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A new *in vitro* method for evaluating the antimicrobial activity of toothpaste

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The purpose of this study was to introduce a new *in vitro* method for evaluating the antimicrobial activity of toothpaste, reflecting the actual toothbrushing time and the dilution of toothpaste by salivation. We designed three experimental groups and one negative control group. The experimental groups were (1) 90 μL of toothpaste + 10 μL 1X phosphate-buffered saline (PBS, 9/10 dilution group), (2) 50 μL of toothpaste + 40 μL 1X PBS (1/2 dilution group), and (3) 25 μL of toothpaste + 65 μL 1X PBS (1/4 dilution group). During toothbrushing, saliva is continuously secreted into the oral cavity and the toothpaste concentration is diluted over time during toothbrushing. Therefore, the 1/2 and 1/4 dilution experimental groups were added. The negative control group was toothpaste diluted 20,000-fold with 1X PBS. Miracle Fresh Doctor toothpaste and *Streptococcus mitis* KCOM 1350, *Prevotella intermedia* KCOM 1107, *Fusobacterium nucleatum* subsp. *polymorphum* KCOM 1322, and *Aggregatibacter actinomycetemcomitans* KCOM 1306 were used as the toothpaste and target bacterial strains, respectively. The number of bacterial cells plated on agar plates in the negative control group was 1,000 CFU. If the number of colonies on the experimental group plate was less than one, the treatment was considered to have > 99.9% bactericidal activity. These results suggest that this new *in vitro* method for antimicrobial evaluation could be used as the standard method for testing the antimicrobial activity of toothpaste.

Keywords: Antimicrobial, *In vitro* evaluation, Toothpastes

Introduction

The major causative agents of oral infectious diseases such as dental caries and periodontitis are oral bacteria. It is well-known that toothbrushing is the most effective method for preventing dental caries and periodontitis. The major mechanism of toothbrushing's effectiveness is the mechanical removal of oral bacteria. Antimicrobial agents are also major ingredients of toothpaste, included to increase the antibacterial effect [1]. For the *in vitro* evaluation of the antimicrobial activity of toothpaste, the disc diffusion assay [2-5] was conducted, targeting the major causative bacterial species of dental caries and/or periodontitis. In these assays, the incubation time after exposing toothpaste to the targeted bacteria was 16 to 24 hours. However, the actual contact time between toothpaste and the bacteria during toothbrushing is just 1 to 3 minutes depending upon the person. Therefore, the purpose of this study was to introduce a new *in vitro* method for evaluating the antimicrobial activity of toothpaste, reflecting the actual toothbrushing time and the dilution of toothpaste by salivation.

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Materials and Methods

1. Bacteria and bacterial culture

Streptococcus mitis KCOM 1350, *Prevotella intermedia* KCOM 1107, *Fusobacterium nucleatum* subsp. *polymorphum* KCOM 1322, and *Aggregatibacter actinomycetemcomitans* KCOM 1306 were used as the target bacteria in this study. These strains were isolated and identified from the oral cavity of a Korean population and obtained from the Korean Collection for Oral Microbiology (Gwangju, Korea).

The *S. mitis* strain used in this study was grown in brain heart infusion (BD Difco Laboratories, Franklin Lakes, NJ, USA) broth in a 37℃ incubator with 5% CO_2. The other strains were cultured in tryptic soy broth (BD Difco Laboratories) supplied with 0.5% yeast extract, 0.05% cysteine HCl-H_2O, 0.5 mg/mL hemin, and 2 μg/mL vitamin K_1 [6] in a 37℃ anaerobic chamber (BACTRONEZ; Sheldon Manufacturing Inc., Cornelius, OR, USA) and anaerobic conditions (10% H_2, 5% CO_2, and 85% N_2).

2. *In vitro* antimicrobial activity test

The bacterial strains were inoculated into each medium and cultured in an incubator at 37℃ for 24–48 hours. The bacterial cell concentration was determined by measuring optical density at 600 nm wavelength (OD_{600}) and by counting CFU/mL using a spectrophotometer. An OD_{600} value of 0.5 might equal approximately 1 × 10^9, 1.4 × 10^9, 0.5 × 10^9, and 0.5 × 10^8 CFU/mL of *S. mitis* KCOM 1350, *P. intermedia* KCOM 1107, *F. nucleatum* subsp. *polymorphum* KCOM 1322, and *A. actinomycetemcomitans* KCOM 1306, respectively. Each bacterial culture solution was concentrated to 2 × 10^{10} CFU/mL. Miracle Fresh Doctor toothpaste (Q GENETICS, Co., Ltd., Seoul, Korea) was used for testing the antimicrobial activity in this study. The experimental groups were: 1) 90 μL of toothpaste + 10 μL 1X phosphate-buffered saline (PBS, 9/10 dilution group), 2) 50 μL of toothpaste + 40 μL 1X PBS (1/2 dilution group), and 3) 25 μL of toothpaste + 65 μL 1X PBS (1/4 dilution group) (Step 1 in Fig. 1). The experimental groups were prepared in 1.5 mL Eppendorf tubes, and then 10 μL of bacterial culture solution (2 × 10^{10} CFU/mL) was added to each tube and vortexed for 30 seconds. The tubes were placed at room temperature for 150 seconds, and then 900 μL of 1X PBS was added to each experimental group (10-fold dilution). A sample (50 μL) of each of the toothpaste mixtures and the bacterial culture solutions was transferred to a 50 mL conical tube containing 9.950 mL of 1X PBS (final 2,000-fold dilution) (Step 2 in Fig. 1). At this time, the concentration of bacteria in each experimental group was 1 × 10^6 CFU/mL. The mixture in each experimental group was diluted 10-fold with 1X PBS and 10 μL of this was plated on an agar medium suitable for the bacterial species (final 20,000-fold dilution) and cultured for 2 days (Step 3 in Fig. 1). For the negative control group, we prepared two Eppendorf tubes. In the first tube, 90 μL of toothpaste and 10 μL of 1X PBS were

