Rapid LED-based fluorescence microscopy distinguishes between live and dead bacteria in oral clinical samples

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ABSTRACT

Despite the existence of several methods for the diagnosis of oral infectious diseases, few rapid and quantitative methods exist for discriminating between live and dead bacterial cells in oral clinical samples. In this study, we characterized a light-emitting diode (LED) fluorescence microscopic technique for quantifying live and dead oral bacterial cells stained with 4',6'-deamidino-2-phenyllindole and propidium iodide. Four bacterial strains representative of the human oral microflora were used in this study. In addition, saliva and subgingival fluid specimens were collected from healthy volunteers. Saliva was obtained from the donors without stimulation, whereas subgingival fluid was obtained by inserting a sterile endodontic paper point into the subgingival sites of the first molar. The samples were cultured on agar plates and subjected to LED microscopy. The correlations between both methods were analyzed. The number of live bacterial cells as determined by LED-based fluorescence microscopy and standard colony counts on agar plates correlated well for the known oral bacterial strains and bacterial cells in the clinical specimens. The LED illumination method characterized in this study can be used for the rapid enumeration of living and dead cells. However, to show specificity, this method requires further innovations.

Although more than 700 phylotypes have been detected in the human oral cavity, fewer than 100 are found in a typical individual (1), and their complex interactions result in the formation of biofilms and oral infectious diseases (4, 11), with dental caries and periodontitis being the major ones (2, 17). Bacterial detection from a particular oral region is important for diagnosis, and to date, various methods for the detection of bacteria have been developed (7, 8, 18). The polymerase chain reaction (PCR) is one of the most sensitive and specific methods for determining the prevalence of periodontal bacteria (5, 10, 20–22), but it cannot distinguish between live and dead bacterial cells and is not suitable for the rapid detection necessary in clinical trials.

Bacterial detection methods for living bacteria include standard microbiological colony counts on agar plates, but most bacterial cells in nature are not recovered using culture techniques and many enter into a viable but non-culturable (VBNC) state (12). Therefore, the need exists for a rapid, culture-independent detection method. Culture-independent counting methods are based primarily on various cell properties including membrane permeability, esterase activity, morphological changes, respiratory activity, and nutrient uptake, among others, and several different dyes that allow for the direct visualization of these properties are available.

The membrane permeability of most ionic dyes is not quantitatively diagnostic of cell death but this property can be used as a practical indicator of cell
viability. As such, propidium iodide (PI) is a frequently used ionic dye that permeates damaged cell membranes and combines with DNA to form an easily detected fluorescent compound (13). Therefore, PI is used to detect damaged and dead cells. In contrast, 4′,6′-deamidino-2-phenyllindole (DAPI) is a nonionic dye that permeates both intact and damaged cell membranes to fluorescently label DNA in living and dead cells (6, 9). Therefore, by co-staining with PI and DAPI, the rapid and direct enumeration of both live and dead bacterial cells should be possible. In recent years, Live/Dead® BacLight™ Bacterial Viability Kits (Molecular Probes, Inc., Eugene, OR, USA) have been used for bacterial enumeration and viability testing based on the assessment of membrane integrity by fluorescence microscopy. However, these kinds of microscopic examinations are tedious, time-consuming, and unsuitable for testing large numbers of samples (16).

In this study, we assessed oral bacteria stained with PI and DAPI using a light-emitting diode (LED) detection apparatus. In addition, the utility of this counting method was evaluated using clinical saliva specimens. We believe that this is the first LED illumination method utilizing both ionic and nonionic dyes for the rapid detection and quantification of living and dead oral bacteria.

