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Monitoring the prevalence of viable and dead cariogenic bacteria in oral specimens and *in vitro* biofilms by qPCR combined with propidium monoazide

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Abstract

Background: *Streptococcus mutans* and *Streptococcus sobrinus* are associated with the development of dental caries in humans. However, previous diagnostic systems are unsuitable for monitoring viable cell numbers in oral specimens. Assessing the relationship between the numbers of viable and dead bacterial cells and oral status is important for understanding oral infectious diseases. Propidium monoazide (PMA) has been reported to penetrate dead cells following membrane damage and to cross-link DNA, thereby inhibiting DNA amplification. In the present study, we established an assay for selective analysis of two viable human cariogenic pathogens, *S. mutans* and *S. sobrinus*, using PMA combined with real-time PCR (PMA-qPCR).

Results: We designed species-specific primer sets for *S. mutans* and *S. sobrinus*, generated standard curves for measuring cell numbers, and evaluated the dynamic range of the assay. To determine the effectiveness of the assay, PMA was added to viable and autoclave-killed cell mixtures. PMA treatment effectively prevented DNA amplification from dead cells. No amplification of DNA from dead cells was observed in these organisms. In addition, we applied this assay to analyze viable cell numbers in oral specimens. A significant correlation was found between the number of viable *S. mutans* cells in saliva and that in plaque among caries-free patients, whereas no correlation was observed between saliva and carious dentin. The total and viable cell numbers in caries-positive saliva were significantly higher than those in caries-free saliva. Finally, we analyzed the usefulness of this assay for *in vitro* oral biofilm analysis. We applied PMA-qPCR for monitoring viable *S. mutans* cell numbers *in vitro* in planktonic cells and oral biofilm treated with hydrogen peroxide (H₂O₂). In planktonic cells, the number of viable cells decreased significantly with increasing H₂O₂ concentration, whereas only a small decrease was observed in biofilm cell numbers.

Conclusions: PMA-qPCR is potentially useful for quantifying viable cariogenic pathogens in oral specimens and is applicable to oral biofilm experiments. This assay will help to elucidate the relationship between the number of viable cells in oral specimens and the oral status.

Keywords: Dental caries, Dental plaque, Biofilm, Propidium monoazide (PMA), Quantification, qPCR (Real-time PCR), *Streptococcus mutans*, *Streptococcus sobrinus*, Viability

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Background

Dental caries represents one of the most common infectious diseases afflicting humans [1]. Of the mutans group of streptococci, *Streptococcus mutans* (serotype *c*, *e*, *f*, and *k* mutans streptococci) and *Streptococcus sobrinus* (serotype *d* and *g* mutans streptococci), which are Gram-positive oral commensal species, are strongly implicated as etiological agents associated with human dental caries. Previous investigations have reported that *S. sobrinus* has a higher acidogenic capacity compared with *S. mutans*, and the prevalence of *S. sobrinus* is more closely associated with high caries activity than is that of *S. mutans* [2,3]. These studies suggest the importance of the diagnoses of infection by these organisms. Previously, several studies have reported methods for diagnosis of these organisms [4,5]. However, DNA-based detection and quantification of specific bacteria cannot distinguish between live and dead bacteria. Bacterial DNA is degraded after the loss of cell viability; thus, the remaining DNA of already dead bacteria can still act as a template DNA for PCR. Consequently, DNA-based detection systems overestimate the cell population. However, we have not differentiated live and dead bacteria within the context of diagnosis of oral infectious diseases, including dental caries. In the present study, we successfully developed and evaluated a discriminative method between live and dead bacteria for the human cariogenic pathogens *S. mutans* and *S. sobrinus* using propidium monoazide (PMA). Previously, ethidium monoazide (EMA) was used for discriminating live from dead bacterial cells [6,7]. EMA is a DNA/RNA intercalating substance that only enters bacterial cells with compromised cell walls and cell membranes. However, EMA was reported to possibly to penetrate viable cells of some bacterial species, resulting in underestimation of viable bacterial numbers [8-11]. Because PMA is less able to penetrate viable cells, more attention has been paid to PMA as an alternative to EMA [8]. In the present study, we examined the population of live and dead bacteria in oral specimens. The relationships of cell viability with saliva and dental plaque or carious dentin were further analyzed. Finally, we analyzed the cell viability of *S. mutans* assessed by this PMA technique after treatment with hydrogen peroxide (H₂O₂) and proposed the usefulness of this technique for biofilm experiments. This is the first report to apply the combination of PMA plus real-time PCR (PMA-qPCR) for analysis of the prevalence of live/dead *S. mutans* cells in oral specimens and to reveal the relationship between cell numbers in saliva and cell numbers in dental plaque and/or carious dentin. Additionally, we applied PMA-qPCR for monitoring viable *S. mutans* cell numbers *in vitro* in planktonic cells and biofilm treated with

various concentrations of H₂O₂ for possible application in biofilm experiments.

