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A pilot study of occupational exposure to pathogenic microorganisms through lip cosmetics among dental hygienists

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Abstract

Objectives: In dental clinics, dental hygienists are exposed to aerosolized pathologic bacteria, which can be transmitted to the oral cavity via lip cosmetics. Accordingly, such contamination poses a consistent health risk among staffs. Our study examined the bacterial contamination of lip cosmetics used by dental hygienists while in a clinic setting.

Methods: Sixteen dental hygienists were surveyed regarding their job assignments and habits associated with lip cosmetic. Subsequently, microorganisms were analyzed in collected samples of the hygienists' lip cosmetics using colony-forming unit (CFU) assays, 16s-rDNA polymerase chain reaction, and DNA sequencing.

Results: Notably, 81.3% of the submitted lip cosmetic samples were contaminated, with bacterial CFUs ranging from undetectable to innumerable. Many samples (43.8%) exceeded the microbial limits of cosmetic contamination. Of the lip cosmetic used for more than 6 months, 60% exceeded the microbial limit. When wearing a mask every time, only one of the six samples exceeded the microbial limit. More frequent dental mask changing was associated with a lower likelihood that the cosmetic sample would exceed the microbial limit. No samples from hygienists who changed their masks four times a day exceeded the microbial limit, compared to 33.3% from hygienists who only changed the mask when it became wet. Most isolated bacteria were gram-positive, facultative anaerobic, asporogenic, and opportunistically pathogenic, and the most prevalent species were *Staphylococcus aureus*, *Streptococcus salivarius*, and *Staphylococcus epidermidis*.

Conclusion: Our findings indicate that dental staff, including dental hygienists, should exercise more careful workplace habits, particularly with regard to infection control and cosmetic use.

KEYWORDS

bacteria, cosmetics, dental hygienist, lip, masks

1 | INTRODUCTION

Dental healthcare workers are daily exposed to microorganisms, which originated from the patients' mouths and present in the surrounding environment. Oral cavity contains a variety of microflora in a high concentration, more than 10^8 colonies per 1 mL.[1] Dental procedures frequently use high-speed rotating instruments, such as drills and ultrasound scaler, so large amounts of aerosols and splashes are consistently produced. Such aerosols, however, can travel over a distance of 1.5 m from a patient and can remain for up to 30 minutes after formation.[2,3] Thus the working environment in dental clinics is filled with lots of splatters and aerosols, and they can transmit microorganisms from patients or clinical materials to workers.[4] Even, a study showed a facial contamination with bacteria after aerosol-producing treatment, in which 5.6 cfu/m^3 of bacteria were detected on the worker's nares after 90-minute treatments, despite of using protective masks.[5] Furthemore, such a splash exposure was extremely high in dental healthcare professionals, 87.9% for dentists and 88.6% for dental hygienists, while the rate of nurses was approximately half (42.9%).[6] Therefore, dental healthcare workers face a significant risk of bacterial exposure and occupational infection.

Dental hygienists are healthcare professionals who work in dental clinics to provide oral healthcare services, especially for the prevention of oral diseases.[7] To care patients' health without oral disease, they clean and debride the oral cavity using ultrasound instruments. Concerning with the occupational infection in dental clinics, dental hygienists showed work-related symptoms more than twice and four times as often as administrative staffs and nurses, respectively.[8] In the study, the individuals who reported nasal irritation, running eyes, itchy, or dry skin, spent more time using the ultrasonic scaler, and the air samples taken from the area of oral hygiene care were contained oral commensals and skin bacteria.[8] So, it suggested the high-frequent symptoms of dental hygienists were caused by the occupational exposure to pathogens in dental clinics.

In dental clinic, lots of environmental materials that were contaminated by bacterial aerosol were reported, such as dental unit chairs, lights, drills, spittoon bowls, mobile trays, as well as the circulating air.[9-11] Even worse, those occupational infections were also detected on the clothing of dental staffs, in which most of the dental healthcare workers' white coats (90%) were contaminated with nosocomial bacteria.[12] Reports suggested various materials, such as necktie, stethoscope, pen, lanyard, and identify badge, can be a transmitting carrier of potential pathogens.[13]

