Journal of Advances in Microbiology

9(2): 1-6, 2018; Article no.JAMB.39738
ISSN: 2456-7116

Effect of Sub Minimal Inhibitory Concentration Cetylpyridinium Chloride on Biofilm Formation and Hydropyobicity of Streptococci and Actinomycetes

So Yeon Lee[1] and Si Young Lee[1*]

[1]Department of Oral Microbiology, Research Institute of Oral Science, College of Dentistry, Gangneung-Wonju National University, Gangneung, 210-702, Korea.

Authors' contributions

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

Article Information

DOI: 10.9734/JAMB/2018/39738
Editor(s):
(1) Simone Aquino, Professor, Universidade Nove de Julho, São Paulo, Brazil.
Reviewers:
(1) Elisabetta Blasi, University of Modena and Reggio Emilia, Italy.
(2) Kalima Nzanzu Adelard, Catholic University of Graben and Ruwenzory Official University, Democratic Republic of Congo.
(3) Alba E. Vega, Universidad Nacional de San Luis, Argentina.
Complete Peer review History: http://www.sciencedomain.org/review-history/23666

Original Research Article

Received 5th January 2018
Accepted 10th March 2018
Published 15th March 2018

ABSTRACT

Background and Objectives: If the antimicrobial agent is not administered continuously, the concentration of the antimicrobial agent necessarily becomes lower than the minimum inhibitory concentration (MIC) and maintains the sub- minimum inhibitory concentration (sub-MIC). There were no studies on the biofilm formation of initial attachment bacteria such as streptococci and actinomyces in cetylpyridinium chloride. In this study, we investigated the effect of cetylpyridinium chloride of sub-MIC on biofilm formation and bacterial cell hydrophobicity using *Streptococcus mutans, Streptococcus gordonii, Actinomyces naeslundii* and *Actinomyces odontolyticus*.
Materials and Methods: The degree of biofilm formation was classified into 1-4 according to the degree of adhesion to glass slip. Hydrophobicity was measured to investigate whether differences in biofilm formation were related to differences in bacterial surface hydrophobicity.
Results: When biofilm formation was compared between the control group without cetylpyridinium chloride and the cetylpyridinium chloride sub-MIC, biofilm formation was decreased in the experimental group of *S. mutans* and *A. naeslundii*. Cellular hydrophobicity was observed with regard to adhesion and the hydrophobicity of *S. mutans* and *A. naeslundii* decreased in the

**Corresponding author: E-mail: dlthdus8031@naver.com*

experimental group compared to the control group. However, *S. gordonii* did not change biofilm formation, but cell hydrophobicity was significantly increased in the experimental group compared to the control group.
Conclusion: When cetylpyridinium chloride was applied to oral bacteria, there was a correlation between biofilm formation and cell hydrophobicity in some bacteria.

Keywords: Biofilm; cetylpyridinium chloride; hydrophobicity; oral bacteria; sub-mic.

1. INTRODUCTION

Dental plaque is a major cause of dental diseases. Removal of dental plaque can significantly reduce dental disease, but mechanical plaque removal is limited. Supplemental use of antimicrobials is effective in controlling and removing plaque accumulation [1]. Cetylpyridinium chloride is included a variety of mouthwashes and toothpaste, it is often used in dentistry to control plaque formation and prevent occurrence of oral disease [2,3]. Positively charged cetylpyridinium chloride has antibacterial activity by binding to negative charge bacteria [4,5], degrading lipid bilayer of the cell membrane, and inducing leakage of cell contents [6].

When these antibacterial agents are applied to the oral cavity, minimum inhibitory concentration (MIC) is measured to determine sensitivity of oral bacteria to the antibacterial agent. Minimum inhibitory concentration (MIC) of the antimicrobial agent is the minimum concentration of antimicrobial agent that inhibits bacterial growth. Concentration of antimicrobial agent is maintained at higher concentration than MIC only for a certain period after administration. If the antimicrobial agent is not continuously administered, concentration of the antimicrobial agent is inevitably lower than the MIC, referred to as a sub-minimum inhibitory concentration (sub-MIC) [7,8]. Sub-MICs cannot kill germs, but they interfere with metabolism of bacteria and affect various properties including morphological, biochemical, and attachment characteristics, and are therefore reported to lead to changes in bacterial virulence factors [7,9-13].