Results

Specificities and sensitivities of the qPCR assay

Fifty-two bacterial strains, including *S. mutans* and *S. sobrinus* strains, were tested using primers designed from genome regions specific for the bacterial strains. Each specific primer pair had broad specificity for the *S. mutans* or *S. sobrinus* strains (Table 1). Standard curves for linear regression between the threshold cycle (Ct) values and corresponding colony-forming units (CFU) were obtained by 10-fold serial dilutions of *S. mutans* and *S. sobrinus* cultures. The regression equations for the standard curves for *S. mutans* and *S. sobrinus* were $Y = -2.994X + 35.61$ ($R^2 = 0.9914$) and $Y = -3.230X + 37.73$ ($R^2 = 0.9998$), where $Y = Ct$, $X = \log_{10}x$, and $x = CFU$, respectively (Additional file 1: Figures S1A and S1B). The dynamic ranges were equivalent to 10² to 10⁹ CFU for both *S. mutans* (9.07×10^{-4} to 9.07×10^3 μg of chromosomal DNA) and *S. sobrinus* (2.19×10^{-4} to 2.19×10^3 μg of chromosomal DNA) per reaction mixture.

Effects of PMA and EMA on cell viability

We analyzed the effects of various concentrations of PMA on cell viability. The effects of 2.5 and 25 μM PMA on the viability of 2.77×10^6 CFU of *S. mutans* and 2.85×10^6 CFU of *S. sobrinus* were almost the same as that of 0 μM PMA. After PMA treatment, the bacterial cells were counted. The mean (n=3) values for *S. mutans* and *S. sobrinus* were 2.6×10^6 CFU and 2.4×10^6 CFU, respectively, at 2.5 μM PMA; 2.3×10^6 CFU and 2.27×10^6 CFU, respectively, at 25 μM PMA; and

Table 1 Strains and amplification results for *S. mutans* and *S. sobrinus*

| Strain | Primers used for amplification | | |
|----------------------------|--------------------------------|------------------------------|-----------|
| | <i>S. mutans</i> -specific | <i>S. sobrinus</i> -specific | Universal |
| <i>S. mutans</i> UA159 | + | - | + |
| <i>S. mutans</i> Xc | + | - | + |
| <i>S. mutans</i> MT703R | + | - | + |
| <i>S. mutans</i> MT8148 | + | - | + |
| <i>S. mutans</i> OMZ175 | + | - | + |
| <i>S. mutans</i> NCTC10449 | + | - | + |
| <i>S. mutans</i> Ingbritt | + | - | + |
| <i>S. mutans</i> GS5 | + | - | + |
| <i>S. sobrinus</i> MT8145 | - | + | + |
| <i>S. sobrinus</i> OU8 | - | + | + |
| <i>S. sobrinus</i> OMZ176 | - | + | + |
| <i>S. sobrinus</i> AHT-K | - | + | + |

6.77×10^3 CFU and 1.15×10^6 CFU, respectively, at 250 μ M PMA. Neither 2.5 or 25 μ M PMA treatment had a significant effect on cell viability of either *S. mutans* or *S. sobrinus* (Student's *t*-test; Figure 1A and 1C), whereas 2.5 μ M EMA reduced cell viability of *S. mutans* and *S. sobrinus* by nearly 2.2 log (Figure 1B and 1D). In addition, PCR was not completely inhibited by treatment of dead cells with 2.5 μ M PMA (data not shown). Therefore, we used 25 μ M PMA in this study.

Effect of PMA on defined ratios of viable and heat-killed bacterial suspensions

To examine the effectiveness of PMA treatment at selectively detecting viable cells in the presence of dead cells, various mixtures comprising viable and heat-killed cells were evaluated by qPCR. An aliquot each of *S. mutans* and *S. sobrinus* cells was heated at 121°C for 15 min in an autoclave. The heat-killed cells were mixed with untreated original culture cells in defined ratios, with viable cells representing 0.01%, 0.1%, 1%, or 10% of the total bacteria. In both strains, the signals from 0.01 to 100 μ g of chromosomal DNA were identical in live cells with and without 25 μ M PMA-treated heat-killed cells (Figure 2A and 2B).

Spiking *S. sobrinus* cells with oral specimens

To examine whether PCR was inhibited in the presence of oral specimens, chromosomal DNA from *S. sobrinus*-free saliva and plaque specimens was added to *S. sobrinus* cells. The qPCR analysis of *S. sobrinus* was not inhibited by chromosomal DNA from saliva (Figure 3A) or plaque (Figure 3B).

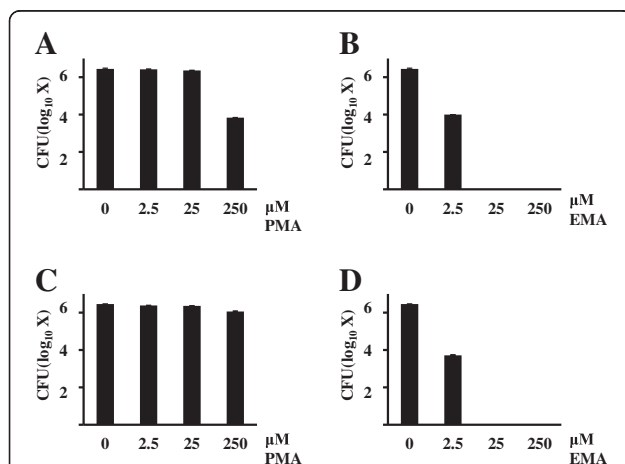


Figure 1 Effects of PMA (A and C) and EMA (B and D) on *S. mutans* and *S. sobrinus* cell viability. A total of (A and B) 2.77×10^6 CFU of *S. mutans* and (C and D) 2.85×10^6 CFU of *S. sobrinus* were treated with 0, 2.5, 25, and 250 μ M PMA and cross-linked. After PMA and EMA treatments, bacterial cells were plated on Mitis-Salivarius agar plates and counted. The mean and S.D. values of independent triplicate data are shown.

