ELSEVIER

FEMS Microbiology Letters 217 (2002) 23–30

FEMS MICROBIOLOGY Letters

www.fems-microbiology.org

Real-time PCR for quantification of *Streptococcus mutans*

Akira Yano [a,*], Noboru Kaneko [b], Hirohisa Ida [c], Toshikazu Yamaguchi [d], Nobuhiro Hanada [a]

[a] *Department of Oral Health, National Institute of Public Health, 1-23-1 Toyama, Shinjuku-ku, Tokyo 162-8640, Japan*
[b] *Department of Oral Health Science, Graduate School of Medical and Dental Sciences, Niigata University, 2-5274 Gakkoutyo-doori, Niigata-shi, Niigata 951-8514, Japan*
[c] *Department of Bacteriology, BML, Inc., 1361-1 Matoba, Kawagoe-shi, Saitama 350-1101, Japan*
[d] *Division of Clinical Development, R&D Center, BML, Inc., 1361-1 Matoba, Kawagoe-shi, Saitama 350-1101, Japan*

Received 18 May 2002; received in revised form 6 September 2002; accepted 11 September 2002

First published online 18 October 2002

Abstract

A real-time polymerase chain reaction (PCR) assay was developed for the quantification of *Streptococcus mutans*. Primers targeting *gtf* genes of *S. mutans* were designed and tested for their specificity using 28 oral streptococcal strains, three other bacterial strains, and human DNA. The primers could amplify specifically the target DNA fragment from a mixture of oral streptococcus genomic DNA containing about 10 fg to 10 ng of *S. mutans* genome DNA. The real-time PCR produced a linear quantitative detection range over concentrations spanning seven exponential values, with a detection limit of a few copies of *S. mutans*' genomic DNA per reaction tube. The results of the real-time PCR assay corresponded well to those of conventional culture assays for *S. mutans* in saliva samples. A real-time PCR assay for *Streptococcus sobrinus* and *Streptococcus downei* was also established and produced results that corresponded well to those from conventional culture assays for *S. sobrinus* in saliva samples. These assays will be useful as a new means to assess one of the important risk factors for caries.

Keywords: Real-time PCR; *S. mutans*; *S. sobrinus*; Saliva; Glucosyltransferase

1. Introduction

Several oral streptococci possess glucosyltransferases (GTFs). The GTFs of mutans streptococci are related to the bacteria's cariogenicity; in particular, water-insoluble glucan-synthesizing GTFs (GTF-I and GTF-SI) are correlated with cariogenicity [1,2]. GTF-I enzymes are encoded by the *gtfB* gene of *Streptococcus mutans* and the *gtfI* gene of *Streptococcus sobrinus* and *Streptococcus downei* [3–6]. GTF-SI is encoded by the *gtfC* gene of *S. mutans* [7]. The products of *gtfB* and *gtfI* synthesize water-insoluble glucans, and the product of *gtfC* synthesizes both water-soluble and -insoluble glucans. These glucans provide firm footholds on the tooth surface for mutans streptococci and contribute to the formation of cariogenic dental plaques [1,2].

The levels of mutans streptococci in saliva have been shown to be a means of predicting both caries activity [8,9] and the transmission risk of the mutans streptococci, which from mother to child can be an important etiological factor of caries [10,11]. However, the quantification of mutans streptococci is laborious, and so far levels of mutans streptococci have not been used as an established index of caries diagnosis. The standard medium used for isolating mutans streptococci, Mitis–Salivarius–Bacitracin (MSB) agar [12], does not have the selectivity, that is necessary for morphological discrimination of the colonies to identify the species of mutans streptococci. Development of a practical assay for mutans streptococci is necessary to establish quantification of these bacteria as a new index of caries risk. Some laboratories have reported improvements of the MSB medium and have discussed new selective media for mutans streptococci [13–15]. Hence, a conventional MSB medium is usually used as the standard method for isolating and quantifying mutans streptococci.

Polymerase chain reaction (PCR) is a powerful tool for the detection and quantification of bacteria. Real-time

* Corresponding author. Fax: +81 (3) 5285 1172.
E-mail address: akiray@nih.go.jp (A. Yano).

0378-1097/02/$22.00
PII: S0378-1097(02)01028-5

PCR techniques are increasingly used in diagnosis, especially for anaerobic bacteria and viruses [16,17]. PCR assays for detecting mutans streptococci are more specific than conventional culture methods [18,19]. However real-time quantitative PCR has not been popular for mutans streptococci. One of the reasons may be the difficulty in isolating DNA from mutans streptococci, owing to the hard outer envelope of these Gram-positive bacteria. In general, rapid methods of DNA isolation, such as those based on boiling and hot phenol, have merits for quick detection by conventional PCR, but the recovery rate and/or quality of the DNA seems to be insufficient for direct quantification by real-time PCR [20,21]. In this study, we have established two quantitative real-time PCR assays targeting *gtf* genes of mutans streptococci, one for *S. mutans* and the other for *S. sobrinus* and *S. downei*, by adapting the glass beads method of DNA isolation.