Several studies have reported that sub-MICs of antibiotics can effectively inhibit biofilm formation, and bacteria grown in sub-MICs of antibiotics have shown that bacterial aggregation is reduced [14,15]. Cell hydrophobicity is known to be closely related to bacterial adhesion, and bacteria with high cellular hydrophobicity have been reported to attach to the acquired pellicle, epithelium or prosthetic dentures of teeth [16-18]. Therefore, in this study, we investigated the effect of sub-MIC cetylpyridinium chloride on the biofilm formation and cellular hydrophobicity of *Streptococcus mutans*, *Streptococcus gordonii*, *Actinomyces naeslundii*, and *Actinomyces odontolyticus*.

2. MATERIALS AND METHODS

2.1 Bacteria and Culture Conditions

In this study, *S. gordonii* KN1, *S. mutans* KN88, *A. naeslundii* KN543 and *A. odontolyticus* KN544 were used. Bacteria were cultured for 18 hours in a 5% CO_2 incubator at 37℃ using Brain Heart Infusion (BHI, Becton, Dickinson and Company, Sparks, MD, USA). Turbidity of the bacteria was measured at 660 nm (OD_{660}) using a spectrophotometer, and the number of bacteria was determined by applying to a predetermined standard curve.

2.2 Measurement of Minimal Inhibitory Concentration (MIC)

The MIC of cetylpyridinium chloride was determined by liquid medium dilution method according to guidelines of the Clinical and Laboratory Standards Institute (CLSI) [19]. The MIC was a two-fold serial macro-dilution method. Cetylpyridinium chloride at a concentration of 1000 μg / ml was continuously diluted with Brain Heart Infusion (BHI) broth (Becton, Dickinson and Company, Sparks, MD, USA) at a ratio of 1/2. Bacteria were incubated to a final concentration of 5×10^5 cells/ml and cultured in a 5% CO_2 incubator at 37℃ for 18 hours. After incubation, concentration at which bacterial growth was inhibited was visually determined by MIC.

2.3 Biofilm Formation Assay

Biofilm formation was assessed by modifying previously reported experimental methods [20]. Bacteria were cultured in a CO_2 incubator for 18 hours, and then absorbance was measured at

OD_{660} using a spectrophotometer. The suspension was diluted with BHI broth to a concentration 1 x 10^9 CFU/ml. In a 12-well plate (SPL Life Sciences, Pocheon-si, Gyeonggi-do, Korea) containing a sterile glass slip (round, 12 mm diameter), 4 ml of the culture medium and 25 µl of prepared bacterial suspension were added and cultured in a CO_2 incubator for 18 hours. After incubation, the glass slip on which the biofilm was formed was washed with phosphate-buffered saline (PBS, pH 7.2) to remove bacteria that did not adhere to the glass slip. Biofilm formation was classified into 1-4 points according to the degree of adhesion to the glass slip, and criteria are revealed in Table 2 below. To investigate the effect of cetylpyridinium chloride on the formation of biofilm, bacteria were cultured in a culture medium containing cetylpyridinium chloride of sub-MIC and conducted in the same method as above.

2.4 Bacterial Cell Surface Hydrophobicity Assay

To investigate if the difference in biofilm formation degree is related to the difference in hydrophobicity of the bacterial surface, hydrophobicity was measured using the previously reported method [21]. Bacterial cell surface hydrophobicity was determined by the degree of adsorption of bacteria to n-hexadecane. Bacteria cultured in sub-MIC cetylpyridinium chloride were centrifuged at 10,000 xg for 5 minutes, washed with PUM buffer [22], suspended in the same buffer, and adjusted to an absorbance of 1 × 10^9 cells/ml at OD_{660}. 2 ml of the bacterial suspension were added to the glass tube (13 mm), 400 µl of n-hexadecane (Sigma chemicals Co., St. Louis, MO, USA) were added, and then vigorously vortexed for 1 minute. After the mixed solution was at room temperature for 15 minutes, absorbance of the solution excluding the supernatant was measured at OD_{550} using a spectrophotometer.

Bacterial cell hydrophobicity was calculated by applying the following formula. % HP = [OD(initial)-OD(expt)] × 100 / OD(initial); OD (expt) refers to the OD_{550} value measured after adding n-hexadecane and incubating for 15 minutes.

2.5 Statistical Analysis

Independent sample t-test was used to determine the difference in hydrophobicity between sub-MIC cetylpyridinium chloride treated groups and untreated groups for each bacterium ($p < 0.05$). Statistical analysis was conducted using Software Package for Social Sciences (SPSS, version 21, IBM Inc., USA).

