for our study. So, we investigated the in vitro activities of nisin and chloramphenicol alone and their combinational effects on *S. aureus* ATCC 6538. Furthermore, we explored the effects of biofilm-related virulencegenes by gene analysis.

II. MATERIALS AND METHODS

A. Strain, Reagents and Antibiotics

The tested bacterial strain was *S. aureus* ATCC 6538. Nisin from *L. lactis* (2.5%; balance sodium chloride) and chloramphenicol (≥98%, HPLC) were purchased from Sigma Aldrich (St. Louis, MO). All other chemicals used in this study were of analytical grade.

B. Minimum Inhibitory Concentration (MIC) Assays

MICs for nisin and chloramphenicol were assigned by using the broth microdilution method according to a previous report [12]. First of all, bacterial culture (100 µL) with a turbidity of 0.5 McFarland standard and different concentrations of nisin and chloramphenicol (2-128 µg/mL) were inoculated in a 96-well plate. The plate was incubated at 37°C for 24 h. The no visible growth well was designated as MIC value.

C. Crystal Violet (CV) Assay

Bacterial culture (100 µL)and increasing concentrations of nisin and chloramphenicol (2-128 µg/mL) in the Mueller-Hinton Broth (MHB) medium were seeded into 96-well plates. The plate was incubated at 37°C for 48 h. After incubation, non-adhered bacterial cells were removed, and the plate was washed three times with PBS. The wells were then subjected to staining with 0.5%CV for 20 min, and each well was washed with PBS. Finally, each well was fixed with 30% acetic acid. OD was read at 590 nm with a spectrometer. In addition, light microscopy images were taken with antibiotic treatment at 1/2 MIC concentration [13].

D. Checkerboard Assay

The checkerboard assay was used to evaluate the synergism between nisin and chloramphenicol against *S. aureus* ATCC 6538[14]. Nisin and chloramphenicol solutions were added at increasing concentrations in the horizontal and vertical directions into a 96-well plate. 0.5 McFarland bacterial suspension (100 µL) was added to each well. The final volume was adjusted to 200 µL. The plates were incubated statically at 37°C for 24 h. After incubation, the fractional inhibitory concentration (FIC) value was calculated as follows:

$$\mathbf{\Sigma\ FIC} = \mathrm{FIC(NISIN)} + \mathrm{FIC(Chloramphenicol)} \quad (1)$$

$$\mathbf{FIC}(\mathrm{NISIN}) = \frac{\text{MIC of drug Nisin in the combination}}{\text{MIC of drug Nisin alone}} \quad (2)$$

$$\mathbf{FIC}(\mathrm{Chloramphenicol}) = \frac{\text{MIC of drug Chloramphenicol in the combination}}{\text{MIC of drug Chloramphenicol alone}} \quad (3)$$

E. Colony Biofilm Assay

For the colony biofilm assay, sterile polycarbonate membranes (Poretics 25-mm-diameter black polycarbonate membranes with a pore size of 0.22 µm, GE) were placed on Petri dishes with and without antibiotics. 0.5 McFarland bacterial suspension (10 µL) was seeded on the membranes. The Petri dishes were changed every 24 hours and incubated statically at 37°C for 48 h to form biofilms [15].

F. RNA isolation and cDNA synthesis

Total RNA from the biofilm samples were isolated with TRIzol reagent (Invitrogen, San Diego, CA) following the manufacturer's guidelines. The concentration and purity of the RNAs were assessed with spectrophotometry using a Multiskan GO (Thermo Fisher Scientific, Inc., Wilmington, DE, USA). 300 ng of RNA was reverse-transcribed using a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems™).

G. Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

The expression levels of *agrA, icaA, saeR* and *spa* were analyzed with qRT-PCR using "HOT FIREPol® EvaGreen® qPCR Mix Plus" (Solis BioDyne, Estonia). The primers were designed using the Primer3 program (https://bioinfo.ut.ee/primer3-0.4.0/) andareshown in Table 1. The GyrB gene, encoding the subunit B of DNA gyrase, was used as the housekeeping gene. The reactions were facilitated with Rotor-Gene (Qiagen, Düsseldorf, Germany) using standard parameters. The expression levels of the genes were determined using the $2^{-(\Delta\Delta Ct)}$ method.