Correlation of viable *S. mutans* cell number assessed by PMA-qPCR and by culture

We compared the *S. mutans* cell number in dental plaque from caries-free patients (n=24) with that from patients with carious dentin (n=21) by qPCR with and without PMA and culture. Positive correlations were observed between the cell number detected by PMA-qPCR and that determined by culture for both caries-free dental plaque (Figure 4A) and carious dentin (Figure 4C). The positive correlations between qPCR and culture are shown in Figure 4B (dental plaque) and 4D (carious dentin). The slopes of the regression equations were lower for qPCR than for PMA-qPCR, indicating that the cell number determined by qPCR was higher than that determined by PMA-qPCR.

Quantitative discrimination of live/dead cariogenic bacterial cells in oral specimens

The numbers of *S. mutans* and *S. sobrinus* cells in carious dentin and saliva were quantified in patients with dental caries. As shown in Figure 5A, the mean totals of *S. mutans* cells (\pm S.D.) calculated by qPCR without PMA were 1.47×10^6 ($\pm 6.88 \times 10^5$) per 1 mg dental plaque (wet weight) from caries-free donors (n=24) and 1.48×10^6 ($\pm 7.80 \times 10^5$) per 1 mg carious dentin (wet weight)

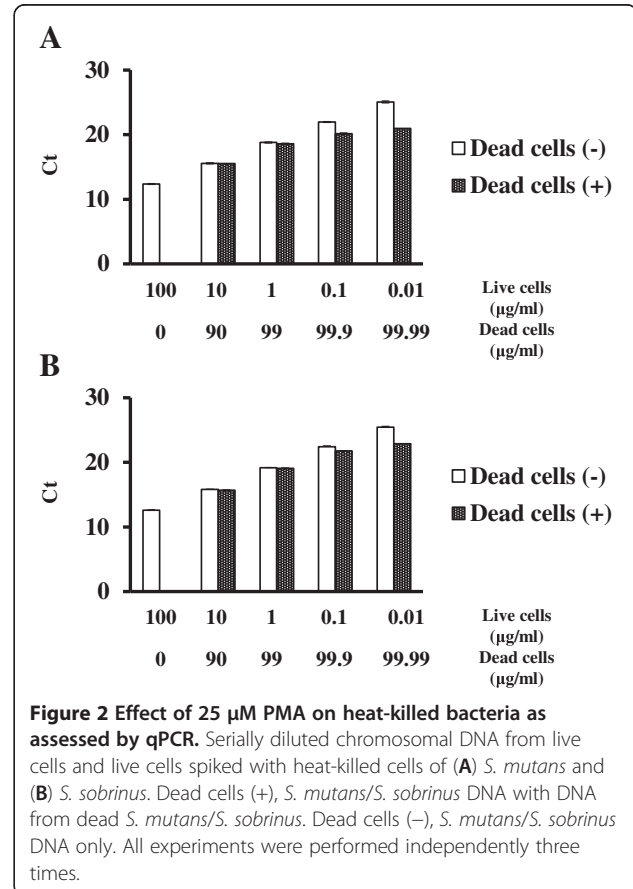
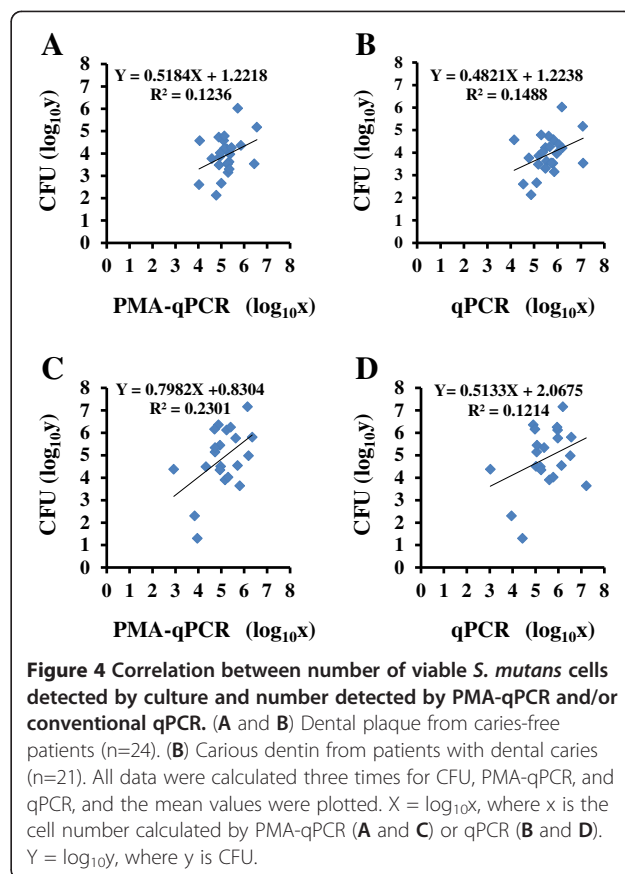
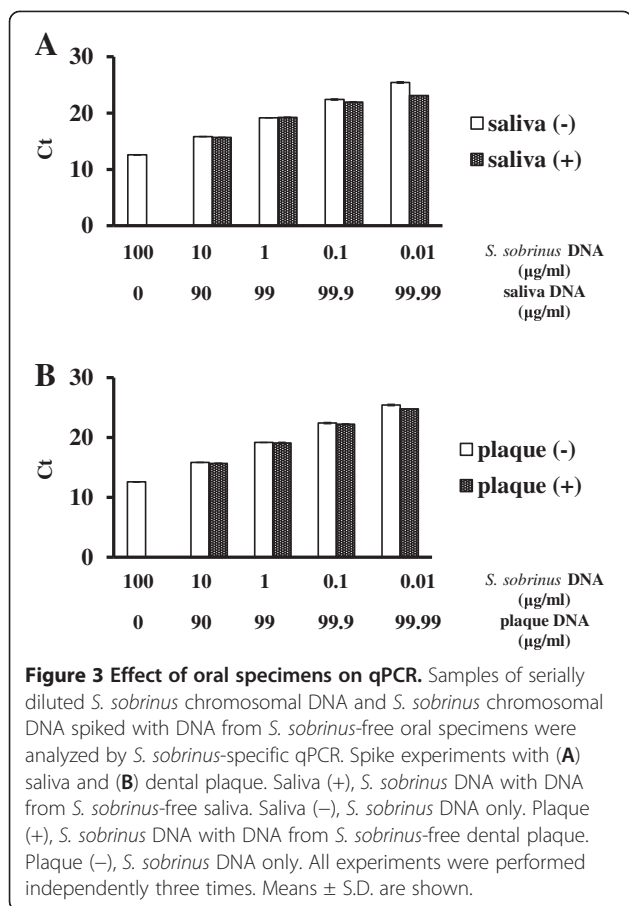