2. Materials and methods

2.1. Bacterial strains and DNA

Bacterial strains used in this study are listed in Table 1. Human genomic DNA was obtained from Promega (Tokyo, Japan). To determine the PCR conditions, we used a DNA mixture that modeled the DNA template extracted from saliva, containing 5 ng of human genomic DNA and 5 ng of extracted DNA from the bacteria listed in Table 1 except target bacteria in a PCR tube. The nucleotide and its deduced amino acid sequences {*gtfB* (accession numbers M17361, D88651, D88654, D88657, D88660, D89977), *gtfC* (M17361, M22054, D88652, D88655, D88661, D89978), and *gtfD* (D89979) of *S. mutans*; *gtfI* (D63570, D13858, D90213) and *gtfT* (D13928) of *S. sobrinus*; *gtfI* (M17391) and *gtfS* (M30943) of *S. downei*; *gtfG* (U12643) of *Streptococcus gordonii*; *gtfI*, *gtfK* (Z11873), *gtfL* (L35495), *gtfM* (L35928), and *gtfN* (AF049609) of *Streptococcus salivarius*; and *gtfR* (AB025228) of *Streptococcus oralis*} were aligned by CLUSTAL X [22] and a primer pair specific to *S. mutans* and a primer pair common to *S. sobrinus* and *S. downei* (Table 2) were selected. PCR primers Sm F5 and Sm R4 anneal to conserved regions of *gtfB* and *gtfC* genes of *S. mutans*, and amplify 415-bp DNA fragments from both genes. PCR primers Ss F3 and Ss R1 anneal to conserved regions of *gtfI* genes of *S. sobrinus* and *S. downei*, and amplify 329-bp DNA fragments from those bacteria (Table 2). Both primer pairs were designed to amplify the regions that correspond to a section of the conserved

Table 1
Bacterial strains used in this study

Strain no.	Species group in streptococci	Species and strain	Note
1	*mutans*	*S. mutans* ATCC25175	serotype c, type strain
2		*S. mutans* MT8148	serotype c
3		*S. mutans* LM7	serotype e
4		*S. mutans* MT6229	serotype f
5		*S. sobrinus* ATCC33478	serotype d, type strain
6		*S. sobrinus* OMZ176	serotype d
7		*S. sobrinus* 6715	serotype g
8		*S. sobrinus* AHT	serotype g
9		*S. cricetus* ATCC19642	serotype a, type strain
10		*S. downei* ATCC33748	serotype h, type strain
11		*S. ferus* ATCC33477	serotype c, type strain
12		*S. macacae* ATCC35911	serotype c, type strain
13		*S. ratti* ATCC19645	serotype b, type strain
14	*anginosus*	*S. anginosus* ATCC33397	type strain
15		*S. constellatus* ATCC27823	type strain
16		*S. intermedius* ATCC27335	type strain
17	*mitis*	*S. gordonii* ATCC10558	type strain
18		*S. mitis* GTC495	type strain
19		*S. mitis* ATCC6249	
20		*S. oralis* ATCC35037	type strain
21		*S. pneumoniae* GTC261	type strain
22		*S. sanguinis* ATCC10556	type strain
23	*pyogenic*	*S. agalactiae* GTC1234	type species, type strain
24		*S. pyogenes* JCM262	type strain
25	*salivarius*	*S. salivarius* JCM5707	type strain
26		*S. salivarius* ATCC9759	
27		*S. salivarius* HHT	
28		*S. salivarius* HT9R	
29		*S. thermophilus* NRIC0256	
30	–	*Enterococcus faecalis* JCM5803	type species, type strain
31	–	*Escherichia coli* DH5α	type species

Table 2
Nucleotide sequences, positions, and melting temperatures of the primers used in this study

Primer	Sequence (5′–3′)	Position	T_m[a] (°C)	Fragment size (bp)
Sm F5	AGCCATGCGCAATCAACAGGTT	2007–2028[b]	69	415
Sm R4	CGCAACGCGAACATCTTGATCAG	2421–2399[b]	70	
Ss F3	GAAACCAACCCAACTTTAGCTTGGAT	2123–2148[c]	69	329
Ss R1	ATGGAGTGATTTTCCATCGGTACTTG	2451–2426[c]	69	

[a]Melting temperature calculated by Primer Express (Applied Biosystems) at 1 μM of each primer.
[b]The position of the nucleotide sequence from the ATG codon of the *gtfB* gene (accession number: D88651).
[c]The position of the nucleotide sequence from the ATG codon of the *gtfI* gene (accession number: M17391).

catalytic domain of GTF-I and -SI [4,6]. Primers were purchased from Invitrogen (Tokyo, Japan).

