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Antibacterial Sensitivity Changes in Mixed Species Biofilm of Oral *Streptococci* against Chlorhexidine and Cetylpyridinium Chloride

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Authors' contributions

This work was carried out in collaboration between both authors. Author So Yeon Lee performed the experiments and wrote the first draft of the manuscript. Author Si Young Lee designed the study, managed the analyses of the study. Both authors read and approved the final manuscript.

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ABSTRACT

Background and Objectives: Although plaques are present in a mixed state of various bacterial species, it is not yet known how antimicrobial efficacy of mixed bacteria against the antimicrobial agent changes compared to susceptibility of individual bacteria to the antimicrobial agent. In this study, antibacterial effects of chlorhexidine and cetylpyridinium chloride were observed in biofilm state after mixing two bacteria to observe the change of susceptibility value when bacteria were mixed with each other.
Materials and Methods: The minimum biofilm inhibitory concentration (MBIC) and the minimum biofilm eradiation concentration (MBEC) were determined to determine the susceptibility of the antimicrobial agent after the formation of the biofilm, and were determined by microtiter plate method according to the previously reported method.
Results: When two bacteria with different susceptibility values were mixed, it was observed that most of the combinations had higher values of susceptibility values of the two bacteria. However, in some bacterial combinations, susceptibility values of the two bacteria revealed various results such

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as following the low value, lower than the low value, or higher than the high value.
Conclusion: This study has demonstrated that antimicrobial susceptibility to mixed bacteria can change in a variety of ways without simply following high values.

Keywords: Biofilm; cetylpyridinium chloride; chlorhexidine; minimum biofilm eradiation concentration; viridans streptococci.

1. INTRODUCTION

There are many kinds of bacteria in dental plaque [1,2]. These bacteria interact with each other in a variety of ways, including using metabolites produced by other species or releasing specific molecules [1]. *Streptococci* are known as the early clusters of oral biofilms [3]. Metabolism of *streptococci* in biofilm state is not the same as that of planktonic state. In addition, it has been reported that biofilms containing various bacterial species have different metabolism including growth and acid production as compared with biofilms of single bacterium [1].

Although plaques are present in a mixed state of various bacterial species, it is not yet known how antimicrobial efficacy is changed by comparing the mixed state of bacteria with susceptibility of individual bacteria to the antimicrobial agent. When bacteria are mixed in comparison with a bacterium, interaction between bacteria including the metabolism of the bacterium is inevitably changed. Studies on the effect of antimicrobial agents on one species of bacteria have been reported, but studies on the susceptibility changes of antimicrobial agents on mixed bacteria are lacking. In some studies, there have been studies of resistance to antimicrobial agents by mixing two different oral bacteria with different metabolic processes [1], but no studies have examined susceptibility by mixing *streptococci* belonging to the same genus.

Various antimicrobial agents included in mouthwashes and toothpastes are used to prevent oral diseases [4]. In general, chlorhexidine and cetylpyridinium chloride, used in dentistry, are effective antimicrobial agents [4-6].

When these antibacterial agents are applied to bacteria, the method used to determine sensitivity of the bacteria to the antimicrobial agent is to determine minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC). The MIC of the antimicrobial agent refers to minimum concentration of the antimicrobial agent inhibiting growth of bacteria, and MBC refers to minimum concentration at which the bacteria are killed by 99.9% [7]. However, since MIC and MBC are ineffective for application to bacterial biofilms [8,9], it is necessary to confirm susceptibility of the antibacterial agent to biofilm bacteria. Recently, a method of measuring a minimum biofilm inhibitory concentration (MBIC) and minimum biofilm eradication concentration (MBEC) has been used to evaluate activity of an antimicrobial agent against a biofilm [10-12]. The MBIC of the antimicrobial agent is defined as the minimum concentration at which growth of biofilm bacteria is inhibited, and the MBEC is minimum concentration at which 99.9% of biofilm bacteria are eradicated [8,10].

In this study, we selected bacterium with various MBIC values. Then, changes of susceptibility for chlorhexidine and cetylpyridinium chloride to biofilm bacteria were investigated by mixing each of the bacteria in various combinations.

2. MATERIALS AND METHODS

2.1 Selection of Bacteria

From the bacterial stock list of isolated oral streptococcal strains of Department of Oral Microbiology, Gangneung-Wonju National University, strains were selected for mixing bacteria in this experiment. Bacterial strains with various MBIC (Fig. 1) were selected. The selected bacteria were mixed with two strains in various combinations.

