Visualization of Oral Bacteria Using a Fluorescent Dye and Its Application for the Assessment of Oral Care

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ABSTRACT

The purpose of this study was to investigate the number of oral bacteria and its changes by visualizing the presence of bacteria using a fluorescent dye to stain bacterial DNA, 4', 6-diamidino-2-phenylindole (DAPI), and examine the usefulness of this method for oral-care assessment. In 10 healthy adults and 10 elderly persons, from whom informed consent was obtained, we investigated diurnal changes in the number of oral bacteria, oral conditions, and their lifestyle. In addition, we measured the number of oral bacteria before and after oral care training in 11 students belonging to Nursing College A. Bacteria were collected by wiping 3 points in the oral cavity (on the tongue, inside of the cheek, and surfaces of upper and lower teeth) with clean swabs. DAPI facilitated visualization of the presence of bacteria in a sample in a short period and counting, suggesting its usefulness for oral-care assessment.

I. Introduction

Oral care is important to prevent aspiration pneumonia in elderly persons, persons with consciousness disorder, and severe-status patients under respiratory control[1][2]. Fungus, *Klebsiella pneumoniae*, *Staphylococcus aureus*, and *Pseudomonas aeruginosa* cause geriatric pneumonia[3]. These bacteria cause chronic respiratory infectious diseases as periodontogenic bacteria by insufficient oral care. In patients who oral ingestion is impossible, aspiration of these bacteria is easy to cause pneumonia related to the reduction of the oral self-cleaning function[4]. As oral indigenous bacteria which do not usually show pathogenicity, cause respiratory infectious diseases in infection-prone persons such as the elderly and severe-status patients, it is necessary to decrease the number of oral bacteria through oral care.

Therefore, bacteriological examination of the oral cavity is essential to evaluate the efficacy of oral care. However, as bacterial medium culture and identification of the bacteria is complex and take for a long time, there have been few studies and evidence involving elderly persons or chronic-phase patients regarding oral care. To clarify these issues, the presence and change of number of oral bacteria must be evaluated using a simple method.

The fluorescent dye, 4', 6-diamidino-2-phenylindole (DAPI)[5] which has been used for nuclear staining of cells and microorganisms, facilitates simple microscopic examination and measurement of the presence, number, and morphology of oral bacteria in a short period. In addition, we established conditions for the visualization of bacteria using DAPI, and examined simply and promptly of the presence, number, and morphology of bacteria in samples[6].

We investigated the number of oral bacteria and its changes by simply visualizing the presence of bacteria using a fluorescent dye DAPI for staining of bacterial DNA, and examined whether this method can be applied for the assessment of oral care.

II. Methods

In this study, to clarify whether the visualization of oral bacterial counts with a fluorescent dye can be applied for the assessment of oral care, we investigated diurnal changes in the number of bacteria in the oral cavity and those before and after oral care using the following two methods.

1. Diurnal changes in the number of bacteria in the oral cavity

a. Subjects

The subjects consisted of 10 healthy adults and 10 elderly persons (total: 20).

b. Sample collection

After wiping 3 points (on the tongue, inside of the cheek, and surfaces of upper and lower teeth) in each subject's oral cavity with clean swabs, respectively, they were stored in a bottle containing a fixative (4% paraformaldehyde). The subjects were instructed to collect samples themselves. Their procedures were confirmed through an author's demonstration based on a leaflet regarding the collection method so that there were no marked differences in the method.

Sampling was performed at night (before bedtime), in the morning (after waking up), and at noon (before lunch) to examine diurnal changes in the number of oral bacteria in each subject. To investigate the association of this parameter with the oral state or lifestyle, a survey regarding the presence or absence of dental disease/denture and tooth brushing was conducted in the subjects.

c. Calculation of the number of bacteria and visualization

Samples were filtered using a syringe filter (proprietary name: Mini Sarto, pore size: 5.0 μm, Sartorius Stedim Japan). After large residues and exfoliating mucosal cells were removed, 10 μL of each sample was collected, dropped on a slide glass, and dried at room temperature for 10 minutes. These operations were performed on a clean bench.