MATERIALS AND METHODS

Bacterial strains and culture conditions. The bacterial strains used in this study are listed in Table 1. The Porphyromonas gingivalis and Prevotella intermedia strains were cultured anaerobically (10% CO₂, 10% H₂, and 80% N₂) at 37°C in Gifu anaerobic medium (GAM) broth (Nissui Medical Co., Tokyo, Japan) supplemented with hemin (5 μg/mL) and menadione (0.5 μg/mL), or on GAM and agar plates supplemented with hemin (5 μg/mL) and menadione (0.5 μg/mL). Streptococcus mutans and Streptococcus sobrinus were cultured anaerobically (10% CO₂, 10% H₂, and 80% N₂) at 37°C in brain heart infusion (BHI) broth (Difco Laboratories, Sparks, MD) or on BHI agar plates. The bacterial cultures and oral specimens were briefly sonicated to disrupt cellular aggregates before being inoculated onto agar plates and analyzed by LED-non culture method (LED-NCM).

Optical system. The LED-illuminated detection apparatus Bioplorer (Matsushita Ecology Systems, Aichi, Japan) was used (14, 16). This system is composed of two sets of LED light sources and filters (Fig. 1). One set is for DAPI (Wako Pure Chemicals Co., Ltd., Osaka, Japan) and consists of four UV light-emitting diodes, a 375-nm excitation filter, and a 435 to 580-nm absorption filter. The other set is for PI and comprises four green light-emitting diodes, a 525-nm excitation filter, and a 580 to 650-nm absorption filter. The excitation light source is illuminated for 6 s through the excitation filter on the cell trap membrane filter on the cell separation unit (CSU). The fluorescent light emitted from one of 30 sections of the cell trap membrane filter passes through an objective lens and absorption filter and is projected onto a charge-coupled device. All 30 sections were scanned and fluorescent spots in each section were counted (14).

Cell trapping and staining. The CSU is composed of a plastic stand and a 0.2-μm pore size membrane coated with gold by ion sputtering to ensure a clean surface. The CSU was placed on a suction unit and 100 μL of Tween 80 (0.1%, vol/vol) was added onto the prefilter to improve filterability. One milliliter of bacterial culture was added to the CSU and filtered by suction, and the prefilter was washed with physiological saline. Then, 100 μL of solution containing DAPI (1.0 μg/mL) and PI (2.5 μg/mL) was placed on the membrane filter. After incubation for 2 min, the dye solution was removed by suction. Another 100 μL of DAPI (1.0 μg/mL) was added onto the membrane filter to compensate for the relatively weak staining for DAPI relative to that for PI. The DAPI solution was removed immediately by suction. Finally, bacterial cells were rinsed with 0.1 mL of physiological saline (14).

### Table 1: Correlation of cell numbers as determined with the fluorescent filter method and direct colony count for four oral bacterial strains

<table>
<thead>
<tr>
<th>Strains</th>
<th>Correlation coefficients</th>
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<tbody>
<tr>
<td>Porphyromonas gingivalis ATCC33277</td>
<td>$Y = 0.3612X - 80.291$ $R^2 = 0.9504$</td>
</tr>
<tr>
<td>Prevotella intermedia ATCC25611</td>
<td>$Y = 0.4951X + 321.11$ $R^2 = 0.9910$</td>
</tr>
<tr>
<td>Streptococcus mutans UA157</td>
<td>$Y = 1.2058X - 623.65$ $R^2 = 0.9787$</td>
</tr>
<tr>
<td>Streptococcus sobrinus 6715</td>
<td>$Y = 0.8932X - 914.83$ $R^2 = 0.9853$</td>
</tr>
</tbody>
</table>
Fluorescence microscopic analysis for oral bacteria

As representative Gram-negative and Gram-positive oral bacteria, the results of *P. ginvialis* ATCC 33277 and *S. mutans* UA159 were shown in Figure 2. The correlation between the two methods for each of these bacterial strains was very high. Similarly, other Gram-negative and Gram-positive oral bacterial samples were assayed using both LED-NCM and standard culture methods with a good correlation (Table 1). In addition to these strains, *Treponema denticola* ATCC 35404, a periodontopathic oral spirochete, was examined. As this strain does not form colonies on agar plates, we were only able to confirm that LED-NCM can distinguish living from dead/injured cells (data not shown).