2.2 Determination of MBIC and MBEC of Antimicrobials against Mixed Bacteria

Chlorhexidine (Sigma-Aldrich Chemical Co., St. Louis, MO, USA) and cetylpyridinium chloride (Sigma-Aldrich Chemical Co.) were used and diluted in Brain Heart Infusion (BHI) broth (Becton, Dickinson and Company, Sparks, MD, USA) to a concentration of 1000 μg/ml. The MBIC and the MBEC were measured to observe

susceptibility of mixed bacteria to the antimicrobial agent after biofilm formation. Sensitivity to antimicrobials after biofilm formation was measured by the previously reported method [8,10,13]. To mix the bacteria, concentration of each bacterial suspension was adjusted to 0.5 McFarland (1×10^8 CFU/ml), and the mixture was used so that combined concentration of the two bacteria was 5×10^5 cells/ml. To form biofilm, 180 µl of BHI medium was added to a 96-well plate (SPL), 20 µl of the turbidity-adjusted bacterial suspension was added, and the plate was incubated for 18 hours at 37℃ in a 5% CO_2 incubator. After incubation, cells were washed 3 times with BHI medium to remove unattached bacteria. In a new microplate, the antimicrobial agent was serially diluted in BHI medium, and the diluted solution was transferred to a biofilm-attached microplate and incubated at 37 ℃ for 18 hours. After incubation, turbidity was observed with naked eyes, and minimum concentration at which the growth of biofilm bacteria was inhibited was determined as MBIC. After MBIC determination, the biofilm was re-cultured in a medium containing no antimicrobial agent. Turbidity of the supernatant was visually observed to determine minimum concentration at which the bacteria no longer grow (MBEC). All experiments were repeated twice. When results were inconsistent, the experiments were repeated, and values revealing the same value twice or more were determined as MBIC and MBEC.

3. RESULTS

The results for mixed biofilm were divided into 5 groups.

a. Group 1: The susceptibility values of the mixed two bacteria follow a high value of two individual bacteria.
b. Group 2: The susceptibility values of the two bacteria follow a low value.
c. Group 3: Following the mean value of the susceptibility values of the two bacteria.
d. Group 4: Lower than the low value of the two bacteria.
e. Group 5: Higher than the high value of the two bacteria.

Figs. 1 and 2 reveal the MBIC and MBEC values for biofilms formed by mixed strains. The highest proportion in the MBIC results for chlorhexidine (Fig. 1) was Group 1 with a high value of the susceptibility values of the two bacteria. There were combinations that revealed interesting results in MBIC results. The difference in MBIC values between S. mutans KN615 and S. salivarius KN292 was 8 times. However, when they were mixed, low value of the MBIC value of the two bacteria was followed. In MBEC results of chlorhexidine (Fig. 2), Group 1 which revealed high value of the susceptibility values was mostly observed.

MBIC and MBEC results of cetylpyridinium chloride were observed in many combinations in various groups. MBIC results revealed Group 3 which represents average value of susceptibility values of two bacteria the most frequently. In MBEC results (Fig. 2), Group 4, which followed low value was the most. In some combination of cetylpyridinium chloride, MBIC results revealed unusual results. S. mitis KN506 and S. mutans KN529 had the same MBIC value, but the MBIC value increased 4 times higher when two bacteria were mixed. MBIC values of S. mitis KN509 and S. oralis KN515 were the same, but MBIC value decreased 4 times lower than MBIC values of individual bacteria when mixed.

4. DISCUSSION

Antibacterial effects of chlorhexidine and cetylpyridinium chloride on oral bacteria have been well known [5,6,14]. Chlorhexidine has long been used as a control agent for dental caries and periodontal diseases [15,16], and is effective in reducing plaque and inhibiting oral microorganisms including *Streptococcus mutans* [17,18]. Chlorhexidine molecules react with negative charge on the surface of bacterial cells and damage cytoplasm and cell membranes [19]. Ammonium salts such as cetylpyridinium chloride are safe and effective amphipathic compounds and have antimicrobial activity in conjunction with negatively charged bacterial surfaces [20]. Cetylpyridinium chloride decomposes lipid bilayers in cell membranes, to induce cell contents leakage, and to inhibit extracellular enzymes that synthesize polysaccharides [21,22].

It is well known that bacteria that persist on biofilm exhibit a wide range of properties, including increased resistance to antimicrobial agents, unlike planktonic bacteria [23]. However, no studies have confirmed sensitivity changes when compared to individual bacteria and mixed bacteria. When two bacteria with different susceptibility values are mixed, the sensitivity value of the two mixed bacteria is thought to follow the high value of two bacteria. In this study, MBIC and MBEC results for chlorhexidine follow

the high values of the two bacterial susceptibility values in the biofilm state. MBEC results of cetylpyridinium chloride also revealed tendency to follow high value or higher values than those of the two bacterial susceptibility values. However, in MBIC results, various observations such as following a low value or revealing a median value were observed besides following high value. The reason why the susceptibility value was changed to lower or higher when the bacteria are mixed is unclear. Although chlorhexidine and cetylpyridinium chloride are antimicrobial agents that have similar actions to bacteria, changes of sensitivity values after bacterial mixing were different in the two antimicrobial agents.