After washing, each sample was stained with 10 μg/mL DAPI solution (phosphate-buffered saline, pH 7.4, Sigma-Aldrich Japan) for 30 minutes, washed in distilled water, covered with a covering glass, and photographed under a fluorescence microscope (filter No. 2, Zeiss Axioplan 2, Zeiss). In addition, the photographed images were input to a computer, and bacteria were counted. The number of all bacteria in unit area (0.013 mm^2) was measured. The total number of bacteria

in 5 areas (0.065 mm^2) per sample was calculated.

d. Analytical method

The data were expressed as the mean±standard deviation (S.D.). For statistical analysis, the Mann-Whitney U test was used. A P-value of <0.05 was regarded as significant.

e. Ethical consideration

The purpose and method of this study, free will-based participation, and the absence of identification of individuals were explained to the subjects. Informed consent was obtained from all subjects. Prior to this study, its protocol was approved by the Ethics Review Board of The Japanese Red Cross Hokkaido College of Nursing.

2. Number of bacteria in the oral cavity before and after oral care

a. Subjects

The subjects were 11 first-year students attending technical training for daily life support in Nursing College A.

b. Collection of samples

Oral bacteria were collected before and after oral care training (gargling and brushing) by students. After wiping 3 points (on the tongue, inside of the cheek, and surfaces of upper and lower teeth) in the oral cavity with clean swabs, respectively, they were stored in a bottle containing a fixative (4% paraformaldehyde). The students' procedures were confirmed through an instructor's demonstration and training by themselves.

c. Calculation of the number of bacteria and visualization

Each sample was treated as described above (1. Diurnal changes in the number of bacteria in the oral cavity).

d. Analytical method

The number of bacteria was expressed as the mean± standard deviation. In each subject, the values before and after oral care were compared.

e. Ethical consideration

The purpose and method of this study, free will-based participation, and the absence of identification of individuals were explained to the subjects. Informed consent was obtained from all subjects. The subjects of this experiment were students, and it was carefully conducted during lessons so that there was no influence on the lessons. Informed consent was obtained after explaining the following points: the results would have no effect on academic evaluation, and there would be no disadvantage even if they did not participate in this study.

III. Results

1. Diurnal changes in the number of bacteria in the oral cavity

There was no dropout case. The data from all subjects were analyzed.

a. Number of bacteria in the oral cavity

(1) Subjects' age and oral state

The subjects consisted of 10 aged 20 to 39 years and 10 aged 70 to 79 years. There were 14 females and 6 males. Concerning the presence or absence of dentures, they were present in 1 of the former and 5 of the latter. Concerning the presence or absence of dental disease, it was present in 6 of the former and 2 of the latter.

The frequency of tooth brushing was once a day or twice a day in 8 of the former and 9 of the latter. It was 3 times or more in 2 and 1, respectively.

Oral discomfort was absent in 6 of the former and 4 of the

Table 1. Subjects

| Subject No.	Age group	Gender	Dentures	Dental disease	Tooth brushing (times/day)	Oral discomfort*
1	20s	Female	Absent	Absent	2	Absent
2	20s	Female	Absent	Absent	2	4, 5
3	20s	Female	Absent	Absent	2	Absent
4	20s	Female	Absent	Present	1	Absent
5	20s	Female	Absent	Present	3	2
6	30s	Male	Absent	Present	1	Absent
7	20s	Male	Absent	Absent	2	Absent
8	20s	Male	Present	Present	1	Absent
9	30s	Male	Absent	Present	2	2, 3, 4
10	20s	Male	Absent	Present	3	1
11	70s	Female	Present	Absent	2	1
12	70s	Female	Present	Absent	1	1
13	70s	Female	Present	Absent	4	Absent
14	70s	Female	Absent	Present	2	3
15	70s	Female	Absent	Present	1	3
16	70s	Female	Present	Absent	1	4
17	70s	Female	Absent	Absent	2	4
18	70s	Female	Present	Absent	2	Absent
19	70s	Male	Absent	Absent	2	Absent
20	70s	Female	Absent	Absent	2	Absent

*Oral discomfort: 1. Food retention between teeth/dentures, 2. Dirty tongue, 3. Halitosis, 4. Dry mouth, 5. Others

latter. Subjects with oral discomfort, aged 20 to 39 years, complained of a dirty tongue, halitosis, dry mouth, and others. Those aged 70 to 79 years complained of food retention between their teeth/dentures, halitosis, and dry mouth (Table 1).