2.2. Human saliva samples

Paraffin-stimulated saliva samples were collected for 3 min from the students of an elementary school. The samples were immediately stored on ice and transferred to the laboratory within 1 day. A portion of each sample was immediately used for the colony counting assay, and the rest was stored at −20°C. Informed consent was received from the children and their parents.

2.3. Enumeration of bacteria by colony counting

MSB [12] and modified MSB agar plates were obtained from BML (Tokyo, Japan). Modified MSB improved the sensitivity and the selectivity of mutans streptococci (Patent No. JP2002027975). 50 μl of saliva and 10-fold dilution series were plated by a spiral-plating machine (EDDY JET, IUL USA Inc., KY, USA). Plates were incubated at 37°C for 48 h using a simple anaerobic culture system (Anero Pack, Mitsubishi Gas Chemical Co. Inc.). Each colony on the modified MSB was tested for its biochemical characteristics [23], as well as its ability to cause glucan-dependent binding to the tube wall [24]. The standard deviations of these culture assays were determined by analysis of five saliva samples for the MSB culture and modified-MSB samples carried out in triplicate before the assays of children's saliva shown in Fig. 5. The maximum standard deviation of triplicate MSB culture assays was ±0.23 (log CFU ml^{-1}) and that of triplicate modified-MSB culture assay was ±0.11 (log CFU ml^{-1}). The culture assays were performed before the real-time PCR assay, and the investigator of the real-time PCR assay was kept blind to these data.

2.4. Real-time PCR assay

Frozen saliva samples were quickly thawed at 37°C, and 250 μl of each sample was spun down. The pellets were resuspended in 570 μl of 20 mg ml^{-1} lysozyme (Sigma, Tokyo, Japan) solution, containing 50 mM Tris–HCl (pH8.0) and 20 mM EDTA, and incubated at 37°C for 30 min. 30 μl of 20 mg ml^{-1} Proteinase K solution (Qiagen, Tokyo, Japan) was added and incubated at 55°C for 10 min. Approximately 0.8 g of acid-washed glass beads (diameter 150–212 μm, Sigma) and 1 μl of 100 mg ml^{-1} RNase A (Qiagen) were then added, and the samples were vigorously shaken in 2 ml Safe-Lock micro test tubes (Eppendorf, Tokyo, Japan) by a Mixer Mill MM300 (Qiagen) at 30 Hz for 10 min. 600 μl of buffer AL from a DNeasy Tissue kit (Qiagen) was added and incubated at 70°C for 30 min. The beads were spun down and the supernates transferred to new tubes. In this process, about one-fifth of the solution was lost with the beads.

A one-third volume of ethanol was added and mixed. DNA was isolated from the solution using a DNeasy tissue column according to the Qiagen instructions. The DNA was eluted with 200 μl of 10 mM Tris–HCl, pH 8.5. Real-time PCR was performed by the ABI PRISM 7700 Sequence Detection System (SDS; Applied Biosystems). Each reaction tube contained 25 μl of reaction mixture, including 1×SYBR Green PCR buffer, 0.625 U of AmpliTaqGold DNA polymerase, 0.25 U of AmpErase UNG (uracil *N*-glycosylase), 0.2 mM of each of the dNTPs with 0.4 mM of dUTP, 3 mM $MgCl_2$ (SYBR Green PCR Core Reagents, Applied Biosystems), 5 μl of DNA extracted from saliva samples and 1 μM of each primer. The cycling conditions were 2 min at 50°C for uracil *N*-glycosylase (this treatment prevents carryover cross-contamination by digesting uracil-containing PCR fragments generated by prior PCR assays), 10 min at 95°C for activation of AmpliTaqGold, 40 cycles of 15 s at 95°C for denaturation and 1 min at 68°C for annealing and extension.

2.5. Conventional PCR assay

Platinum Taq DNA polymerase (Invitrogen) or AmpliTaqGold (Applied Biosystems, Tokyo, Japan) was used for conventional PCR. The other components of the reaction mixture were as in the real-time PCR reaction mixture except for SYBR green. Amplification was performed in an iCycler Thermal Cycler (Bio-Rad, Tokyo, Japan). The cycles of the two-step PCR were the same as those of the real-time PCR. To determine the optimal PCR conditions, annealing and elongation temperatures were shifted to 66, 68, 70 and 72°C. Three-step PCR was performed for 30 cycles of denaturation at 95°C for 15 s, annealing at 55°C