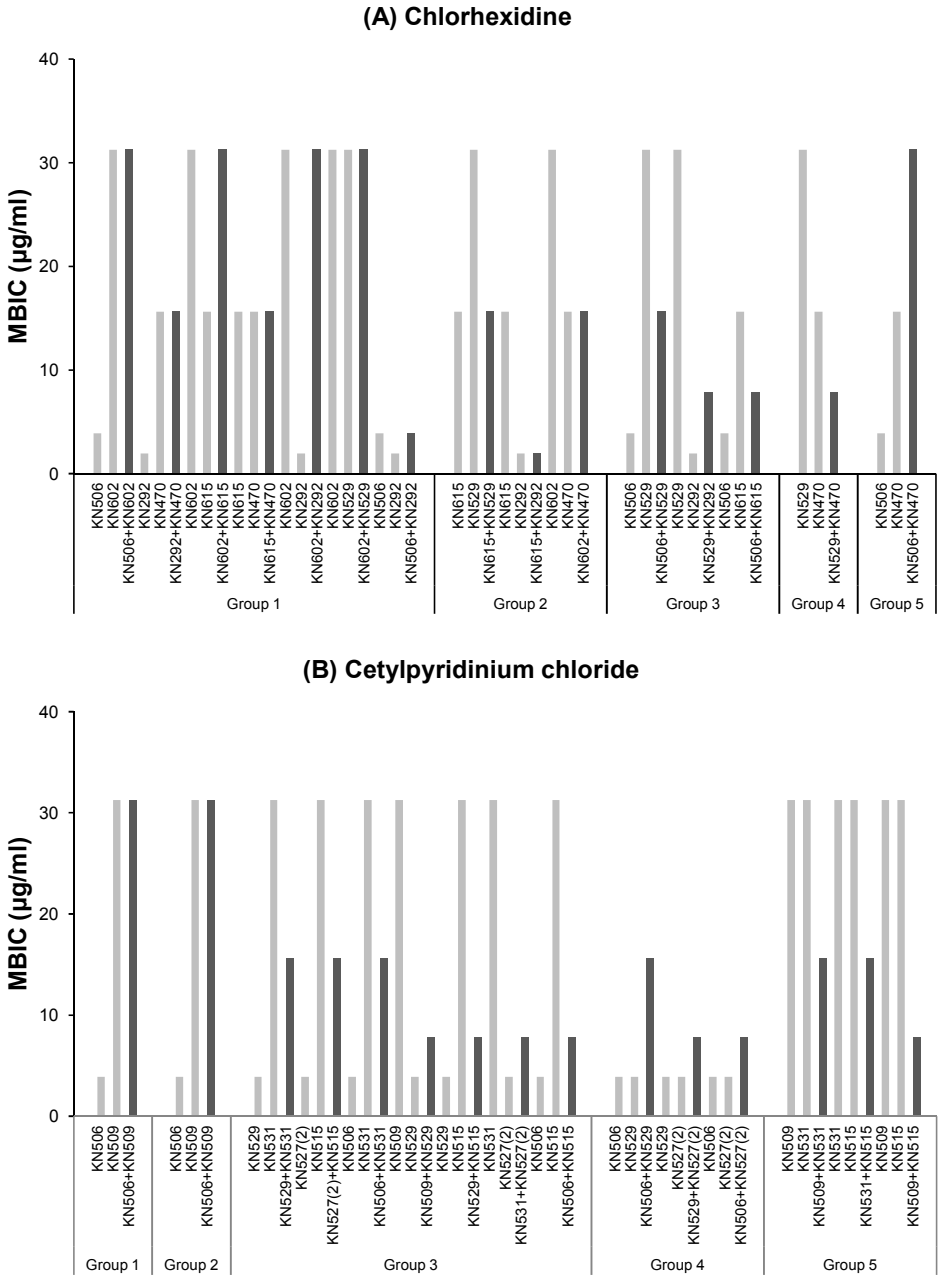

Fig 1. MBIC values before and after bacterial mix against chlorhexidine(A) and cetylpyridinium chloride(B)