Most of women apply facial cosmetics, and a majority of dental hygienists in the world is women.[14,15] During oral hygiene works, dental hygienists contaminate their face unintentionally with bacteria from the workplace, and their facial cosmetics can also be infected by contacting with their exposed skin. However, cosmetics cannot be washed when they infected, while clinical areas, badges or clothing infected by bacterial aerosols can be washed. Unless discarding, the propagated bacteria in cosmetics cannot be removed. Therefore, if the dental staffs' cosmetics were contaminated by the nosocomial dental pathogens, it might be a consistent and occupational health risk of dental healthcare professionals. In Korea, a large number of female adults use lip cosmetics (72.7%,[16]); which is more than the world (63%,[17]). And, lip cosmetic is the most frequent corrective make-up tool, almost 2.8 times per day in 44.2% of respondants, talking and eating all day long.[16] Recently, thus, the infection through ingestion of contaminated lip cosmetics become concerned, since they are used in direct contact with skin, thereby easily contaminated from skin-originate bacterial flora, and applied near the oral cavity.[18-20]

To our knowledge, there is no previous study investigated the bacterial contamination of dental staffs' cosmetics, including lip cosmetic. In the present study, therefore, we examined the microbial contamination of lip cosmetics used by dental hygienists in their workplaces, and surveyed their usage habits of the lip cosmetics and the clinical protective equipment.

2 | MATERIALS AND METHODS

The present study was divided into two stages: a pre-examination survey and the microbiological testing of collected samples.

2.1 | Survey of working patterns and lip cosmetic usage

Sixteen dental hygienists who worked at a large dental hospital and reported that more than 60% of their working hours spend in a clinical setting participated in this cross-sectional survey. The participants completed a self-administered structured questionnaire intended to assess their habits regarding lip cosmetic use and infection control.[21] Frequency of changing dental masks in terms of time, it is expressed as the number of times of mask changing during the 8-hour work time.[22]

2.2 | Sample collection

Samples of lip cosmetics currently used by the subjects in the clinical setting were collected. An area from the used surface of each lip cosmetic was aseptically collected and weighed, after which 0.1 g of each sample was dispersed in 5 mL of bacterial culture medium (BBL™ Brain Heart Infusion, BHI; Becton and Dickinson, Annapolis, MD) containing 0.1% of Tween 20 and mixed in a sonicator for

10 minutes to fully suspend the sample. Each suspension was then diluted via 10-fold serial dilution (10^{-1} or 10^{-3}) in BHI medium, and a 500-μL aliquot was used to inoculate each BHI agar plate with the aim of obtaining single colonies. The plates were incubated at 37°C under aerobic conditions (5% CO_2).

2.3 | Quantification of bacterial colonies

Colony-forming units (CFU, cfu) were used to estimate the number of bacteria per sample. To determine this common microbiological unit, every bacterial group formed from a single viable bacterium is counted on a culture plate. In this study, the numbers of colonies on each BHI agar plate were counted after a 48-hour incubation and reported as the colony CFU per 0.1-g lip cosmetic sample. Two examiners counted each plate and calculated the values. When CFU was countless, it was calculated as 15 000 for analysis.

2.4 | Identification of each bacterium

Collected bacteria were identified using a polymerase chain reaction (PCR)-based analysis to identify common sequences in bacterial deoxyribonucleic acid (DNA) and 16s ribosomal DNA (rDNA). To identify the bacterial species from each sample, a 500-μL aliquot of a liquid sample from a 24-hour incubation was used to inoculate a pure BHI agar plate. After a subsequent 24-hour incubation, several (three to four) representative colonies from each plate were collected separately and suspended in 500 μL of distilled water. To extract genomic DNA, the bacterial suspensions were boiled and held at 99°C for 3 minutes, followed by 4°C for 5 minutes. To amplify 16s rDNA, 2 μL of extracted DNA template per sample, 10 pmol of primer pairs, and 6 μL of distilled water were combined in a premix-tube for PCR containing Taq polymerase and deoxynucleotide tri-phosphate (dNTP) (Maxime PCR PreMix Kit; iNtRON Biotechnology, Seoul, Republic of Korea). The following universal primers for these reactions were produced by Cosmo Genetech Inc (Seoul, Republic of Korea): 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-GGTTACCTTGTTACGACTT-3'). The reaction mixtures were subjected to 35 cycles of denaturation and annealing at 52°C in an automated thermal cycler (Bio-Rad, Hercules, CA).