Figure 2 Effect of 25 μ M PMA on heat-killed bacteria as assessed by qPCR. Serially diluted chromosomal DNA from live cells and live cells spiked with heat-killed cells of (A) *S. mutans* and (B) *S. sobrinus*. Dead cells (+), *S. mutans*/*S. sobrinus* DNA with DNA from dead *S. mutans*/*S. sobrinus*. Dead cells (-), *S. mutans*/*S. sobrinus* DNA only. All experiments were performed independently three times.



(n=21); viable cell numbers calculated by qPCR with PMA were $3.98 \times 10^5 (\pm 1.27 \times 10^5)$ per 1 mg carious dentin (wet weight) and $3.86 \times 10^5 (\pm 1.33 \times 10^5)$ per 1 mg dental plaque (wet weight), representing 26.9% and 29.5% of the total cells, respectively (Figure 5A). There was no significant difference in viable cell number or total cell number between caries dentin and plaque (Mann–Whitney test).

Next, we compared the number of cells in saliva from patients with and without dental caries. The mean totals of *S. mutans* cells (\pm S.D.) calculated by qPCR were $4.24 \times 10^8 (\pm 2.38 \times 10^8)$ per 1 ml of saliva from patients with dental caries (n=21) and $1.02 \times 10^8 (\pm 5.07 \times 10^7)$ per 1 ml of saliva from caries-free donors (n=24); viable cell numbers calculated by qPCR with PMA were $1.68 \times 10^8 (\pm 1.06 \times 10^8)$ per 1 ml of saliva from patients with dental caries (n=21) and $4.45 \times 10^7 (\pm 3.31 \times 10^7)$ per 1 ml of saliva from caries-free donors (n=24), representing 39.6% and 43.6% of the total cells, respectively (Figure 5B). Total cell number and viable cell number differed significantly between caries-positive and -negative saliva ($p < 0.05$ for each; Mann–Whitney test).

Streptococcus sobrinus was detected in only one patient with dental caries (data not shown). The total numbers of *S. sobrinus* cells calculated by qPCR without PMA were

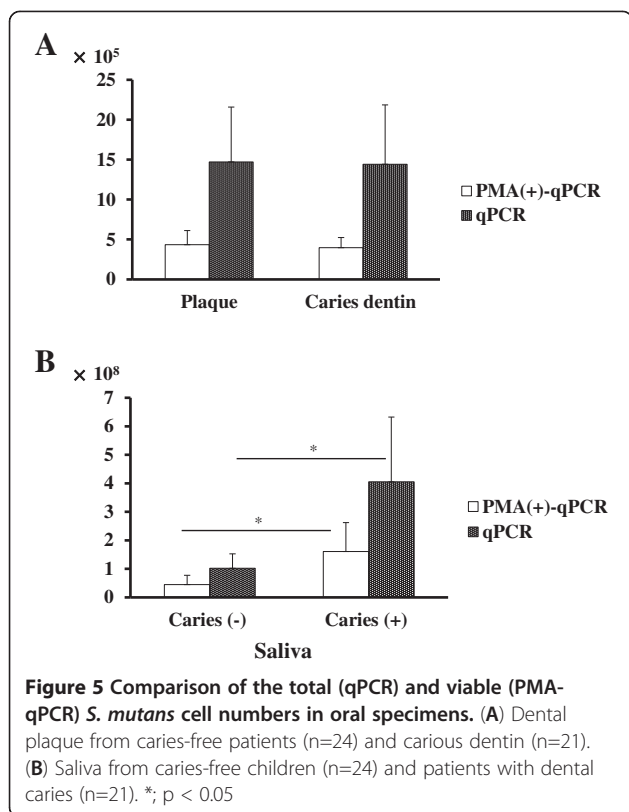
8.14×10^7 CFU per 1 ml of saliva (32.5% were live cells) and 1.58×10^9 CFU per 1 mg carious dentin (7.84% were live cells).