(2) Visualization of oral bacteria using a fluorescence microscope

Oral bacteria were stained with DAPI, and examined using a fluorescence microscope. The bacteria measured approximately 2 to 3 μm, being globular or in chains. Bacteria were observed singly or in colonies. Figure 1 shows fluorescence microscopy photographs of diurnal changes in oral bacteria in subjects aged 20 to 39 and 70 to 79 years, respectively (Subject Nos. 7 and 14). Thus, oral bacteria were fluorescently visualized on DAPI staining in all subjects.

age: 20-30s

age: 70s
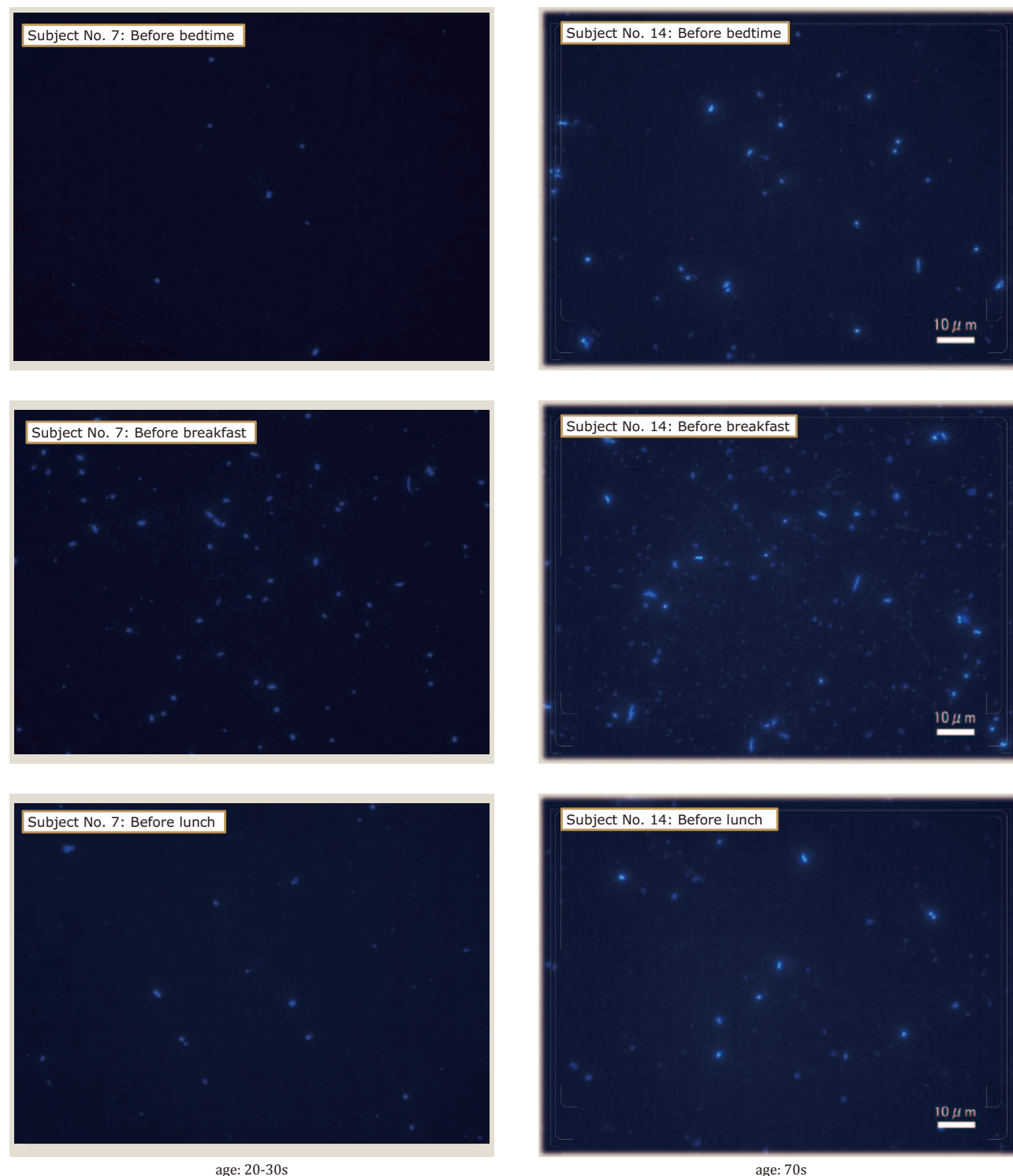
Figure 1. Visualization of oral bacteria using a fluorescence microscope