Following amplification, the PCR products were resolved by electrophoresis on a 1% agarose gel containing ethidium bromide. For sequencing analysis, the final PCR products were purified using a MEGAquick-spin kit (iNtRON Biotechnology) as instructed by the manufacturer. Briefly, each amplified PCR product was mixed with DNA binding buffer (supplied with the kit) and applied to a purification spin column. After a 1-minute centrifugation step at 13 000 rpm, each column was washed twice with washing buffer, after which highly pure DNA was eluted by the addition of elution buffer, a 1-minute incubation, and a 1-minute centrifugation at 13 000 rpm. The PCR products were sent for automated sequencing using a BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Beverly, MA), ABI 3730XL sequencing machine (Applied Biosystems), and Sequencher 5.1 analysis program (Gene Codes Corp., Ann Arbor, MI), which was conducted by Cosmo Genetech Inc, Republic of Korea. Subsequently, the newly aligned 16s rDNA sequences were compared with the bacterial genes deposited in the GenBank database (National Center for Biotechnology Information, Bethesda, MD), and bacterial strains with 99% matches were searched using the Basic Local Alignment Search Tool for nucleotides (BLAST; National Center for Biotechnology Information).

2.5 | Microbiological limit

Guidelines published by many countries have set cosmetic microbiological limits.[23-26] Generally, these guidelines recommend that adult cosmetics contain <1000 CFU of aerobic bacteria per gram, with no known opportunistic pathogens (eg, *Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Candida albicans*, and other yeast/mold). This study therefore analyzed and evaluated the submitted lip cosmetic samples according to these international microbial cosmetic guidelines.[23,25,26]

2.6 | Statistical analysis

The statistical analysis of the data was based on Frequency analysis, chi-square. A probability cut-off level of 0.05 was used to indicate statistical significance. The statistical analysis was processed using the statistical software package SPSS, version 16.0 (IBM Corp., Armonk, NY).

3 | RESULTS

3.1 | Personal information of subjects

On average, the hygienists worked for 38.9 hours per week and cared for 10.6 patients per day (Table 1). Furthermore, 56.2% of the participants used lipstick (Table 2). Regarding facial protection usage, only 37.5% and 62.5% of the

TABLE 1 General characteristics of the subjects (n = 16)

Variables	M ± SD
Age (years)	24.9 ± 2.9
Work Experience (years)	3.8 ± 2.9
Working hours per week (hours)	38.9 ± 4.5
Patients attended per day (person)	10.6 ± 3.4

TABLE 2 Statistical analysis of the relationship between lip cosmetic usage habits and bacterial detection

Variable		Total number of sample, N (%)[a]	CFU M ± SD, unit/mL	Exceeding samples[b] N (%)[c]
Type of product	Lipstick	9 (56.2)	2288.3 ± 5092.6	5 (55.6)
	Lip gloss	7 (43.8)	1717.6 ± 4534.1	2 (28.6)
Usage period (months)	6>	6 (37.5)	16.8 ± 30.9	1 (16.7)
	6≤	10 (62.5)	3251.7 ± 5704.4	6 (60.0)
Re-use frequency during work time	0	10 (62.5)	2046.6 ± 4861.5	4 (40.0)
	1≤	6 (37.5)	2025.3 ± 4886.7	3 (50.0)
Frequency of lip cleaning before use	Always	1 (6.3)	1.0 ± 0	0 (0.0)
	Sometimes	2 (12.5)	2726.0 ± 3838.2	2 (100.0)
	Never	13 (81.3)	2089.6 ± 5101.0	5 (38.5)
Frequency of product entrance cleaning after use	Sometimes	10 (62.5)	1214.4 ± 3789.8	3 (30.0)
	Never	6 (37.5)	33 412.3 ± 4705.4	4 (66.7)

CFU, colony-forming unit.
[a]Calculated in column percentages.
[b]"Exceeding sample" indicates a sample that exceeded the microbial limit for cosmetics (ie, cosmetics should not contain *Escherichia Coli*, *Pseudomonas aeruginosa*, or *Staphylococcus aureus* and should contain aerobic bacteria at a level below 1000 bacterium/mL).
[c]Calculated in row percentages.

participants always or frequently wore dental masks, respectively. Moreover, 12.5% and 68.8% of participants did not use or only occasionally used facial protectors like face shield, respectively (Table 3).