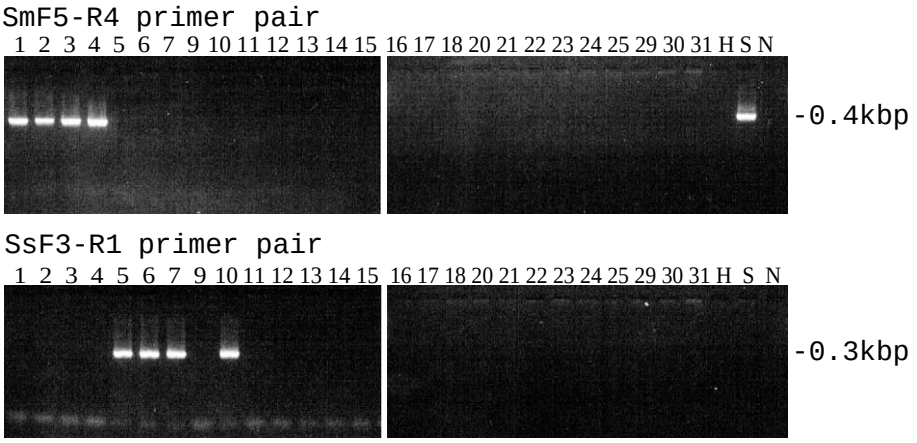

Fig. 1. Specificity of the Sm F5/R4 and Ss F3/R1 primer pairs. PCR products amplified products from 50 ng template DNAs were analyzed by agarose gel (3.5%) electrophoresis. The number above each lane corresponds to the number of the bacterial strain listed in Table 1 (H: human DNA obtained from Promega; S: saliva; N: no-template control) that was used as the PCR template. Template DNAs were extracted by the bead protocol from bacterial cultures and *S. sobrinus*-free saliva.

for 15 s and elongation at 72°C for 30 s. PCR products were analyzed by 3.5% agarose gel electrophoresis (NuSieve 3:1 agarose, BioWhittaker Molecular Applications, Inc., ME, USA).

2.6. Calculation of copy number of streptococcal genome DNA

The raw data of real-time PCR indicated that femtograms of target DNA were present in each PCR tube. After accounting for sample losses caused by the dead volume of beads during the extraction of DNA, the DNA in each PCR tube was considered to correspond to 5 μl of saliva sample. The genome size of mutans streptococci is reported to be about 2 Mbp [25,26], which corresponds to 2.6 fg of DNA. Thus, we used the following equation to calculate the copy number of bacterial DNA per ml of a saliva sample: (raw data: fg/tube)×1 ml (saliva)/5 μl (in tube)/2.6 fg.

In order to compare the real-time PCR assay with conventional culture assays, Spearman's correlation coefficients were calculated with the statistical softwear SPSS ver.10.0J (SPSS Japan Inc., Tokyo, Japan).

3. Results

3.1. Specificity of PCR primers

To test the specificity of the primers, bacterial and human genomic DNA and a human saliva DNA were used as templates for three-step PCR (Fig. 1). The Sm F5/R4 primer pair amplified the predicted size of DNA fragments only when the genomic DNA of *S. mutans* or DNA from *S. mutans* containing saliva were used as a template. The Ss F3/R1 primers amplified the predicted size of DNA fragments only when the genomic DNA of *S. sobrinus* or *S. downei* was used as a template. No other bands, such as primer dimers, were detected except the unreacted primers with intercalating ethidium bromide dye.

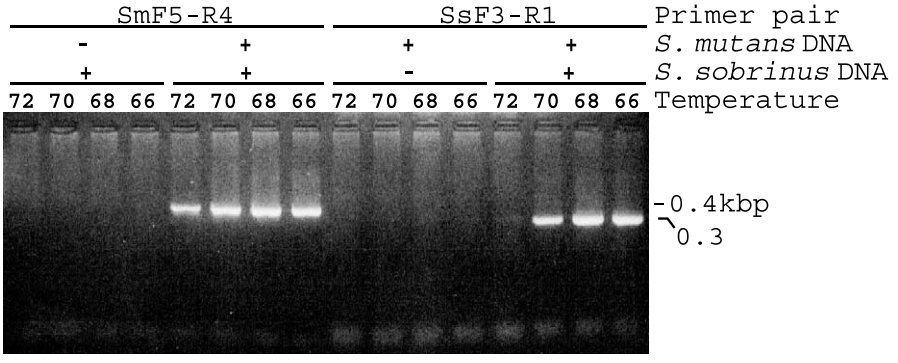

Fig. 2. Optimization of PCR conditions. PCR products amplified from 4 pg of target DNA and excessive non-target DNA were analyzed by agarose gel (3.5%) electrophoresis. 5 ng of DNA extracted from each cultured strain (numbers 8, 10–31 in Table 1) and human DNA were mixed in one PCR reaction tube. 5 ng of extracted DNA from *S. sobrinus* was added with the Sm F5/R4 primer pair, and 5 ng of extracted DNA from *S. mutans* was added with the Ss F3/R1 primer pair. Annealing and polymerizing temperatures are indicated above each lane.