(3) Diurnal changes in the number of oral bacteria

The diurnal changes in the number of oral bacteria in each subject are shown in Figs. 2 and 3. In most subjects aged 20 to 39 and 70 to 79 years, the number of oral bacteria was the highest in the morning, followed by those at night and at noon. In 1 of the young subjects and 1 of the elderly subjects, the changes were reversed in comparison with the other subjects. In 1 of the former, the number of oral bacteria was the highest at noon.

We examined diurnal changes in the number of oral bacteria with respect to age. In most subjects aged 20 to 39 years, the mean number of oral bacteria in the morning was 2 to 3 (maximum: 5) times higher than at night and at noon. In those
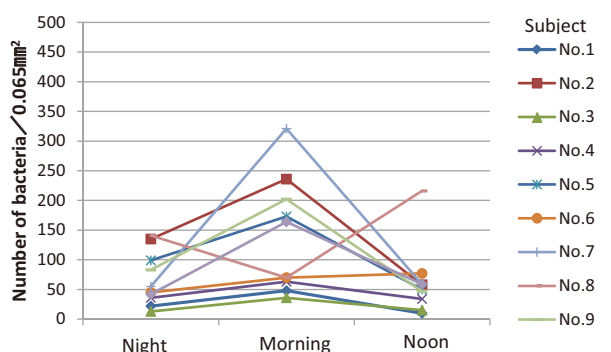
aged 70 to 79 years, it was 1.7 to 1.9 times higher than at night and at noon; the increase was less marked than in the young subjects. The elderly subjects tended to show a high number of oral bacteria all day.

(4) Number of oral bacteria with respect to age

The mean number of oral bacteria with respect to age is presented in Fig. 4. In subjects aged 20 to 39 years, the mean number of oral bacteria at night, in the morning, and at noon was 67.145.4, 138.3±95.7, and 62.5±57.8, respectively. In those aged 70 to 79 years, the values were 113.6±69.5, 184.5±115.0, and 122.2±86.8, respectively. At all points, the number of oral bacteria in the latter was higher than in

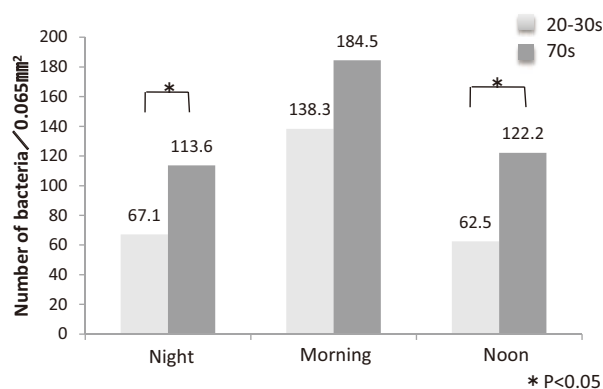**Figure 2. Diurnal changes in the number of oral bacteria (age: 20-39)**

Figure 4. Mean number of oral bacteria with respect to age

Figure 3. Diurnal changes in the number of oral bacteria (age: 70s)
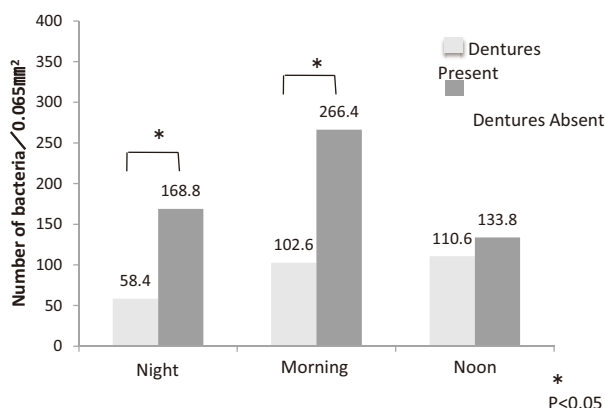