3.2 | Cross-analysis of the participant's usage behaviors and detected bacteria

Tables 2 and 3 demonstrate the results of analyses to determine the relationships between the usage patterns of lip cosmetic and facial protectives, respectively, and the detection of bacteria in cosmetic samples. Regarding the lip cosmetic usage habits, a longer usage duration was associated with an increase in the bacterial CFUs, as well as an increase in the number of samples that exceeded the cosmetic microbial standard. Microbial limit was exceeded by 60% among lip cosmetic used for more than 6 months (Table 2).

Conversely, more frequent dental mask wearing was associated with a lower likelihood that the cosmetic sample would exceed the microbial limit. When wearing a mask every time, only

TABLE 3 Statistical analysis of the relationship between facial protective device usage and bacterial detection

Variable		Total number of sample, N (%)[a]	CFU M ± SD, unit/mL	Exceeding samples[b] N (%)[c]
Frequency of wearing dental masks	Always	6 (37.5)	10.7 ± 24.2	1 (16.7)
	Sometimes	10 (62.5)	3255.4 ± 5702.1	6 (60.0)
Frequency of changing dental masks	When a mask gets wet	6 (37.5)	2513.7 ± 6117.1	2 (33.3)
	Four times a day	2 (12.5)	30.0 ± 42.4	0 (0.0)
	Two times a day	0 (0)	0 ± 0	0 (0.0)
	Once a day	7 (43.8)	2493.7 ± 4655.9	4 (57.1)
	After surgical treatment	1 (6.3)	20.0 ± 0.0	1 (100.0)
Frequency of wearing facial protective equipment (face shield)	Often	3 (18.8)	4007.0 ± 6922.2	2 (66.7)
	Sometimes	11 (68.8)	1872.1 ± 4648.1	3 (27.3)
	Never	2 (12.5)	2.0 ± 0.0	2 (100.0)

CFU, colony-forming unit.
[a]Calculated in column percentages.
[b]"Exceeding sample" indicates a sample that exceeded the microbial limit for cosmetics (ie, cosmetics should not contain *Escherichia Coli*, *Pseudomonas aeruginosa*, or *Staphylococcus aureus* and should contain aerobic bacteria at a level below 1000 bacterium/mL).
[c]Calculated in row percentages.

one of the six samples exceeded the microbial limit. If mask was changed four times a day, there was no exceed microbial limit the lip cosmetic. In the case of facial protective equipment (face shield), bacterial contamination was not consistently observed according to the frequency of wearing (Table 3).

3.3 | Microbial examination of the submitted lip cosmetics

According to the results of the CFU and PCR analyses, 81.3% of the samples (13/16) were contaminated with bacteria (Table 4). The bacterial CFU counts ranged from undetectable to innumerable; although colonies did not grow from samples #1, #2, and #10-14, the plate corresponding to sample #9 contained a large number of colonies that could not be counted by the examiner. The PCR analysis identified 1-3 types of bacteria per sample, except samples #2, #10, and #11. Twelve bacterial species were isolated; of these, the most common were *S aureus* (five samples), *Streptococcus salivarius* (six samples), and *Staphylococcus epidermidis* (six samples). *Enterococcus faecalis*, *Pseudomonas putida*, *Paenibacillus pasadenensis*, *Bacillus niabensis*, *Bacillus pumilus* group, *Bacillus subtilis*, *Staphylococcus sciuri*, *Staphylococcus warneri*, and *Streptococcus mitis* were also detected. Additionally, many samples (43.8%) exceeded the microbial limits for cosmetics: samples #3-5, #8, and #15 contained *S aureus* and samples #8-9 and #16 contained more than 1000 aerobic bacteria/mL *S aureus* and more than 1000 aerobic bacteria/m was found in one(#15) of the three samples (#7, #14, and #15) used for more than 5 months, but not in samples used for 1-4 months (Table 4).