Correlation of viable *S. mutans* cell number among oral specimens

The correlations of viable cell number between saliva and caries-free plaque and/or carious dentin were examined. Among caries-free patients, the number of viable *S. mutans* cells in saliva was significantly correlated with the number in plaque (n=24, Figure 6A). No correlation was observed between saliva and carious dentin (n=21, Figure 6B).

Application of PMA-qPCR for monitoring live bacteria in biofilm and the planktonic phase

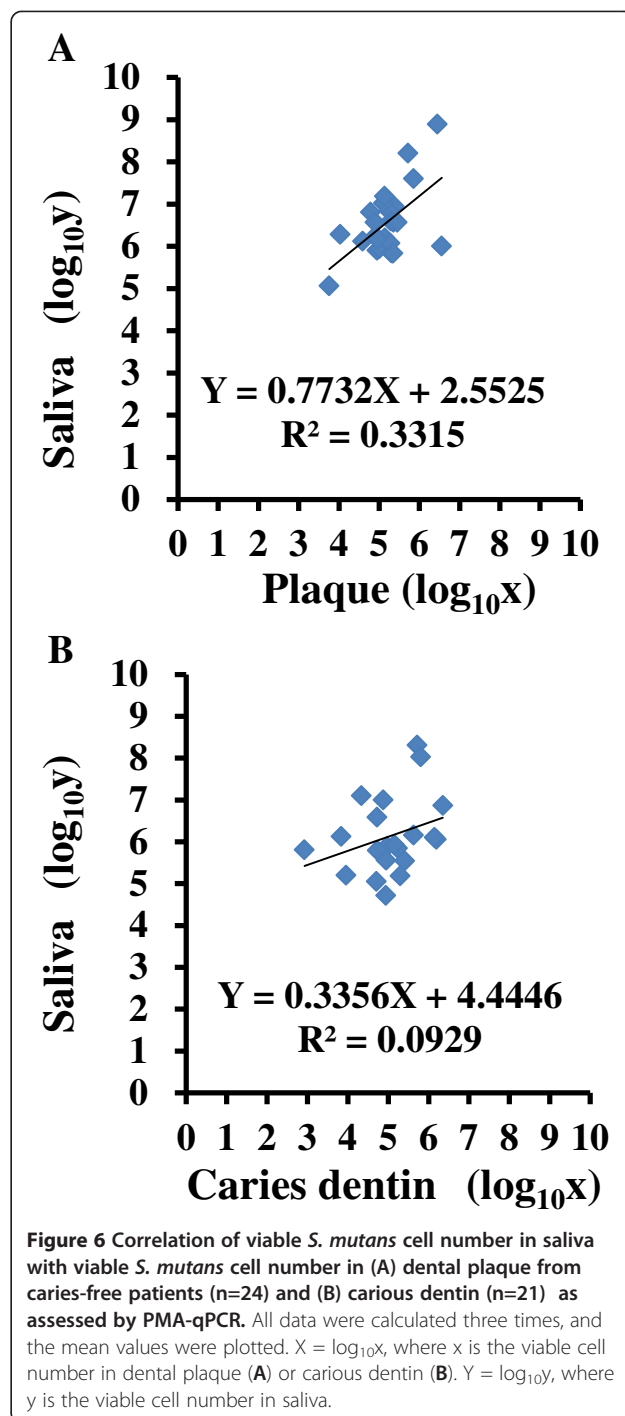
One purpose for the development of this assay was to monitor the viable cell number in biofilm. To evaluate the *S. mutans* cell number in both planktonic and biofilm forms, the cells were exposed to various concentrations of H_2O_2 . In the planktonic phase, the number of viable *S. mutans* cells in 0.0003% H_2O_2 was only 10.0% of the number in H_2O_2 -untreated cells, whereas the number in 0.003% H_2O_2 was 34.7% of that in H_2O_2 -untreated cells (Figure 7A). There was a significant



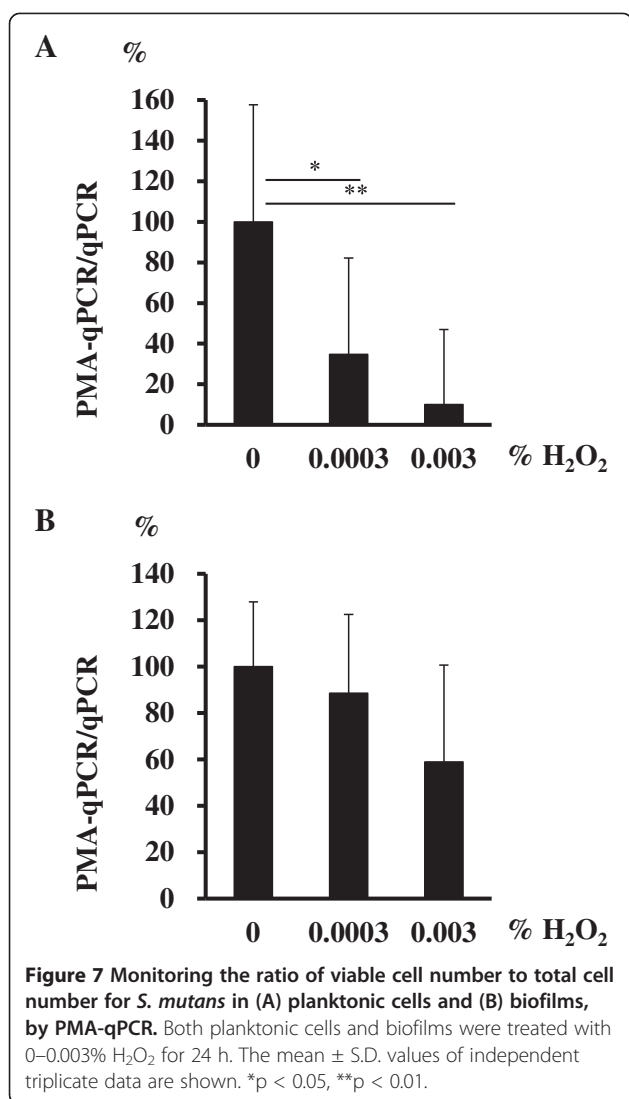
difference in the viable/total cell ratio between 0% and 0.0003% H₂O₂ (Bonferroni test; p < 0.05) and between 0% and 0.003% H₂O₂ (Bonferroni test; p < 0.01). In biofilm, the number of viable *S. mutans* cells in 0.0003% H₂O₂ was 88.6% of the number in H₂O₂-untreated cells, whereas that in 0.003% H₂O₂ was 58.9% of that in H₂O₂-untreated cells (Figure 7B). There was no significant difference in the viable/total cell ratio between 0% and 0.0003% H₂O₂ or between 0% and 0.003% H₂O₂.