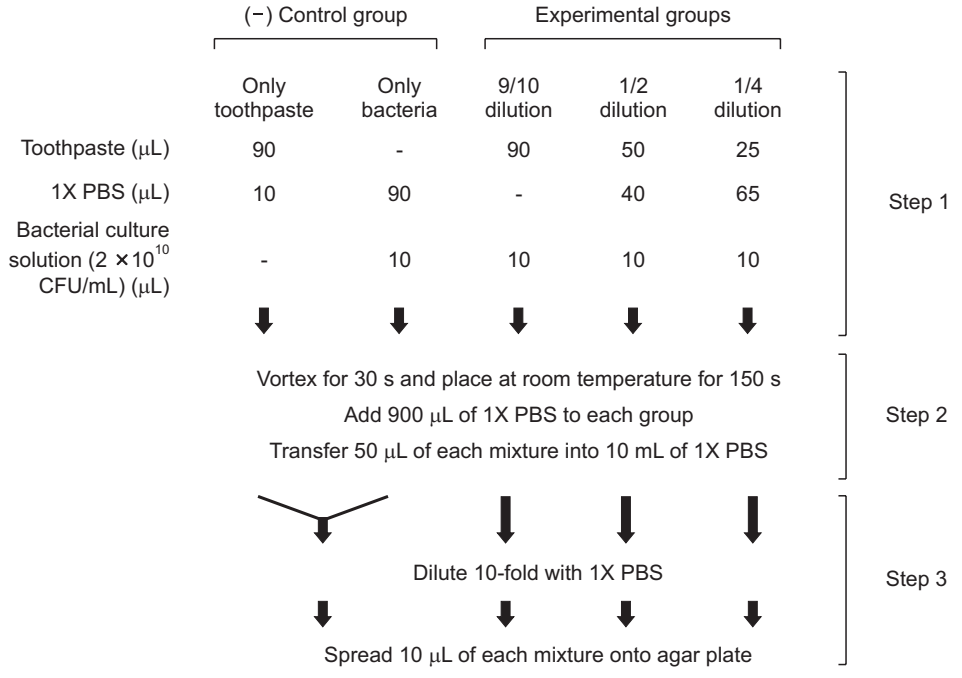

Fig. 1. Schematic diagram of the *in vitro* procedure for evaluating the antimicrobial activity of toothpaste.
PBS, phosphate-buffered saline.

added and in the second tube, 90 μL 1X PBS and 10 μL of bacterial culture solution (2×10^{10} CFU/mL) were added (Step 1 in Fig. 1). They were treated the same as the experimental groups in the second step and then mixed in one Eppendorf tube (Fig. 1). The third step of the control group experiment was the same as that of each experimental group (Fig. 1). The control and experimental group mixtures were diluted 10-fold more with 1X PBS and 10 μL was plated on an agar medium suitable for the bacterial species (final 20,000-fold diluted) (Fig. 1). When the number of colonies on the plate of the negative control group was 1,000, and the number of colonies on the plate of the experimental group was less than one, the tested toothpaste was determined to have > 99.9% bactericidal activity. Each experiment was repeated three times.