4 | DISCUSSION

In dental clinics, commonly used equipment such as drills, compressed air, and ultrasonic instrument result in the aerosolization of small water drops throughout the treatment area. These aerosols contain microorganisms, such as oral bacteria from patient, and then diffuse in the air of clinic.[2,4] These bacterial aerosols can be transmitted not

TABLE 4 Microbial isolates from each submitted lip cosmetic sample

Sample	Usage period (month)	Type of product	Species of bacteria from samples	CFU (unit/mL)	Exceed microbial limit[a]
#1	1	Lip gloss	-	NT	X
#2	3	Lip stick	*Pseudomonas putida* group	NT	X
#3	4	Lip gloss	*Staphylococcus warneri* group, *Streptococcus salivarius*	NT	X
#4	5	Lip gloss	*Staphylococcus epidermidis*, *Bacillus pumilus* group	NT	X
#5	5	Lip gloss	*Streptococcus salivarius*, ***Staphylococcus aureus***, *Staphylococcus epidermidis*	20	O
#6	5	Lip stick	*Streptococcus salivarius*	78	X
#7	6	Lip gloss	*Bacillus subtilis*	NT	X
#8	6	Lip stick	-	NT	X
#9	6	Lip stick	*Staphylococcus epidermidis*, ***Staphylococcus aureus***, *Streptococcus salivarius*	2	O
#10	6	Lip stick	***Staphylococcus aureus***	5440	O
#11	8	Lip gloss	*Staphylococcus sciuri*, *Enterococcus faecalis*	12 000	O
#12	8	Lip stick	*Bacillus niabensis*, ***Staphylococcus aureus***	2	O
#13	10	Lip stick	*Paenibacillus pasadenensis*, *Staphylococcus epidermidis*	countless	O
#14	14	Lip gloss	-	NT	X
#15	14	Lip stick	*Staphylococcus epidermidis*, *Streptococcus mitis*, *Streptococcus salivarius*	60	X
#16	18	Lip stick	***Staphylococcus aureus***, *Staphylococcus epidermidis*, *Streptococcus salivarius*	12	O

CFU, colony-forming unit. NT, not detectable.

[a]Exceed microbial limit indicates a sample that exceeded the microbial limit for cosmetics (ie, cosmetics should not contain *Escherichia Coli*, *Pseudomonas aeruginosa*, or *Staphylococcus aureus* and should contain aerobic bacteria at a level below 1000 bacterium/mL).

Samples # 5, # 9, # 10, # 12 and # 16 contains *Staphylococcus aureus*

only on the surfaces of clinical equipment, but also on the bodies of dental staffs. In a previous report, several pathogenic bacteria, including *Staphylococcus*, *Streptococcus*, *Enterobacterium*, and anaerobic gram-negative bacteria, were detected on the clinical surfaces of dental units, drill, and spittoon bowls.[9] Another study even found that more than 50% of the contact lenses worn during dental treatments exhibited fungal and bacterial contamination, despite wearing goggles.[27]

Lip cosmetics are used in direct contact with skins and it leads them easily infected by microorganisms.[18,19] Moreover, those bacteria still survive and proliferate within under suitable condition, though lip products contain greater amounts of preservatives than other cosmetics.[19,28] Thus, given the frequency and amount of the application, 2.35 times at amount of 24 mg per day on average,[20] the propagation of pathogens within a lip product can be a health risk to users. On the other hand, the 81.3% of our lip samples were contaminated with bacteria at varying counts, from none to countless, and 43.8% of the samples exceeded the international microbial limits for cosmetics (Tables 2 and 3). And it was partly associated with the longer duration of lip cosmetic use. This is consistent with previous studies in which varied amounts of bacterial contamination were observed in 100% of the sampled lip products, in which a longer duration of lip product use was also associated with an increased bacterial contamination.[18,19] In our study, from one of the three samples (#7, #14, and #15) which was used for more than 5 months, exceeded microbial limit and *S aureus* (#15) was found (Table 4). However, the samples used for 1-4 months were considered safe.

In the present study, lots of species of bacteria were detected; *S aureus*, *S salivarius*, and *S epidermidis*, *E faecalis*, *P putida*, *P pasadenensis*, *B niabensis*, *B pumilus* group, *B subtilis*, *S sciuri*, *S warneri*, and *S mitis*. From only 16 samples, the authors found various bacterial strains at least 12 species, and it was a little unlike previous studies. The studies for used lip cosmetics in markets or of individuals also showed bacterial contaminations, but the number of detected microorganisms were just 3 to 5; *S arlettae*, *Proteus penneri*, and *Providencia vermicola*[19]; *Bacillus*, *Micrococcus sedentarius*, *S saprophyticus*, *S aureus*, *S epidermidis*[28]; *S aureus*, *E coli*, and *S epidermidis*.[29] Such a different pattern to our results suggested because the objects were the samples of common personals who do not work in dental clinic, and their workplace do not polluted with bacterial aerosols.