3. RESULTS

3.1 Minimum Inhibitory Concentration (Mic)

Minimum inhibitory concentrations of cetylpyridinium chloride are revealed in Table 1 below. The sub-MIC concentration of cetylpyridinium chloride used in the experiment was 1/2 of the MIC.

Table 1. Minimal Inhibitory Concentrations Of Cetylpyridinium Chloride

	MIC (µg/ml)
S. gordonii	0.2441
S. mutans	0.2441
A. naeslundii	0.1221
A. odontolyticus	0.1221

3.2 Biofilm Formation Assay

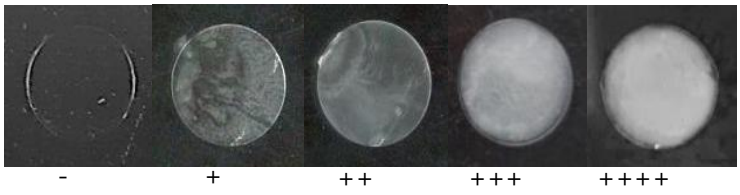
Each bacterial strain without the addition of cetylpyridinium chloride revealed different biofilm formation patterns (Table 2). *S. gordonii* and *A. odontolyticus* revealed no difference in biofilm formation between the group without cetylpyridinium chloride and the group cultured with cetylpyridinium chloride sub-MIC. However, when the sub-MIC of cetylpyridinium chloride was added in *S. mutans* and *A. naeslundii*, the biofilm formation pattern was changed. *S. mutans* cultured in the presence of cetylpyridinium chloride of the sub-MIC revealed decrease in the amount of biofilm from +3 to +2 as compared with the control. Similarly, when cultured in the presence of sub-MIC of cetylpyridinium chloride, *A. naeslundii* decreased biofilm formation from +4 to +2 as compared to the control.

3.3 Bacterial Cell Surface Hydrophobicity Assay

The difference in hydrophobicity between each experimental strain is shown in Fig. 1. When compared to the control group without cetylpyridinium chloride, *A. naeslundii* revealed high hydrophobicity of 87%, *S. gordonii* (approximately 60%), *S. mutans* and

Table 2. The effect of sub-mic cetylpyridinium chloride on biofilm formation

	No cetylpyridinium chloride	Cetylpyridinium chloride
S. gordonii	++	++
S. mutans	+++	++
A. naeslundii	++++	++
A. odontolyticus	+	+



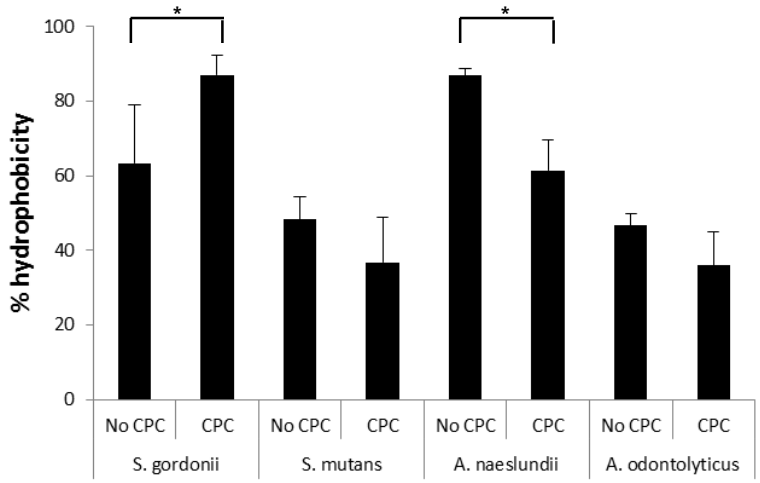


Fig. 1. The effect of sub-MIC cetylpyridinium chloride on hydrophobicity of *S. gordonii, S. mutans, A. naeslundii* and *A. odontolyticus*. The bacteria were cultured in the sub-MIC cetylpyridinium chloride and hydrophobicity was measured. Values indicate means of three experiments; standard deviations of the mean (error bars) are indicated by vertical lines.
**, $p < 0.05$ (CPC = cetylpyridinium chloride)*

A. odontolyticus (approximately 50%) revealed relatively low hydrophobicity. When cetylpyridinium chloride of sub-MIC was added, *S. mutans* and *A. odontolyticus* revealed no significant difference in hydrophobicity compared to the control group. However, *S. gordonii* increased hydrophobicity to approximately 87% in the experimental group grown in presence of sub-MIC cetylpyridinium chloride compared with the control group, and revealed statistically significant difference ($p < 0.05$). In *A. naesundii*, hydrophobicity of the experimental group was reduced to 60% as compared with the control group, revealing statistically significant difference ($p < 0.05$).