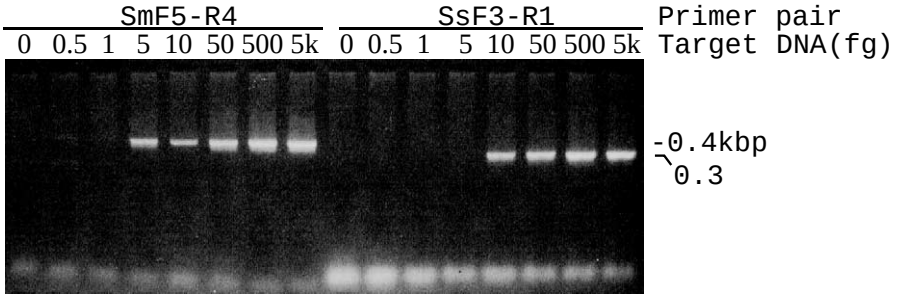

Fig. 3. Sensitivity test of the PCR assay. PCR was performed with 0–5000 fg of a mixture of target DNA and non-target DNA (5 ng of human DNA and 5 ng of extracted DNA from strains 5–7, 9–18, 20–25, and 29–31 for the Sm primers, or from strains 1–4, 9, 11–18, 20–25, and 29–31 for the Ss primers) in a volume of 25 μl. 10 μl of the amplified DNA solution was applied to each lane of agarose gel (3.5%) for electrophoresis.

3.2. Optimization of PCR

The PCR cycles for the Sm and Ss primer pairs were optimized for the best performance. In order to minimize the reaction time, PCR cycles were designed as two-step reactions. Because saliva contains a variety of DNA sources, such as human cells and several Gram-positive and -negative bacteria, for optimization the DNA templates should have a similar complexity to that of extracted DNA solution from saliva. Therefore, we included a DNA mixture along with the PCR template. We carried out two-step PCR using the DNA mixture and 4 pg of DNA from *S. mutans* ATCC25175-type strain as a PCR template with the Sm F5/R4 primer pair (Fig. 2A), or the DNA mixture and 4 pg of DNA from *S. sobrinus* ATCC33478-type strain as a PCR template with Ss F3/R1 primer pair (Fig. 2B). The amplified DNA bands were weak at reaction temperatures of 72 and 70°C. The optimum temperature for both primer pairs was 68°C; this temperature provided efficient signals, minimum reaction time, and no non-specific signals.

The specificities of PCR assays were tested with a few

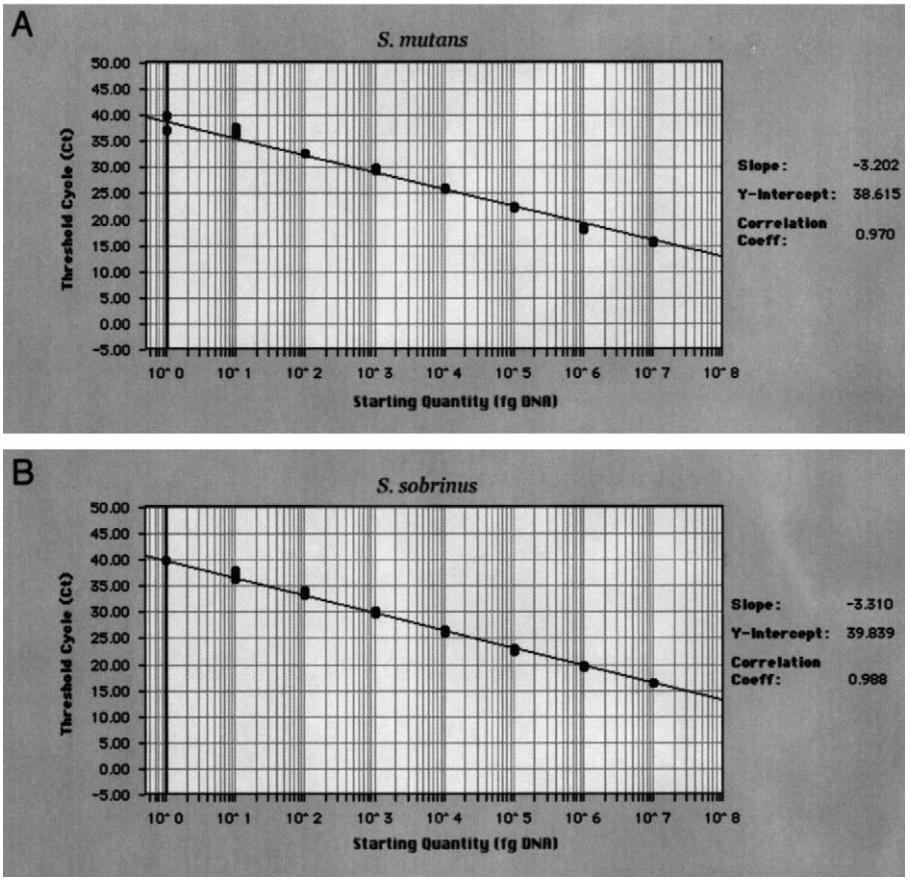

Fig. 4. Standard curves of the real-time PCR assays. A: The graph is a standard curve generated by ABI PRISM™ SDS from the threshold cycle numbers of a 10-fold dilution series of *S. mutans* with non-target DNA (5 ng of human DNA and 5 ng of extracted DNA from strains 5–7, 9–18, 20–25, and 29–31). B: The graph is a standard curve generated by SDS from the threshold cycle numbers of a 10-fold dilution series of *S. sobrinus* with non-target DNA (5 ng of human DNA and 5 ng of extracted DNA from strains 1–4, 9, 11–18, 20–25, and 29–31).