**Image analysis program.** The number of DAPI-fluorescent cells \(N_{\text{DAPI}}\) indicated the total number of live and dead cells, whereas the number of PI-fluorescent microbial cells \(N_{\text{PI}}\) indicated only the number of dead cells in a sample. Therefore, the number of live cells was given by \(N_{\text{DAPI}} - N_{\text{PI}}\). \(N_{\text{DAPI}}\) and \(N_{\text{PI}}\) were determined for all 30 sections per membrane (14). Therefore, the total number of viable \(A\), and dead and injured cells \(B\) per membrane were given by the following formulas, respectively:

\[
2 \times \sum_{i=1}^{30} (N_{\text{DAPI}} - N_{\text{PI}}) \quad (A)
\]

\[
2 \times \sum_{i=1}^{30} (N_{\text{PI}}) \quad (B)
\]

**Clinical specimens.** Saliva was obtained from ten healthy donors without stimulation and diluted with phosphate-buffered saline (PBS; 1 : 10). These clinical samples were used for both LED microscopy and culture method (LED-CM) agar plate detections. For the colony counting, a BHI plate (BHI supplemented with 2% agar) was used. Subgingival fluid was obtained from ten healthy donors by inserting a sterile endodontic paper point into the subgingival sites of the first molar for 10 s. The paper point was transferred to 250 μL of PBS in a 1.7-mL tube and vortexed for 5 s. LED and colony counting were then performed on identical samples.

**RESULTS**

**Evaluation of oral bacteria by LED-illuminated detection**

We evaluated the LED-illuminated detection and enumeration technique by comparing the cell numbers detected using the LED non-culture method (LED-NCM) and the traditional counting of colonies on agar plates. As representative Gram-negative and Gram-positive oral bacteria, the results of *P. ginvialis* ATCC 33277 and *S. mutans* UA159 were shown in Figure 2. The correlation between the two methods for each of these bacterial strains was very high. Similarly, other Gram-negative and Gram-positive oral bacterial samples were assayed using both LED-NCM and standard culture methods with a good correlation (Table 1). In addition to these strains, *Treponema denticola* ATCC 35404, a periodontopathic oral spirochete, was examined. As this strain does not form colonies on agar plates, we were only able to confirm that LED-NCM can distinguish living from dead/injured cells (data not shown).

**Fluorescence imaging of oral bacteria from oral specimens**

Human saliva diluted with PBS (1 : 10) was examined by LED-NCM, and the trapped cells were stained with DAPI (Fig. 3A) and PI (Fig. 3B). Cells that emitted both DAPI and PI fluorescence were considered dead while cells that emitted only DAPI fluorescence were considered viable. Our results demonstrate that the number of viable and dead cells in an oral saliva specimen can be rapidly determined simultaneously by LED-NCM.

**Enumeration of the bacterial cells in oral specimens**

Initially, we confirmed the inhibitory effects of saliva on LED-NCM-based enumeration. However, we observed no inhibition of LED-NCM-based enumeration using 10% (vol/vol) saliva (data not shown). Therefore, we concluded that LED-NCM is applicable to enumerating bacteria from saliva. The viable cell numbers determined by LED-NCM and standard culture methods (colony formation units; CFUs) in the same samples were determined with a strong correlation between the two methods (Fig. 4). Using LED-NCM, the average percentage of viable bacterial cells in 52 saliva samples was 55.7 ± 13.3 (mean ± S.D.).

In addition, we analyzed the relationship between the numbers of viable cells and CFUs using subgingival fluid (Fig. 5). The average percentage of viable bacterial cells in 27 subgingival fluid samples was 33.6 ± 22.1 (mean ± S.D.).