Figure 5. Mean number of oral bacteria with respect to the presence or absence of dentures
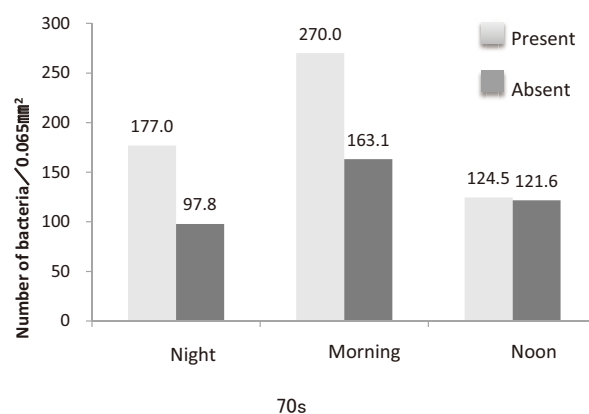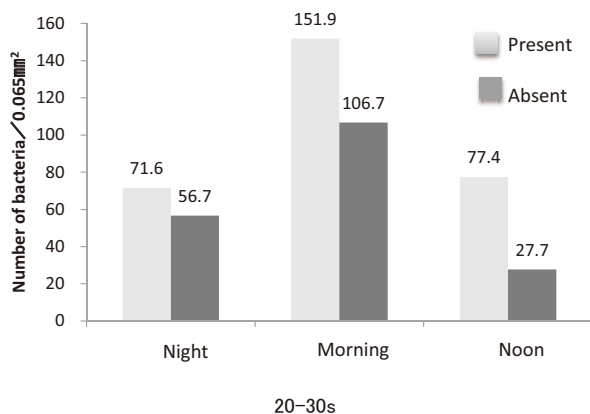

20−30s

70s

Figure 6. Mean number of oral bacteria with respect to the presence or absence of dental disease
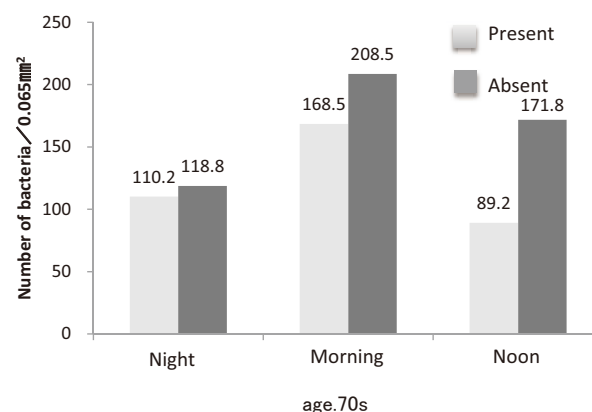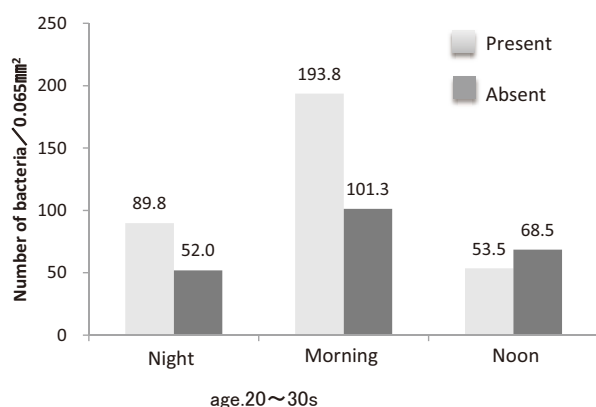
4