Table 1. Primer sequences used in the study

Gene Name	Primer Sequence
AgrA	F: 5'-CCTATGGAAATTGCCCTCGC-3' R: 5'-CCAACTGGGTCATGCTTACG-3'
Spa	F:5'-GCGTAACACCTGCTGCAAAT-3' R: 5'-AGAAGGCGCTTTGTTGATCT-3'
Ica A	F: 5'-ACACTTGCTGGCGCAGTCAA-3' R: 5'-TCTGGAACCAACATCCAACA-3'
Sae R	F: 5'-GTAACAACGACAACTAGCGGT-3' R: 5'-CGAGTTCCCTTGGACTAAATGG-3'
gyr B	F: 5'-ATATTGCACAGCCACCGTTG-3' R: 5'-CCGCTTCAATCGCATCTTCA-3'

H. Statistical Analysis

The results were examined by using a one-way analysis of variance (ANOVA) test with three independent replicates. $P<0.05$ was regarded as statistically significant.

III. RESULTS

A. Antimicrobial and Antibiofilm Activity

In this study, we investigated the effects of chloramphenicol, nisin and their combinations against *S. aureus* (ATCC 6538). The MIC values of chloramphenicol and nisin were 32 µg/mL and >64 µg/mL, respectively. Thus, chloramphenicol had a higher-level antimicrobial effect than nisin against *S. aureus* (ATCC 6538). Nisin and chloramphenicol showed antibiofilm activity at increasing concentrations (Fig.1).

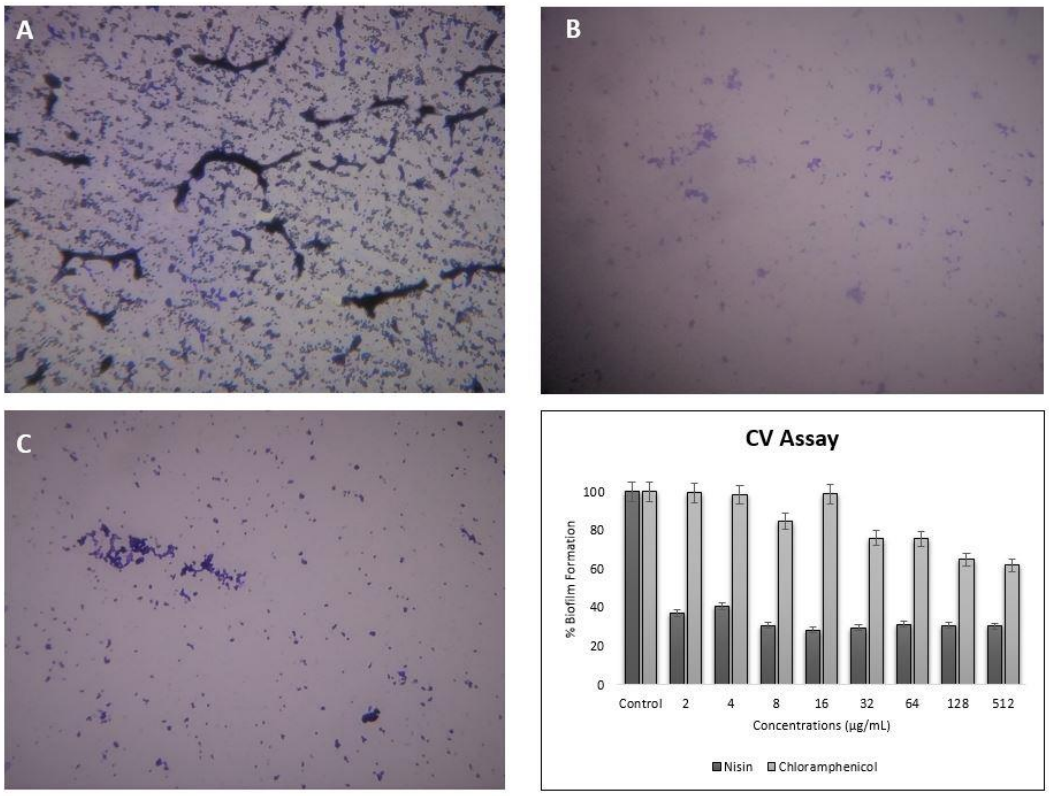

Figure 1. Light microscopic images of crystal violet stained *S. aureus* (ATCC 6538) biofilm. A. Control B. ½ MIC concentration of nisin treatment C. ½ MIC concentration of chloramphenicol treatment. D. Antibiofilm activity of nisin and chloramphenicol

B. Checkerboard Assay

The combined effects of chloramphenicol and nisin were calculated by the FIC index. The MIC value of chloramphenicol was determined as 8 µg/mL in combination. On the other hand, the MIC value of nisin was calculated as 5 µg/mL in combination. The FIC index calculated with these results was 0.32. A FIC index value of less than 0.5 (Σ FIC<0.5) indicates a synergistic interaction between two antimicrobial agents. Therefore, the combination of nisin and chloramphenicol showed a synergistic effect against *S. aureus* (ATCC 6538).