On the other hand, most of the strains detected in current study are gram-positive, aerobic or facultative aerobic, and asporogenic. And, the result confirm previous findings, in which gram-positive cocci were the predominant strain.[30] As commensal human microbiota, the bacteria detected in the present study are usually harmless to human. Under some conditions associated with a poor or immunocompromised status, however, reports noted that theses microbes can cause a lot of diseases such as scaled skin syndrome, surgical site infection, bacteremia, catheter-related infection, endocarditis, and so on.[31] Especially for *S aureus* and *E faecalis*, they are strongly associated with oral diseases; *S aureus* was identified as an etiological agent of angular cheilitis, parotitis, and staphylococcal mucositis,[32] and *E faecalis* was the most frequently isolated nosocomial genus in chronic periodontitis[33,34] and the failure of root canal therapy.[35,36] Therefore, such a latent bacterial strain should be monitored closely.

In our study, *S aureus* is one of the most frequent species detected in the lip samples of dental hygienists. Similar to our results, *S aureus* was reported as the organism commonly found most on the dental professionals' apron sleeves, and moreover it contaminated the 50%-75% of the dental staffs' clothing.[5,12] Moreover, 52.4% the of dental prosthetic patients were reported to have *S aureus* around their appliances,[37] and 60% of the dental procedures can create aerosols of *S aureus*.[5] Therefore, given its colonization in dental patients' oral cavities, *S aureus* in the current study is supposed to be aerosolized during oral treatment, floated generally in the air of clinics, and subsequently transmitted into the lip cosmetic samples through the body of the subjects, dental hygienists.

Furthermore, the high rate of lip cosmetic contamination might be attributed to the habits regarding protective gear use of participants. According to previous studies, if a mask remains wet for more than 15 minutes, the protective function may be lost and the user may be exposed to bacterial contamination.[38] All the respondents (ie, dental hygienists) in our study had performed work-specific tasks, including tooth cleaning with ultrasonic scalers. However, only 37.5% participants wore masks always and only 37.5% would change a wet used mask with a new one. Our study shows that exceeding sample was not found when the mask was changed four times a day, but was found in 33.3% when the mask was changed every time it got wet. The mask being wet was judged subjectively. Therefore, in order to be safe from bacterial contamination of lip cosmetics, it is necessary to change the mask for each patient or set the replacement cycle. Under such circumstances, therefore, the subjects were supposed at a high risk of facial contamination and consequent transmission of their lip cosmetics.

Although statistical differences were not been considered, this study found that the CFU of bacterial was 1 aerobic bacteria/mL and the bacterial limits were not exceeded when hygienists always cleaned their lips before applying cosmetics. However, bacterial excess was observed if the lips were sometimes cleaned (100%) or never cleaned (38.5%). The CFU of bacterial was more than 2.000 aerobic bacteria/mL (Table 2). Furthermore, all samples from those who reported never wearing facial protective equipment (face shield) exceeded the bacterial limits, compared to 66.7% from those

who often wore such equipment (Table 3). This study included all employees working at a single dental clinic. The sample was small, and statistical significance might not have been achievable. Further research is required on areas that are not clearly identified in the study results.

In conclusion, this study was the first to examine bacterial contamination in the lip cosmetics used by dental hygienists who are exposed to large amounts of bacterial aerosols. We note that this study was limited to a small number of surveyed dental hygienists and to aerobic bacterial analysis. Thus, further studies are needed to supplement our findings. Our next experiment should be expanded to examine bacterial contamination in other types of cosmetics. However, our results definitely suggest that the lip cosmetics used in dental clinic can be cross-contaminated by several nosocomial bacteria, so dental hygienists should pay considerable attention to the use of both their cosmetics and protective equipment.

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DISCLOSURE

Approval of the research protocol: All protocols used in the present study were approved by the Ethics Committee for Research of Yonsei University, Wonju College of Medicine. *Informed Consent:* The subjects provided signed informed consent to participate in the study after receiving an explanation of the study purpose. *Registry and the Registration No. of the study/trial:* YWDR-15-2-062. *Animal Studies:* N/A. *Conflict of interest:* None declared.

AUTHOR CONTRIBUTIONS

HN: research design and organize the whole process and writing paper. I-hJ: experiment design and writing paper. J-hK: conduct experiment and writing paper. Y-JY: design and support the experiment. B-yP: conduct sample collection, experiment and analyze the data. E-sC: analyze the data.

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