target molecules, which correlated to the sensitivity of the assays, on each of the primer sets using 68°C as the annealing and elongation temperature. The DNA mixture and 0 to 5000 fg of target genome DNA were used as the PCR template (Fig. 3). The certain detection limits of the Sm and Ss primer pairs by electrophoresis were 10 fg of *S. mutans* or *S. sobrinus* DNA. When 5 fg of target DNA was used, a clear signal was visible sometimes, but not always. As shown in Fig. 3, extra bands were limited to the unreacted primers with intercalating ethidium bromide dye under all PCR conditions tested.

3.3. Real-time PCR

The Sm F5/R4 and Ss F3/R1 primer pairs performed well in conventional PCR analysis, showing high sensitivity, high specificity, and low noise. These properties satisfy the criteria for quantitative real-time PCR using SYBR Green [27]. Fig. 4A shows a standard curve for quantification of *S. mutans* DNA. The standard curves were generated on ABI PRISM™ 7700 SDS with a 10-fold dilution series of DNA from an *S. mutans* strain (from 1 ag to 10 ng DNA of *S. mutans* with DNA mixture including 5 ng of each DNA listed in Table 1 except for the strains of *S. mutans*). A standard curve of *S. sobrinus* was also generated (Fig. 4B). The detection limit of the real-time PCR assays was deduced to be 10 fg of templates in the reaction tube, because the threshold cycle (C_t) for 10 fg of templates was significantly different from the C_t of the 'no template' controls and so was on the standard curve generated by the SDS software, whereas the C_t for 1 fg of template did not differ from the C_t for the 'no template' controls. The linearity of the quantifications was demonstrated over a range of six to seven exponential values.

3.4. Comparison of real-time PCR and conventional culture methods

In order to assess the applicability of real-time PCR to quantifying mutans streptococci in humans, we examined the bacteria in several saliva samples from children. In Fig. 5 the CFUs determined by classical culture quantification of bacteria are compared with the copy numbers of the bacterial genome obtained by real-time PCR assay. The colony number determined from four plates of the 10-fold dilution series of each saliva sample is shown on the graph. Real-time PCR assays were performed in triplicate and the mean ± standard deviation is indicated on a graph. The Spearman's rank correlation coefficients were calculated between the cultural methods and the real-time PCR assays. Data for *S. mutans* (Fig. 5A) showed a strong correlation between the real-time PCR assay (copy number ml^{-1}) and both the modified MSB assay (CFU ml^{-1}), $r = 0.976$ ($P < 0.01$), or MSB assay (CFU ml^{-1}), $r = 0.914$ ($P < 0.01$). Data for *S. sobrinus* (Fig. 5B) also showed a strong correlation between the real-time PCR assay and

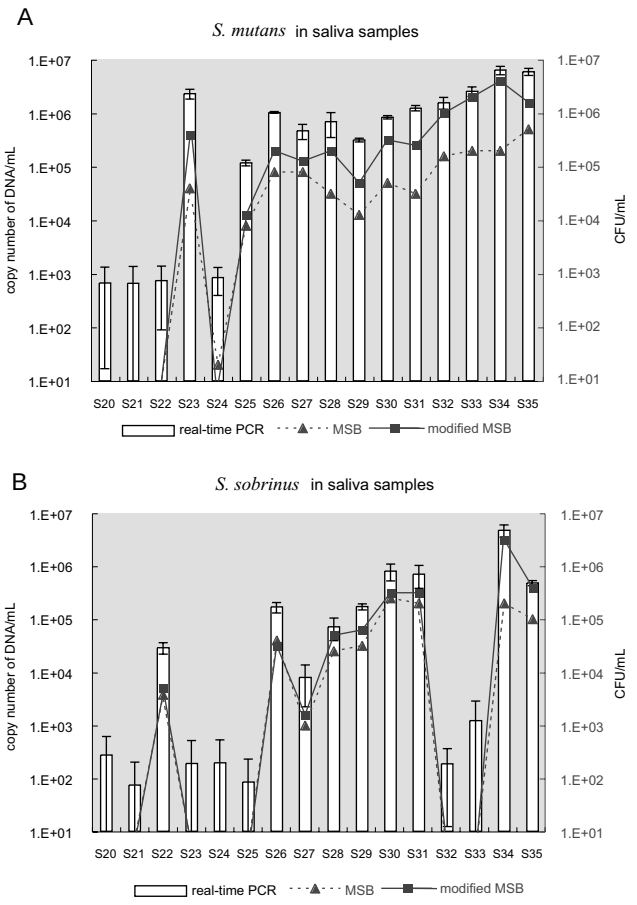

Fig. 5. Quantification of *S. mutans* and *S. sobrinus* in saliva from children. The real-time PCR assay was performed in triplicate. A: Quantification of *S. mutans* in children's saliva. B: Quantification of *S. sobrinus* in children's saliva.