C. Gene Expression Analysis

To evaluate the expression levels of biofilm-related genes, the ½ MIC values of nisin, chloramphenicol and their combination were applied in the colony biofilm assay. The total RNA obtained from the biofilm sample was extracted, and the expressions of the *agrA, spa, icaA* and *saeR* genes were determined by qRT-PCR. The total RNA of the untreated biofilm sample was used as the control, and the *gyrB* gene was used to normalize the expression results of the virulence genes.

As shown in Fig. 2a, the analysis of *agrA* expression levels in the treatments with nisin alone, chloramphenicol alone and their combination demonstrated that the expression of this gene decreased significantly compared to the control group. However, it was found that the expressions of the *icaA, saeR* and *spa* genes were upregulated in all groups compared to the control (Figure 2b-d).

The expressions of the *spa* (Figure 2c) and *saeR* (Figure 2d) genes were upregulated 16- and 30-fold in the chloramphenicol application, respectively. In the combination of the two agents, the expression of *agrA* was significantly reduced compared to the control group (Figure 2a). However, the expressions of the *icaA, saeR* and *spa*genes were found to be significantly upregulated in response to this combination, most notably in the case of the *icaA* gene, whose expression was upregulated 820-fold. The expressions of *Spa* and *saeR* were upregulated 108 and 29-fold, respectively.

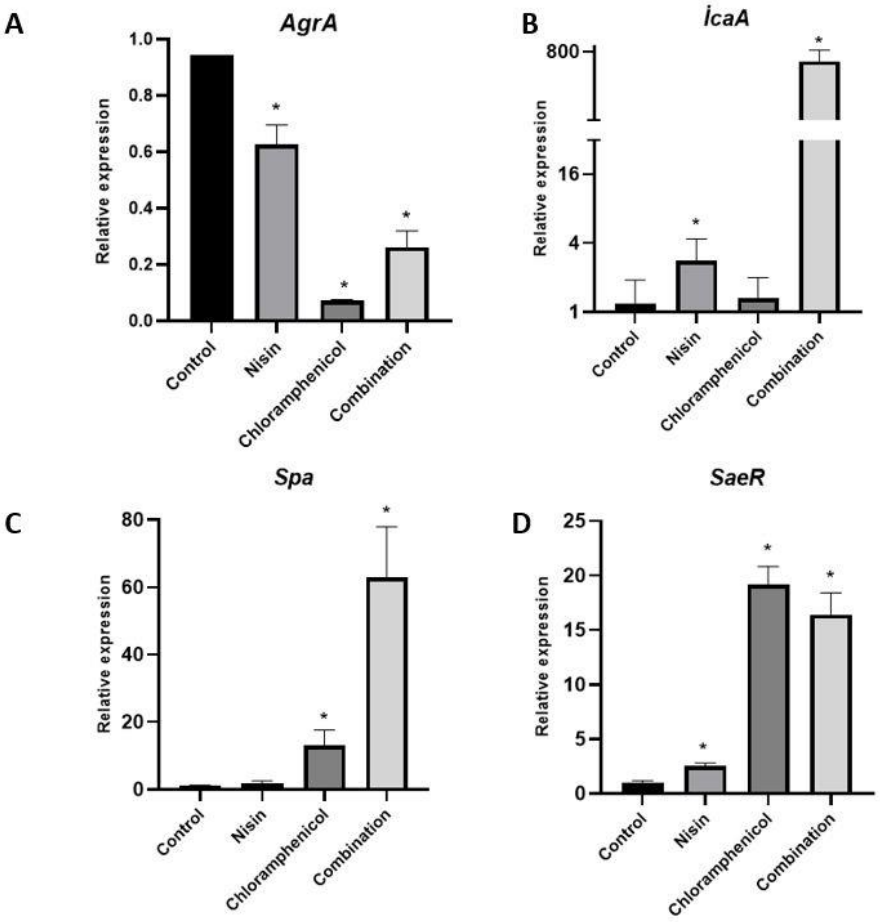

Figure 2. qRT-PCR analysis of *agrA, spa, icaA* and *saeR* which were significantly differentially expressed in control. Data were normalized to the *GyrB* gene, encoding the subunit B of DNA gyrase.Expression and fold-change was calculated by the $2^{(-\Delta\Delta CT)}$ method. *p < 0.05

IV. DISCUSSION

Infections caused by *S. aureus* are a serious problem worldwide. They lead to various severe infections, such as pneumonia, toxic shock syndrome, bacteremia, soft tissue and bloodstream infections in which bacterial virulence genes and toxins are significant mediators [16]. Antibiotic combinations may offer solutions to antibiotic resistance. Going forward, there is growing interest in the combination of antibiotics and antimicrobial peptides as a means of preventing infection.