Figure 7. Mean number of oral bacteria with respect to the presence or absence of oral discomfort
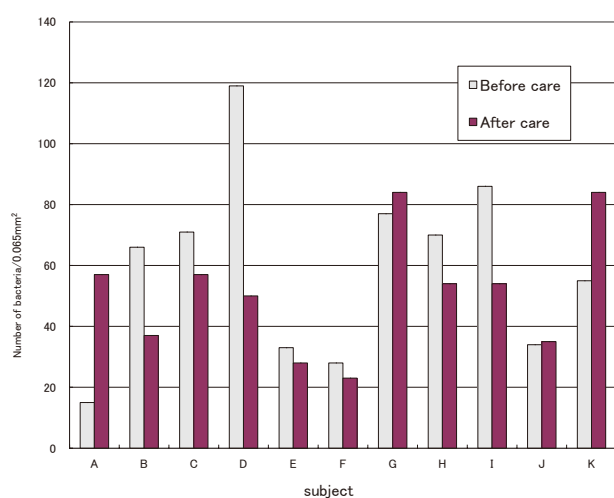

Figure 8. Number of oral bacteria before and after oral care

Figure 9. Oral bacteria on fluorescence microscopy before and after oral care

the former. It was significantly higher at night and at noon (p<0.05).

(5) Number of oral bacteria with respect to the oral state

The mean number of oral bacteria with respect to the presence or absence of dentures in subjects aged 70 to 79 years is shown in Fig. 5. In those with dentures, it was 58.4±24.6 at night, 102.6±38.0 in the morning, and 110.6±119.2 at noon. In those without dentures, the values were 168.8±51.5, 266.4±107.4, and 133.8±48.8, respectively. At all points, the number of oral bacteria in those without dentures was higher than in those with dentures. It was significantly higher at night and at noon (p<0.05).

The mean number of oral bacteria with respect to the presence or absence of dental disease in each age group is shown in Fig. 6. Dental diseases were present in 6 subjects aged 20 to 39 years, but absent in 4. They were present in 2 subjects aged 70 to 79 years, but absent in 8. There were no significant differences. However, in the two age groups, the number of oral bacteria in subjects with dental diseases was higher than in those without dental diseases.

The mean number of oral bacteria with respect to the presence or absence of oral discomfort in each age group is shown in Fig. 7. Oral discomfort was present in 4 subjects aged 20 to 39 years, but absent in 6. It was present in 6 subjects aged

70 to 79 years, but absent in 4. There were no significant differences. However, among subjects aged 20 to 39 years, the number of oral bacteria at night and in the morning in those with oral discomfort was higher than in those without oral discomfort. Among those aged 70 to 79 years, the number of oral bacteria in those without oral discomfort was higher than in those with oral discomfort.

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2. Number of oral bacteria before and after oral care

All required samples were collected, and the data from all subjects were analyzed.

The subjects were 11 female students, aged 20 to 39 years, belonging to Nursing College A. The number of bacteria before oral care ranged from 15 to 119, with a mean of 59.5±9.1. After oral care, it ranged from 23 to 84, with a mean of 51.2±6.1. After oral care, the number of bacteria decreased in 7 students, and increased in 4 (Fig. 8).

Representative photographs of oral bacteria on fluorescence microscopy before and after oral care are presented in Fig. 9. Thus, the number of bacteria before and after oral care and its increase/decrease were visualized.

IV. Discussion

Oral care influences oral cleanliness, oral/swallowing functions, and activities of daily living (ADL). Thus, although the oral care is important, it is difficult to standardize its techniques and procedures and its standardization is not yet established.

In addition, neither the necessity nor assessment of oral care has been standardized. Although there are several parameters for oral assessment, most are based on subjective assessment. Concerning subjective assessment, a previous study reported the results of assessment related to differences on occupation/specialty of nurses and dental hygienists and their reliability[7]. However, it is difficult to apply them in clinical practice. As bacterial culture for research as a method of objective assessment requires many hours and is costly, it is impossible to use it for the assessment of oral care in clinical practice.

In this study, we used a fluorescent dye DAPI as a marker for the visualization of bacteria and measurement and as an objective evaluation method available for the assessment of oral care.

1. Diurnal changes in oral bacteria

With respect to diurnal changes in oral bacteria, we investigated the presence of oral bacteria in healthy subjects aged 20 to 39 and 70 to 79 years. In the present study, oral bacteria were stained with DAPI and it was possible to confirm the presence of bacteria and count them using a fluorescence microscope. Furthermore, the fluorescence of bacterial DNA was observed in all bacteria in the oral cavity. Their shape and relative number could be observed and measured. It was also possible to accurately evaluate diurnal changes in the number of oral bacteria. The following results were consistent with those using the bacterial culture method: (1) the number of oral bacteria before meals was the highest, and it decreased after meals, but gradually increased thereafter; and (2) the number of oral bacteria increased at night, reaching a maximum at the time of waking-up[8].