4. DISCUSSION

Ammonium salts such as cetylpyridinium chloride have antimicrobial activity through various mechanisms. Cetylpyridinium chloride is positively charged and reacts with negative bacterial surfaces to increase possibility of interacting with biofilm cells and matrix [5]. Another mechanism involves degradation of lipid bilayers in the cell membrane, leaking cell contents [6], and inhibiting extracellular enzymes that synthesize polysaccharides, that play a key role in the progression of dental caries [23]. The concentration of cetylpyridinium chloride used in mouthwashes is a higher concentration than the

sub-MIC concentration used in this experiment. However, cetylpyridinium chloride is continuously diluted by saliva in the oral cavity, and cetylpyridinium chloride is present at a concentration of less than MIC in the first few hours after application, if not continuously administered [24,25].

Joe, et al. studied the effect of cetylpyridinium chloride on dental plaque of hydroxyapatite disc surface [26]. According to them, the addition of cetylpyridinium chloride inhibited plaque formation as compared with the control without cetylpyridinium chloride. In our study, biofilm formation of *S. mutans* and *A. naeslundii* decreased when cetylpyridinium chloride of sub-MIC was added.

Cellular hydrophobicity is closely related to bacterial adhesion, and highly cellular hydrophobic bacteria can attach to the acquired pellicle, epithelium or prosthetic teeth [16-18]. Goldberg, et al. reported that cetylpyridinium chloride increased bacterial cell hydrophobicity and bacterial adhesion of *Candida albicans* [27]. In our study, the addition of cetylpyridinium chloride to *A. naeslundii* decreased biofilm formation and decreased bacterial hydrophobicity statistically compared to the control. *S. mutans* was also not statistically significant, but showed the same results. Our results were in contrast to Goldberg, but we confirmed that there is a correlation between hydrophobicity and adhesion. However, in the case of *S. gordonii*, the addition of cetylpyridinium chloride revealed no change in biofilm formation compared to the control group, but bacterial cell hydrophobicity increased. Results are inconsistent with the fact that bacteria with high bacterial cell hydrophobicity form more biofilms. This study has limitations on the reasons why hydrophobicity and biofilm formation results do not show the same pattern. Although it should be clarified by future studies, it is thought that the attachment mechanism influenced by cetylpyridinium chloride is not only cell adhesion by cell hydrophobicity but also another mechanism.

In this study, we examined effects of sub-MIC cetylpyridinium chloride on the biofilm formation and bacterial surface hydrophobicity were investigated in *S. mutans* causing dental caries and *S. gordonii* and actinomyces in initial attachment bacteria. In this experiment, a simple method of observing biofilm formation using a glass slip was used. However, it is necessary to investigate the effect of cetylpyridinium chloride on biofilm after using a hydroxyapatite disc like a tooth surface or using a biofilm reactor to form a biofilm. In addition, the effect of cetylpyridinium chloride on coaggregation between oral bacteria should be further investigated.

5. CONCLUSIONS

When cetylpyridinium chloride was applied to oral bacteria, some bacteria showed a decrease in biofilm and a correlation between biofilm formation and cell hydrophobicity.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

REFERENCES

1. Addy M, Slayne MA, Wade WG. The formation and control of dental plaque--an overview. J Appl Bacteriol. 1992;73:269-78.
2. Mandel ID. Chemotherapeutic agents for controlling plaque and gingivitis. J Clin Periodontol. 1988;15:488-98.
3. Addy M, Moran J. The effect of a cetylpyridinium chloride (CPC) detergent foam compared to a conventional toothpaste on plaque and gingivitis. A single blind crossover study. J Clin Periodontol. 1989;16:87-91.
4. Ramalingam K, Amaechi BT, Ralph RH, Lee VA. Antimicrobial activity of nanoemulsion on cariogenic planktonic and biofilm organisms. Arch Oral Biol. 2012;57:15-22.
5. Myc A, Vanhecke T, Landers JJ, Hamouda T, Baker JR Jr. The fungicidal activity of novel nanoemulsion (X8W60PC) against clinically important yeast and filamentous fungi. Mycopathologia. 2002;155:195-201.
6. Sutherland IW. Biosynthesis and composition of gram-negative bacterial extracellular and wall polysaccharides. Annu Rev Microbiol. 1985;39:243-70.
7. Braga PC, Sasso MD, Sala MT. Sub-MIC concentrations of cefodizime interfere with various factors affecting bacterial virulence. J Antimicrob Chemother. 2000;45:15-25.
8. Lorian V. Medical relevance of low concentrations of antibiotics. J Antimicrob Chemother. 1993;31(Suppl D):137-48.