the modified MSB assay, $r = 0.945$ ($P < 0.01$), and the MSB assay, $r = 0.949$ ($P < 0.01$). Samples for which no colonies were detected were found to contain a low copy number of DNA with, for the most part, a very large standard deviation. By contrast, the S24 sample, for which a small number of colonies of *S. mutans* were detected, had a low copy number of DNA with a small standard deviation.

4. Discussion

In comparing the data from the culture assay with those from the real-time PCR assay, we should consider the difference in units between the CFU and the copy number of the DNA. Generally, one colony is not necessarily derived from one single bacterial cell when the cells are aggregated, and rapidly growing bacterial cells usually contain more than one chromosome [28]. The copy numbers of genome DNA per CFU are influenced by the bacterial species, strain, sample condition and many other factors. Thus, the applicability of the real-time PCR assay is assessed only in terms of the contrast in data determined

from both culture and real-time PCR assays on each sample. Fig. 5 shows that the results from both assays follow almost the same trends. The real-time PCR data from the samples containing little or no *S. mutans* and *S. sobrinus* showed large standard deviations.

The detection limit of the real-time PCR assay is about 800 copies of genomic DNA per ml, because the detection limit of streptococcal DNA was about 10 fg, as shown in Fig. 4, which is equivalent to four genome copies, per tube [25,26]. As each tube contained 5 μl of extracted DNA solution, corresponding to 5 μl of saliva sample, the detection limit per ml of saliva was about 800 copies of DNA. Thus, data around this limit, that is, from 10^2 to 10^3 copies, are not reliable. The theoretical detection limit of the culture assay is one CFU per 50 μl of saliva sample, which is equivalent to 20 CFU per ml, but the actual limit is around 10^2 CFU per ml. Thus, the culture assay, especially that using modified MSB agar, seems to have a similar detection level to that of the real-time PCR assay. Those detection limits will change depending on the sample volumes applied to the culture plates and PCR reactions. However real-time PCR assays seem to be a little more sensitive than culture assays under our conditions.

The Ss F1/R3 primer pair amplifies a fragment of the *gtfI* gene of *S. sobrinus* and *S. downei*. Initially, we had planned to develop primers for quantification of representative species of mutans streptococci, namely *S. mutans* and *S. sobrinus*, in order to use in the epidemiological studies of the caries risks [10,11]. However, the design of primers specific to *S. sobrinus* proved difficult as the nucleotide sequences of *gtfI* from *S. sobrinus* show several intra-species variations (see Section 2), making it hard to select specific primers common to all strains of *S. sobrinus*. The Ss F3/R1 primer pair that we used corresponds to the catalytic domain of the GTF enzyme. The nucleotide sequences of this region are conserved in some *S. sobrinus* strains and, except for the *gtfI* gene of *S. downei*, show inter-species variation. *S. downei* was originally detected in macaque monkeys and was first identified as *S. sobrinus* [29,30]. Although the detailed distribution of *S. downei* in human oral cavities is unknown, it seems unlikely that *S. downei* will be found in human oral cavities as it was originally identified in monkeys [29,30]. Indeed, it was not detected during a serological survey of mutans streptococci among Japanese children [31]. If *S. downei* is in the human oral cavities, however, it is likely to contribute to the formation of caries to a similar extent as *S. sobrinus* [32]. Taking these observations together, the cross detection of *S. downei* was not thought to be a critical problem for caries risk diagnosis.

In general, real-time PCR possesses several advantages over culture assays. The real-time PCR can easily reveal the individual quantities of *S. mutans* and *S. sobrinus* independently. A contrast culture assay needs many steps for separately quantifying these two mutans streptococci. Some reports have indicated that *S. sobrinus* is more cariogenic than *S. mutans*, if so, then an assay for quantifying levels of *S. sobrinus* independently may have potential as another important means of assessing caries risks [33,34]. Microorganisms have to be alive and culturable for detection by plate counting. Sample conditions before plating directly affect the number of colonies. In contrast, PCR detects the DNA of microorganisms whether they are alive or not [35]. This difference influences the way in which samples are transported and stored. Samples remain stable over long periods of freezing for PCR assays. Easy sample handling is a benefit for large-scale screening.