In addition, the number of oral bacteria in the elderly group was higher than in the young group. This may be due to the oral self-cleaning function reduced with a decrease in saliva secretion at an advanced age.

Concerning dentures which are commonly used by elderly persons, the number of oral bacteria in subjects without dentures was higher than in those with dentures. Dentures may become a reservoir for bacteria. A study involving individuals requiring nursing indicated that bacteria that cause aspiration pneumonia, opportunistic infection, or endocarditis were present on dentures[9]. In this survey, the number of oral bacteria was estimated to be higher in subjects with dentures. However, the healthy subjects of this study washed their dentures after meals, and many subjects removed them before sleep. This may have contributed to the finding that the number of oral bacteria on waking-up in subjects with dentures was significantly lower than in those without dentures.

Concerning changes in the number of oral bacteria with respect to the presence or absence of dental disease, the number of oral bacteria in both young and elderly subjects with dental diseases was slightly higher than in those without dental diseases. As dental diseases such as caries and periodontal disease are primarily caused by periodontal disease-associated bacteria[10], this may have resulted in the finding that the number of bacteria was higher in subjects with dental diseases.

Concerning changes in the number of oral bacteria with respect to the presence or absence of oral discomfort, the number of oral bacteria in young subjects with oral discomfort was slightly higher than in those without oral discomfort. Halitosis, a dirty tongue, and dry mouth reflect oral uncleanliness or a reduction in the oral self-cleaning function by saliva; the number of oral bacteria may have been high. However, the oral state was not confirmed, and its association with the number of bacteria was unclear.

Fluorescent staining of bacteria with DAPI based on the presence of oral bacteria, their count, and diurnal changes in young and elderly subjects facilitates simple, prompt measurement of the presence of bacteria and their relative count. This method may be applicable for bacteriological assessment of the oral state.

2. Number of oral bacteria before and after oral care

In the students specializing in nursing, we confirmed an oral care-related increase or decrease in the number of oral bacteria. Appropriate brushing and gargling cleaned the oral cavity, decreasing the number of oral bacteria.

However, the number of bacteria increased after oral care in some subjects. This was possibly because gargling was insufficient after brushing to promote the exfoliation of biofilms, such as acquired capsules and plaque, on the tooth surfaces. This was consistent with the results presented by Sakota et al.[11], who performed bacterial culture to evaluate the efficacy of oral care in students specializing in nursing. Therefore, it may be possible to bacteriologically evaluate the effects of oral care using DAPI.

In addition, the evaluation method used in this study facilitates observation of fluorescence microscopy photographs, in which bacteria are visualized, on a personal computer; therefore, it can also be applied to confirm effective procedures for oral care in nursing education.

3. Significance of this study and indication for nursing

This study showed that the observation of bacteria with DAPI facilitated the objective assessment of the oral state and efficacy of oral care, as it is simple and makes the visualization of the presence of bacteria possible.

Medium culture and identification of bacteria are complex, requiring much time. They do not reflect the oral state

at sampling. Therefore, it is difficult to apply these procedures for clinical care. However, the observation of bacteria with DAPI made it possible to confirm the presence of bacteria and a care-related increase/decrease in the number of bacteria in a short period, suggesting its usefulness for oral-care assessment and the examination of methods/procedures.

Effective oral care and assessment are also important to prevent respiratory infection in elderly persons and severe-status patients. Simple bacteriological evaluation may be significant as an objective oral assessment method.

4. Limitations of this study and future issues

In this study, most oral bacteria were globular or in chains, suggesting the presence of cocci or *Streptococcus*. However, it was impossible to accurately identify the type of bacteria, which is a limitation of this study, raising a future issue.

In the future, the usefulness of our method for oral-care assessment should be established by clarifying changes in the number of bacteria before and after oral care in clinical practice.

V. Conclusion

Using a fluorescent dye, DAPI, oral bacteria were investigated. It was possible to visualize the presence of bacteria in a sample in a short period and count them. This method may be applicable for oral-care assessment.

Acknowledgments

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