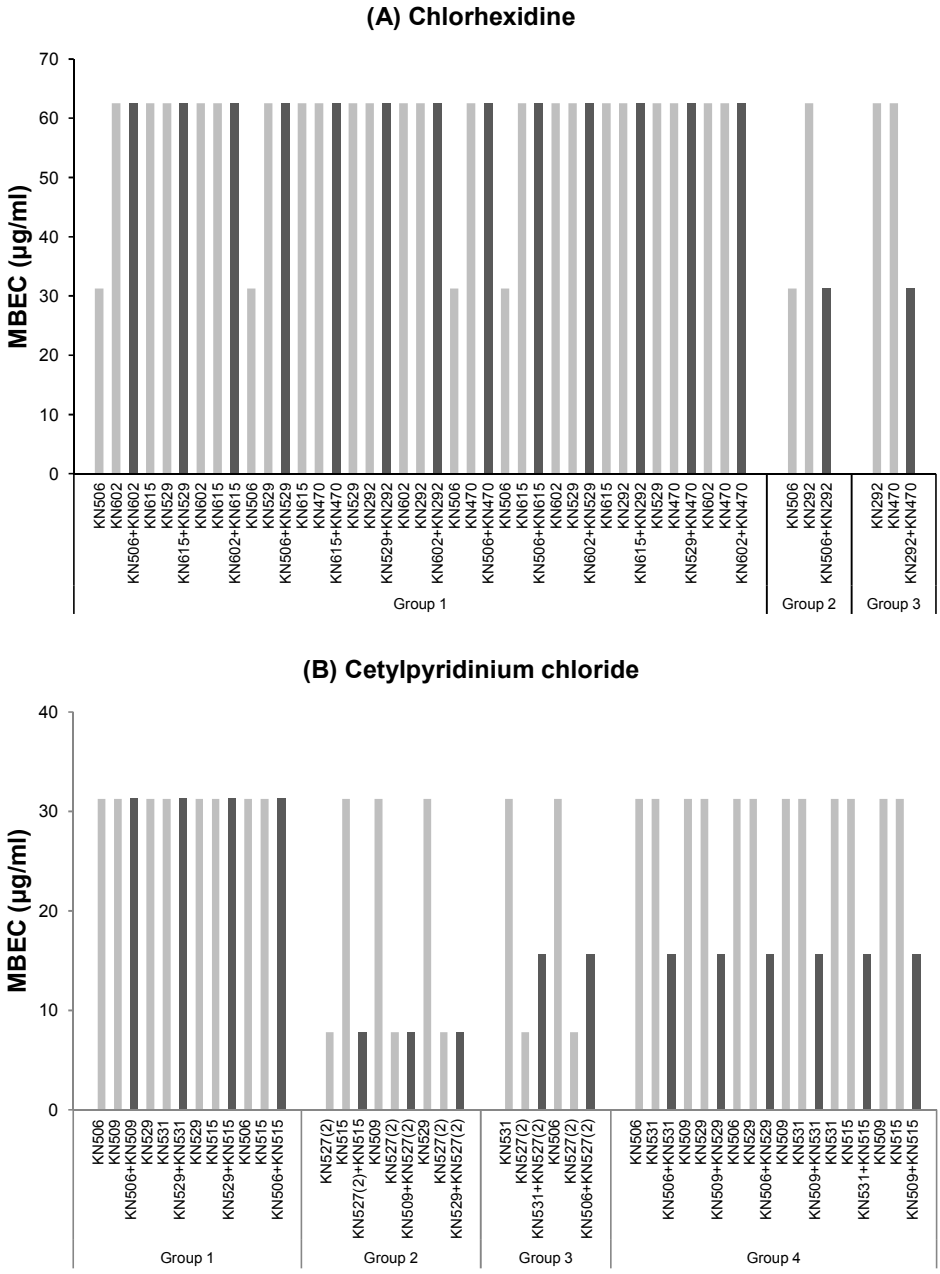

Fig. 2. MBEC values before and after bacterial mix against chlorhexidine(A) and cetylpyridinium chloride(B)

When an antimicrobial agent is applied to a biofilm, various factors such as growth rate, exopolysaccharide matrix, gene expression and resistance gene production may be changed [24]. The unusual results observed in some combinations reveal the possibility of various factors including metabolism and genetic changes of each bacterium when forming biofilm, but it should be revealed through further studies. In this study, we demonstrated that antibacterial susceptibility of mixed bacteria did not simply follow the high value and may change in various ways. Unlike in vitro, in vivo can produce different results for various reasons, such as the environment or nutrient supply. In this experiment, two kinds of bacteria were mixed to form a biofilm. However, since many kinds of bacteria are mixed in the oral cavity, it is

necessary to confirm the sensitivity by mixing various bacteria. Despite these limitations, MBIC and MBEC will help determine the concentration of chlorhexidine and cetylpyridinium chloride in the mouthwash.

5. CONCLUSION

This study has shown that antimicrobial susceptibility to mixed bacteria does not follow a high value among the two bacteria but can vary in a variety of ways. In addition, when the susceptibility to two antimicrobials having similar functions was examined, the two antimicrobials showed different sensitivity susceptibility results.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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