9. Fonseca AP, Extremina C, Fonseca AF, Sousa JC. Effect of subinhibitory concentration of piperacillin/tazobactam on *Pseudomonas aeruginosa*. J Med Microbiol. 2004;53:903-10.
10. Lorian V, Atkinson B. Abnormal forms of bacteria produced by antibiotics. Am J Clin Pathol. 1975;64:678-88.
11. Raponi G, Keller N, Overbeek BP, Rozenberg-Arska M, van Kessel KP, Verhoef J. Enhanced phagocytosis of encapsulated *Escherichia coli* strains after exposure to sub-MICs of antibiotics is correlated to changes of the bacterial cell surface. Antimicrob Agents Chemother. 1990;34:332-6.
12. Chopra I, Linton A. The antibacterial effects of low concentrations of antibiotics. Adv Microb Physiol. 1986;28:211-59.
13. Marsh PD. Microbiological aspects of the chemical control of plaque and gingivitis. J Dent Res. 1992;71:1431-8.
14. Cerca N, Martins S, Sillankorva S, Jefferson KK, Pier GB, Oliveira R, Azeredo J. Effects of growth in the presence of subinhibitory concentrations of dicloxacillin on *Staphylococcus epidermidis* and *Staphylococcus haemolyticus* biofilms. Appl Environ Microbiol. 2005;71:8677-82.
15. Gibbons RJ. Adherent interactions which may affect microbial ecology in the mouth. J Dent Res. 1984;63:378-85.
16. Ellepola AN, Samaranayake LP. The effect of limited exposure to antimycotics on the relative cell-surface hydrophobicity and the adhesion of oral *Candida albicans* to buccal epithelial cells. Arch Oral Biol. 1998;43:879-87.
17. Gibbons RJ, Etherden I. Comparative hydrophobicities of oral bacteria and their adherence to salivary pellicles. Infect Immun. 1983;41:1190-6.
18. A na C O, Elerson G, Victor E A, Mario J A. Influence of subinhibitory concentrations of antimicrobials on hydrophobicity, adherence and ultra-structure of *Fusobacterium nucleatum*. Braz J Microbiol. 2002;33:178-84.
19. Clinical and Laboratory Standards Institute. Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically, M07-A8, 8th ed., CLSI. Wayne, PA. 2009;29(2).
20. Park JH, Lee JK, Um HS, Chang BS, Lee SY. A periodontitis-associated multispecies model of an oral biofilm. J Periodontal Implant Sci. 2014;44:79-84.
21. Lee SY, Kim YJ, Kim KK, Choe SJ. Effects of subinhibitory antibiotic concentrations on *Porphyromonas gingivalis* fibrinogen and hemin binding. 1999;24:121-7.
22. Hamada N, Watanabe K, Sasakawa C, Yoshikawa M, Yoshimura F, Umemoto T. Construction and characterization of a fimA mutant of *Porphyromonas gingivalis*. Infect Immun. 1994;62:1696-704.
23. Ioannou CJ, Hanlon GW, Denyer SP. Action of disinfectant quaternary ammonium compounds against *Staphylococcus aureus*. Antimicrob Agents Chemother. 2007;51:296-306.
24. Bonesvoll P, Lokken P, Rolla G, Paus PN. Retention of chlorhexidine in the human oral cavity after mouth rinses. Arch Oral Biol. 1974;19:209-12.
25. Kara D, Luppens SB, Cate JM. Differences between single- and dual-species biofilms of *Streptococcus mutans* and *Veillonella parvula* in growth, acidogenicity and susceptibility to chlorhexidine. Eur J Oral Sci. 2006;114:58-63.
26. Latimer J, Munday JL, Buzza KM, Forbes S, Sreenivasan PK, McBain AJ. Antibacterial and anti-biofilm activity of mouthrinses containing cetylpyridinium chloride and sodium fluoride. BMC Microbiol. 2015;15:169,015-0501-x.
27. Goldberg S, Konis Y, Rosenberg M. Effect of cetylpyridinium chloride on microbial adhesion to hexadecane and polystyrene. Appl Environ Microbiol. 1990;56:1678-82.

Peer-review history:
The peer review history for this paper can be accessed here:
http://www.sciencedomain.org/review-history/23666