**DISCUSSION**

Establishing the relationship between the number of living and/or dead bacterial cells and inflammation
status is needed to obtain a better understanding of oral infectious diseases, and quantifying the bacteria in the oral cavity is essential for achieving this goal. Previously, NCM based on fluorescent staining was reported to be a reliable and useful method for dealing with clean samples containing only microbial cells (6, 15, 19), but few reports on the NCM-based quantification of oral bacteria from oral samples (3). Our study evaluated the enumeration of live and dead cells from oral specimens under a fluorescence microscope.

Initially, we analyzed the relationship between the number of viable cells determined using LED-NMC and standard cell culture methods for four representative strains of oral bacteria. The two methods exhibited linear relationships and high correlation coefficients for each of the four bacterial species. The slopes of the expression ranged from 0.36 to 0.49 for the Gram-negative species and were particularly close to 1 (0.89–1.2) for the Gram-positive species. One possible reason for the discrepancy is that PI stained both dead and injured cells, and the injured cells could still grow on the agar plates.

This would mean that the LED-NMC counted only the viable cells whereas our culture method counted viable and injured cells. Generally, Gram-negative bacteria are more easily injured than Gram-positive bacteria. Consequently, a higher proportion of injured cells would be expected among the Gram-negative than the Gram-positive strains. Previously, Shimakita et al. reported the relationship between cell number determined by LED-NMC and CFU in food samples (14). They also showed the linear relationships and high correlation coefficients for each of the Gram-negative species (including *Escherichia coli*, *Salmonella enteritidis*, *Klebsiella pneumonia* and so on) species. The slopes of the expression ranged from 0.86 to 1.14 for the Gram-negative and Gram-positive species (14). The differences of slopes between oral bacteria and food pathogens are unknown. The differences of treatment condition of specimens might be affected.

After we characterized the LED-NMC, we applied our technique to the detection and quantification of oral bacteria in saliva. Initially, we examined the inhibitory effect of saliva on the number of oral...
bacteria, and no significant differences were observed between bacteria cultured with and without saliva (data not shown). Therefore, we compared the numbers of viable bacterial cells in saliva specimens using LED-NCM and standard culture methods. Both linear relationships and high correlation coefficients were obtained when the two methods were compared for several different saliva samples. Similarly, positive correlations were obtained following the application of our method to subgingival fluid samples. In this study, the number of viable cells determined by LED-NCM was lower than that determined using standard culture methods. As some oral bacteria tend to aggregate in the oral cavity, the samples were briefly sonicated (< 5 s) prior to inoculation onto agar plates. However, many of the cells may have been injured during sonication, and LED-based methods are unable to distinguish between injured cells and dead cells. Thus, the cell counts obtained by LED-NCM may have been too low. To solve these problems, sonication for specimens should be avoided.

Previous studies have not emphasized the viability of bacteria isolated from various oral regions (16), but clarification of the live/dead cell numbers is essential for understanding the relationship between bacterial viability and infectious disease. Therefore, we employed the LED-NCM using DAPI and PI staining. Rapid enumeration is one of the major advantages of LED-NCM. In clinical aspects, rapid diagnosis is essential for treatment trials, but a major disadvantage of LED-NCM is the lack of bacterial specificity. Therefore, the system is probably more suitable for endodontic treatments, especially for fillings, rather than for the diagnosis of periodontal sites. The LED-NCM system also appears applicable to confirming aseptic treatments and/or sterilizations in dentistry.

Finally, the ability to distinguish viable oral bacteria from dead ones is essential for understanding the relationship of live and dead cells with the development, progression, and treatment of oral diseases. For this purpose, the bacterial specificity of LED-NCM must be improved to provide more practical applications in the future.

Acknowledgment

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REFERENCES


Fig. 4 Relationship between the number of bacteria in saliva determined using LED-NCM and standard culture methods ($y = 0.1781x - 1306689, R^2 = 0.9772$).

Fig. 5 Relationship between the number of bacteria in subgingival fluid determined using LED-NCM and standard culture methods ($y = 0.1218x - 5962.9, R^2 = 0.9747$).