S. aureus ATCC6538 is a clinical isolate, and it is used for infectious disease research and quality control. It also forms a strong biofilm under in vitro conditions [17,18]. Nisin which is an antimicrobial peptide and chloramphenicol is a protein synthesis inhibitor have been used in previous studies [19,20]. In this study, the interaction of nisin and chloramphenicol against this strain was assessed by using the checkerboard assay. The unit

of calculation in this test is the FIC index, which is used to evaluate the presence of synergistic, additive and antagonistic interactions of two antimicrobial agents. If the FIC index is less than 0.5 (Σ FIC<0.5), there is a synergistic interaction between the two antimicrobial agents. If the FIC index is between 0.5 and 4 ($0.5 \leq \Sigma$ FIC $\leq$ 4), there is an additive effect. If the FIC index is greater than 4 (Σ FIC>4), there is an antagonistic effect [14,21].With this evaluation, the effects that may occur when antimicrobial drugs are used together are tested under in vitro conditions. Brumfitt et al. (2002) demonstrated the interaction between nisin and chloramphenicol with 20 clinical *S. aureus* isolates. They found antagonistic interactions in 18 isolates [22]. In our study, a synergistic interaction was observed between nisin and chloramphenicol in the reference strain we used. One possible reason is that different strains show distinct responses to antibiotics and their combinations. Additionally, the study by Tong et al. (2014) showed that nisin and chloramphenicol exert a synergistic effect against *Enterococcus faecalis* OG1RF, ATCC 29212, and strain E [23].

Biofilms are microbial communities that consist of the extracellular matrix, polysaccharides, extracellular DNA (e-DNA), and proteins. Microorganisms within a biofilm structure show different gene expression patterns in comparison to planktonic cells [24]. The biofilm-producing *S. aureus* generally causes chronic infections due to its ability to resist antibiotics [25]. We investigated the expressions of virulence genes in *S. aureus* (ATCC 6538) using the colony biofilm assay involving incubation with sub-MICs of nisin, chloramphenicol and their combinations. In our gene expression analysis, first, we created biofilm layers with the colony biofilm assay. It is a static method that allows the examination of the biofilm structure and components [15]. Furthermore, it determines the bacteria killing effects of the tested antimicrobial agents. The development of bacterial cells on a semipermeable membrane is provided. Bacteria growing on the membrane are taken onto the new medium at certain periods [15]. Therefore, the ability of cells to detach, migrate and get away from the biofilm is limited. This method is frequently preferred in studies carried out on antibiotic resistance and biofilm-related resistance.

In this study, the relative expression levels of *agrA, spa, icaA* and *saeR* were determined via qRT-PCR. The accessory gene regulator (Agr*)* operon is a global regulator associated with quorum sensing which is a cell-to-cell communication system. The Agr system has been shown to regulate the expression of a variety of virulence factors in *S. aureus*, such as delta-hemolysin toxin (*hld*), alpha toxin (*hla*), serin protease (*SplA-F*), and surface protein A (*Spa*) [26-28]. Agr operonactivity has been shown to be particularly important in skin and soft tissue infections [29]. The decrease in the expression level of *agrA* compared to the untreated group indicated that it reduces biofilm formation and facilitates the dispersal of the established biofilm (Fig.2a). Staphylococcal protein A (*spa*) is another significant virulence factor that especially causes pneumonia, bloodstream infections and septic arthritis [26]. *Spa* is downregulated by RNAIII which is an Agr-dependent regulator [30]. Polysaccharide intercellular adhesin (PIA), which is important in biofilm formation, is encoded by the ica operon [31].*IcaA* is a transmembrane protein which requires the *icaD* protein [31]. Interestingly, the expression levels of the *spa, icaA,* and *saeR* genes were upregulated in all applications. The production of virulence factors in *S. aureus* occurs by complex pathways. Additionally, antimicrobial applications at sub-MIC concentrations may cause bacterial resistance.

It has been reported that ica operongenes are not always required in biofilm formation, and antibiotics that affect the protein synthesis pathway do not have much effect on biofilm formation. For example, tetracyclines and streptogramin have been shown to increase the expression level of the *icaA* gene in *Staphylococcus epidermidis* [32]. However, chloramphenicol does not induce *icaA* expression changes in *S. epidermidis*. Similarly, in our study, it was found that the chloramphenicol and nisin applications increased the expression levels of the *ica A* gene. More information is needed on antimicrobial agent combinations and their effects on virulence genes to fight antibiotic resistance.

V. CONCLUSION

In conclusion, we assessed the effects of nisin and chloramphenicol alone and in combination. It was determined that nisin and chloramphenicol had a synergistic effect against the ATCC 6538 strain of *S. aureus* under in vitro conditions. Nisin can be used in antimicrobial therapy to enhance the antibiofilm effect of chloramphenicol. To test these results, similar studies should be conducted with different isolates of *S. aureus*.

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