Results and Discussion

This study was conducted to develop a new *in vitro* method for evaluating the antimicrobial activity of toothpaste, reflecting actual toothbrushing time and the dilution of toothpaste by salivation. The first step for the experimental groups was mixing toothpaste and the targeted bacterial culture solution. Generally, toothpaste is available as a gel or paste. Therefore, it is difficult to evaluate the antimicrobial activity of 1X toothpaste because the bacterial culture solution should be mixed with toothpaste for antimicrobial activity testing. Therefore, we designed the highest toothpaste concentration group to be 90% toothpaste, the 9/10-dilution group. During toothbrushing, saliva is continuously secreted into the oral cavity and the toothpaste concentration is diluted over time while toothbrushing. Therefore, we added the 1/2- and 1/4-dilution groups to reflect the toothpaste concentration reduced by salivation during toothbrushing. The toothpaste and bacterial culture solution mixtures were vortexed for 30 seconds to mix them thoroughly. Then, the mixture was placed at room temperature for 150 seconds more to reproduce a toothbrushing time of 3 minutes and was diluted with 1X PBS (final 2,000-fold dilution). The third step of all groups was to dilute the toothpaste and bacterial culture solution mixture 20,000-fold to reduce the concentration of toothpaste and the target bacteria. Since it was possible that the final diluted concentration of toothpaste could affect the antibacterial activity in each experimental group, the same amount of bacterial culture solution (2×10^5 CFU/mL) and a 10,000-fold diluted solution of toothpaste were mixed and plated on an agar medium and designated as the control group (Step 3 in Fig. 1). In our experience, the antimicrobial activity of toothpaste diluted 20,000 times was 0 to 77% higher than that of 1X PBS, depending upon the type of toothpaste and the target bacterial species. For example, the antimicrobial activity of six types of 20,000-fold diluted toothpaste were 0 to 30% and 50 to 77% higher than those of 1X PBS against *P. intermedia* KCOM 1107 and *F. nucleatum* subsp. *polymorphum* KCOM 1322, respectively (data not shown). These results suggest that this new *in vitro* method for evaluating antimicrobial activity could be used as the standard method for testing the antimicrobial activity of toothpaste.

The data of the antimicrobial activity of Miracle Fresh Doctor toothpaste (Q GENETICS, Co., Ltd.) showed that it was > 99.8 or > 99.9% bactericidal against the four bacterial strains tested in all experimental groups (Fig. 2, Table 1). In a previous study, Miracle Fresh Doctor toothpaste (Q GENETICS, Co., Ltd.) had > 99.9% bactericidal effects against *Streptococcus mutans* KCOM 1054 and *Porphyromonas gingivalis* KCOM 2796 in the 1/4-dilution group (data not shown). *S. mutans* and *P. gingivalis* have been known to be the major causative agents of dental caries and periodontal diseases [7,8]. The targeted bacterial species used in this study are closely related to systemic as well as dental infectious diseases [9-11]. These results suggest that Miracle Fresh Doctor toothpaste (Q GENETICS, Co., Ltd.) could be used as a toothpaste to prevent systemic as well as dental infectious diseases through toothbrushing.

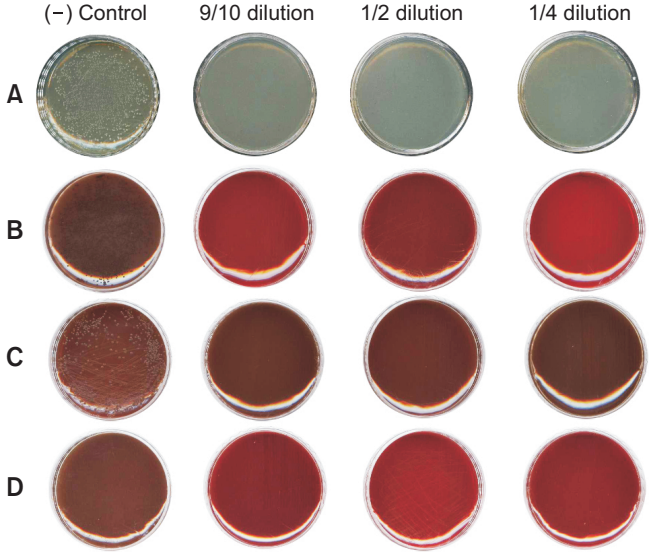

Fig. 2. Bacteria were grown on agar plates. The antimicrobial activity of Miracle Fresh Doctor toothpaste against (A) *Streptococcus mitis* KCOM 1350, (B) *Prevotella intermedia* KCOM 1107, (C) *Fusobacterium nucleatum* subsp. *polymorphum* KCOM 1322, and (D) *Aggregatibacter actinomycetemcomitans* KCOM 1306.

Table 1. Summary of the antimicrobial activity of Miracle Fresh Doctor toothpaste against oral bacteria

Dilution fold	Number of colonies on the plates (average, CFU/plate)			
	Smi KCOM 1350	Pi KCOM 1107	Fnp KCOM 1232	Aa KCOM 1306
9/10	0	0	0	0
1/2	0	0	0	0
1/4	0	0	0	0
(–) Control	1,330	> 2,000	584	> 2,000

Smi, *Streptococcus mitis*; Pi, *Prevotella intermedia*; Fnp, *Fusobacterium nucleatum* subsp. *polymorphum*; Aa, *Aggregatibacter actinomycetemcomitans*.

Antimicrobial agents and surfactants are ingredients of toothpaste. The antibacterial activity of toothpaste might differ depending upon the kinds of antimicrobial agents and surfactants. Additionally, these ingredients might have cytotoxic effects on human oral tissue cells depending upon the concentration. Therefore, it may also be necessary to conduct cytotoxicity tests of the antimicrobial agents and surfactants when evaluating the antimicrobial activity of toothpaste.

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Conflicts of Interest

No potential conflict of interest relevant to this article was reported.

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