Discussion

Streptococcus mutans and *S. sobrinus* are considered to be cariogenic pathogens in humans [12]. Various studies have monitored the prevalence of caries-related organisms in oral specimens [13]. However, attempts to differentiate between viable and dead bacteria in oral specimens in relation to dental caries have not been reported. In the present study, we initially developed a quantification method for discriminating live and dead cariogenic bacteria, specifically for *S. mutans* and *S. sobrinus*. Previous investigations have reported that EMA has a strong inhibitory effect on the amplification of genomic DNA from viable cells [11], and our study confirmed that EMA itself decreases cell viability. Therefore, all experiments were conducted with PMA, which penetrates a damaged cell membrane and intercalates into DNA, resulting in the inhibition of PCR, in combination with qPCR to



quantitatively differentiate between viable and dead cells. We further performed a spiking experiment to evaluate whether this assay was applicable to oral specimens. In general, obtaining oral specimens that do not contain *S. mutans* is challenging, whereas obtaining *S. sobrinus*-free oral samples is relatively easy. For this reason, we performed PMA-qPCR amplification specific for *S. sobrinus* using *S. sobrinus*-free saliva and *S. sobrinus*-free



dental plaque as an alternative in the spiking experiment. As shown in Figure 3, neither saliva nor dental plaque inhibited the PCR, indicating that this assay is applicable for measuring cariogenic bacteria in oral specimens.

We next examined the correlation between the numbers of viable *S. mutans* cells in oral specimens as detected by PMA-qPCR and by culture. We found a positive correlation between these quantification methods for both carious dentin and dental plaque. Compared with culture, the number of viable *S. mutans* cells was overestimated by PMA-qPCR. It may be that the culture method usually underestimates the cell number. The cell number determined by conventional qPCR correlated with the cell number determined by culture. Several previous investigations have reported that the cell number determined by qPCR correlated with CFU [14,15]. However, compared with PMA-qPCR, conventional qPCR overestimated the cell number to a greater extent in both types of clinical

specimens. Therefore, the cell culture count was closer to the number determined by PMA-qPCR than to that determined by conventional qPCR in the present study.

Monitoring viable bacterial cells in oral specimens provides information to help understand oral infectious diseases. When we compared the total and viable cell numbers in carious dentin from patients with dental caries and dental plaque from caries-free children, there was no significant difference between carious dentin and dental plaque in terms of either total number *S. mutans* cells or number of viable cells. We may not be able to simply compare the cell numbers in these specimens because the contents are not identical. Nevertheless, there was no significant difference in the percentage of viable cells between the specimens. However, there was a significant difference in total cell number and viable cell number between saliva from patients with dental caries and saliva from caries-free children. Monitoring of the viable cell number in relation to the total cell number in oral specimens has not previously been performed. To understand the variation in the viable cell number, both the viable and total cell numbers must be determined. To further understand cell viability in relation to dental caries, a greater number of specimens should be analyzed.

When the relationship between the number of viable *S. mutans* cells in saliva and in dental plaque from caries-free children was analyzed using PMA-qPCR, a positive correlation was found between viable *S. mutans* cells in saliva and in dental plaque. This result was consistent with previous reports [16]. There was no significant correlation between the number of viable *S. mutans* cells in saliva and that in carious dentin from caries patients in the present study. Our data suggest that saliva reflects the number of viable cells in caries-free plaque, but not in carious dentin. Previous studies have found that the microbial biota in the dental plaque of children with severe early-childhood caries was significantly less diverse and less complex than that in caries-free children [17]. Therefore, the viability of cariogenic bacteria in saliva may differ between caries-active and caries-free patients. This possibility should be explored in future studies.

Finally, we evaluated the number of viable of *S. mutans* cells in the planktonic phase and in biofilm. In the planktonic phase, the ratio of viable cells to total bacteria decreased with an increase in H₂O₂ concentration (34.7% at 0.0003% H₂O₂ and 10.0% at 0.003% H₂O₂). There was a significant difference in the viable/total bacterial ratio between 0% and 0.0003 and between 0% and 0.003% H₂O₂. However, the decreases in the viable/total cell ratio in biofilm at these concentrations were smaller (88.6% at 0.0003% H₂O₂ and 58.9% at 0.003% H₂O₂), and there was no significant difference between 0% and 0.0003 or 0.003% H₂O₂. These results suggest that PMA-qPCR is applicable for monitoring the numbers of viable and dead cells in

biofilm. In biofilm experiments, a live/dead stain is sometimes used to distinguish visually between live and dead bacteria [18]. Although PMA-qPCR is advantageous for quantifying viable cells, it does not provide the visualization obtained with live/dead staining. PMA-qPCR may be a powerful tool for monitoring the number of viable cells in oral biofilms.

Conclusions

We developed a discriminative quantification method for viable and dead *S. mutans* and *S. sobrinus* cells. We evaluated the potential of this assay and applied it to analyze the prevalence of live/dead cariogenic bacteria in oral specimens and to monitor live/dead cells in biofilm experiments. The ability to discriminate between live and dead bacterial cells in biofilm is essential for studying biofilm, and this assay will be helpful for oral biofilm research. Our assay will contribute to elucidating the role of viable bacteria in oral biofilm and saliva in relation to disease activities.

Methods

Reference strains

The 52 reference strains used in the present study were *S. mutans* UA159, *S. mutans* Xc, *S. mutans* MT703R, *S. mutans* MT8148, *S. mutans* OMZ175, *S. mutans* NCTC10449, *S. mutans* Ingbritt, *S. mutans* GS5, *S. sobrinus* MT8145, *S. sobrinus* OU8, *S. sobrinus* OMZ176, *S. sobrinus* AHT-K, *Streptococcus S. downei* Mfe28, *S. downei* S28, *Streptococcus ratti* BHT, *S. ratti* FA1, *Streptococcus cricentus* HS1, *S. cricentus* E49, *Streptococcus mitis* 903, *Streptococcus sanguinis* ATCC 10556, *S. sanguinis* ATCC 10557, *S. sanguinis* OMZ9, *Streptococcus gordonii* DL1, *Streptococcus oralis* ATCC 557, *Streptococcus salivarius* HHT, *Streptococcus anginosus* FW73, *Streptococcus milleri* NCTC10707, *Lactobacillus rhamnosus* JCM1136, *L. rhamnosus* JCM1561, *L. rhamnosus* JCM1563, *L. rhamnosus* JCM8134, *L. rhamnosus* JCM8135, *L. rhamnosus* JCM8135, *Lactobacillus casei* JCM8132, *Porphyromonas gingivalis* W83, *P. gingivalis* 33277, *Treponema denticola* ATCC 35405, *Treponema medium* ATCC 700293, *Treponema vincenti* ATCC 35580, *Treponema pectinovorum* ATCC 33768, *Aggregatibacter actinomycetemcomitans* Y4, *A. actinomycetemcomitans* JCM8577, *A. actinomycetemcomitans* SUNYaB67, *A. actinomycetemcomitans* SUNYaB75, *Aggregatibacter naeslundii* JCM8350, *Prevotella loescheii* JCM8530, *Prevotella denticola* JCM8525, *Prevotella bivia* JCM6331, *Prevotella pallens* JCM1140, *Prevotella veroralis* JCM6290, and *Prevotella oralis* ATCC 33322.

Ethics statement

All patients were treated in accordance with the Helsinki Declaration regarding the participation of human subjects in medical research. Ethics clearance for the study was obtained from the Ethics Committee of Kyushu

Dental University Hospital (reference number 11–40). The parents of participants were fully informed about the study and signed informed consent forms.

Study subjects and oral specimen sampling

Twenty-one subjects ranging in age from 3 to 10 years and who had dental caries were included in the caries group (mean age \pm S.D. = 7.86 ± 0.43 years; 11 males and 10 females). A healthy (completely caries-free) control group consisted of 24 subjects (ages 3 to 12 years; mean age \pm S.D. = 7.29 ± 0.56 years; 13 males and 11 females). The carious dentin was excavated from cavitated lesions. Before excavation of the carious dentin, the plaque on the surfaces of cavitated lesions was swiped. The dental plaque samples from healthy subjects were collected from the buccal or lingual surface of the second primary molar. Collected carious dentin and dental plaque were placed in 200 μ l of PBS in a sterile 1.5-ml microcentrifuge tube. These samples were washed and placed in PBS solution adjusted to 1 mg per 100 μ l. Saliva was collected from both the caries and healthy control groups. Fifty microliters of saliva was washed with PBS and used for analysis.

Bacterial counting from oral specimens on an agar plate

Serially diluted carious dentin or dental plaque was plated on a Mitis-Salivarius agar plate (Becton Dickinson, Franklin Lakes, NJ) supplemented with 150 g/l sucrose and 200 U/l bacitracin for selection of mutans streptococci (MSB agar). Bacterial counting was performed using a magnifying loupe.

Propidium monoazide treatment

For only viable cell quantification, PMA (3-amino-8-azido-5-[3-(diethylmethylammonio)propyl]-6-phenyl dichloride; Wako Pure Chemical, Osaka, Japan) treatment was performed for bacterial cells prior to DNA extraction, as previously described [19]. Briefly, PMA was dissolved in 20% DMSO to produce a 25-mM stock solution. Following incubation with the dye for 5 min in the dark, similarly prepared cells were exposed for 5 min to a 500-W halogen light placed 15 cm above 500- μ l samples in open microcentrifuge tubes on ice. The toxicity

Table 2 Oligonucleotide primers used in this study

| Primer | Sequence (5'→3') | Locus | Reference |
|-------------|--------------------|----------|-----------|
| Sm F | GTATGCAGGTGAAGAAGC | Sm3-15 | [5] |
| Sm R | TGGCCACACAATGACCAT | | |
| Ss F | TGGCATTGCCGTAGGAAT | Ss6 | [5] |
| Ss R | TTGTGCACGGGTTCTAGT | | |
| Universal F | TCCTACGGGAGGCAGCAG | 16S rRNA | [21] |
| Universal R | GGACTACCAGGGTATCTA | 16S rRNA | |

F forward primer; R reverse primer.

of PMA at 2.5–250 μM to *S. mutans* and *S. sobrinus* was analyzed at 37°C. In the present study, 25 μM PMA was employed for the analysis. All data presented are from triplicate independent cultures and/or biofilms.

DNA techniques

Routine molecular biology techniques were performed as described by Sambrook *et al.* [20]. Chromosomal DNA was isolated from the bacteria using a Puregene DNA isolation kit (Gentra Systems, Minneapolis, MN). Bacterial chromosomal DNA from oral specimens was isolated using MORA-extract (Cosmo Bio, Tokyo, Japan). Next, 150 μl of lysis buffer was added to the pellet. The lysed bacteria were transferred to a tube with glass beads and heated at 90°C for 10 min. The bacterial mixture was then disrupted using a Mini-Bead Beater (BioSpec Products, Bartlesville, OK) with 0.1-mm-diameter glass beads at 4,800 rpm for 2 min. Thereafter, 200 μl of SDS solution was added and heated at 90°C for 10 min. Next, 400 μl of phenol solution was added and mixed for 1 min. After centrifugation, the aliquot was subjected to ethanol precipitation and dissolved in 20 μl of TE buffer.

qPCR

To monitor cell numbers, qPCR was performed with *S. mutans*- and *S. sobrinus*-specific primers designed using Primer Express 3.0 software (Applied Biosystems, Foster City, CA). The primers specific for *S. mutans* and *S. gordonii* are shown in Table 2. A universal primer was used for confirmation of the presence of chromosomal DNA (Table 2). For confirmation of primer specificities, conventional PCR was performed using the following thermocycle: 95°C for 5 min, followed by 25 cycles of 95°C for 30 s, 47°C for 30 s, and 72°C for 1 min. Quantification of these cells in oral specimens and *in vitro* biofilm was performed using qPCR with the SYBR green dye to detect the Sm3-15 locus (for *S. mutans*) and Ss6 locus (for *S. sobrinus*) amplicons [5]. Bacterial chromosomal DNA was amplified using LightCycler FastStart DNA Master^{PLUS} SYBR Green I (Roche Diagnostics GmbH, Mannheim, Germany). Each reaction mixture (total 20 μl) contained 5 μl of DNA (10 ng/ μl), 4 μl of 5 \times Master Mix, 2 μl each of forward and reverse primer (500 nM each), and 9 μl of pure water. The mixtures were applied to a LightCycler Capillary (Roche Diagnostics). Amplification and detection of specific products were performed using the LightCycler Carousel-based System (Roche Diagnostics) and the following thermocycle: 95°C for 10 min, followed by 45 cycles of 95°C for 10 s, 58°C for 10 s, and 72°C for 12 s. Dissociation curves were generated using the following conditions: 95°C for 1 min, 55°C for 1 min, and then an increase in temperature from 55.0 to 95.0°C with a heating rate of 0.5°C per 10 s. The melting

curves with both primer sets showed a single sharp peak (data not shown). DNA concentrations were calculated based on standard curves obtained using 10-fold serial dilutions of bacterial DNA. All data are shown as the mean of triplicate experiments.

Preparation of biofilms and planktonic cells

To examine *S. mutans* strains for the ability to form biofilm under various H₂O₂ concentrations (serially diluted from 0–3%), the biofilm assay was performed. Bacterial cells were precultured overnight in chemically defined medium (CDM) supplemented with 0.5% sucrose, inoculated into 1 ml of 0.5% sucrose CDM (culture:CDM ratio, 1:50), and then incubated for 24 h under anaerobic conditions at 37°C in polystyrene 24-well plates (Corning, Inc., Corning, NY) with final H₂O₂ concentrations of 0–0.03% [22]. The viable cell/total cell ratio in 0% H₂O₂ was considered to be 100%.

Statistics

The Mann–Whitney test and Bonferroni's test were used to determine statistical significance. A difference was deemed significant at $P < 0.05$.

Additional file

Additional file 1: Figure S1. Standard curves for the qPCR assay were generated by the bacterial cell number and Ct value. (A) *S. mutans*. (B) *S. sobrinus*. The mean values of independent triplicate data are shown.

Abbreviations

EMA: Ethidium monoazide; PMA: Propidium monoazide; qPCR: quantitative polymerase chain reaction.

Authors' contributions

AYo participated in the study design, wrote the manuscript, and was responsible for the overall coordination of the study. AYa and SN performed the microbiological analysis, DNA manipulation, and PMA-qPCR analysis. KMo and KMa performed clinical examinations and sampling of oral specimens. IS and SA conducted statistical analyses. TA supervised this study.

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