Quantification by PCR depends on the efficiency of the lysis and DNA extraction procedures. Especially for Gram-positive bacteria, DNA extraction is often difficult and can easily lead to underestimates of the number of bacteria. Although mutanolysin is a good reagent for DNA isolation from mutans streptococci [36], it is expensive for the analysis of large sample numbers. Here, we used lysozyme coupled with disruption by glass beads. This DNA isolation method seemed to work well in reference to the results of the real-time PCR assay. As this isolation method can also be used for the analysis of other bacteria, it might apply generally to the analysis of the oral bacterial flora.

In conclusion, we have developed a real-time PCR assay for the quantification of mutans streptococci. This assay worked well on saliva samples of children when compared to a standard culture assay. Our real-time PCR assay will be useful as a practical diagnosis system not only for the risk of caries but also for other oral bacteria.

Acknowledgements

We thank Dr. Sasaki, Dr. Narikiyo (National Institute of Cancer Research, Tokyo, Japan) and Mr. Nishimura (Morinaga Seika Co., Tokyo, Japan) for allowing us the use of the ABI PRISM 7700 SDS and the Primer Express Software. We also thank Dr. Morita (National Institute of Public Health) for the support during the entire study.

References

[1] Yamashita, Y., Bowen, W.E., Burne, R.A. and Kuramitsu, H.K. (1997) Role of *Streptococcus mutans gtf* genes in caries induction in the specific-pathogen free rat model. Infect. Immun. 61, 3811–3817.
[2] Hamilton, I.R. (2000) Ecological basis for dental caries. In: Oral Bacterial Ecology: The Molecular Basis (Kuramitsu, H.K., and Ellen, R.P., Eds.), pp. 219–274. Horizon Sci. Press, England.
[3] Fukui, K., Moriyama, Y., Miyake, Y., Mizutani, K. and Tanaka, O. (1982) Purification and properties of glucosyltransferase responsible for water-insoluble glucan synthesis from *Streptococcus mutans*. Infect. Immun. 37, 1–9.
[4] Shiroza, T., Ueda, S. and Kuramitsu, H.K. (1987) Sequence analysis of the *gtfB* gene from *Streptococcus mutans*. J. Bacteriol. 169, 4263–4270.
[5] Shimamura, A., Tsumori, H. and Mukasa, H. (1983) Three kinds of

extracellular glucosyltransferase from *Streptococcus mutans* 6715 (serotype g). FEBS Lett. 157, 79–83.
[6] Ferretti, J., Gilpin, M. and Russell, R.R.B. (1987) Nucleotide sequence of a glucosyltransferase gene from *Streptococcus sobrinus* MFe28. J. Bacteriol. 169, 4271–4278.
[7] Hanada, N. and Kuramitsu, H.K. (1988) Isolation and characterization of the *Streptococcus mutans gtfC* gene coding for synthesis of both soluble and insoluble glucan. Infect. Immun. 56, 1999–2005.
[8] Klock, B. and Krasse, B. (1979) A comparison between different methods for prediction of caries activity. Scand. J. Dent. Res. 87, 129–139.
[9] Köhler, B., Pettersson, B. and Bratthall, D. (1981) *Streptococcus mutans* in plaque and saliva and the development of caries. Scand. J. Dent. Res. 89, 19–25.
[10] Köhler, B. and Bratthall, D. (1978) Intrafamilial levels of *Streptococcus mutans* and some aspects of the bacterial transmission. Scand. J. Dent. Res. 86, 35–42.
[11] Smith, R.E., Badner, V.M., Morse, D.E. and Freeman, K. (2002) Material risk indicators for childhood caries in an inner city population. Community Dent. Oral Epidemiol. 30, 176–181.
[12] Gold, O., Jordan, H.V. and van Houte, J. (1973) A selective medium for *Streptococcus mutans*. Arch. Oral. Biol. 18, 1357–1364.
[13] Kimmel, L. and Tinanoff, N. (1991) A modified mitis salivarius medium for a caries diagnostic test. Oral Microbiol. Immunol. 6, 275–279.
[14] Wade, W.G., Aldred, M.J. and Walker, D.M. (1986) An improved medium for isolation of *Streptococcus mutans*. J. Med. Microbiol. 22, 319–323.
[15] Schaeken, M.J., van der Hoeven, J.S. and Franken, H.C. (1986) Comparative recovery of *Streptococcus mutans* on five isolation media, including a new simple selective medium. J. Dent. Res. 65, 906–908.
[16] Sakamoto, M., Takeuchi, Y., Umeda, M., Ishikawa, I. and Benno, Y. (2001) Rapid detection and quantification of five periodontopathic bacteria by real-time PCR. Microbiol. Immunol. 45, 39–44.
[17] Kimura, H., Morita, M., Yabuta, Y., Kuzushima, K., Kato, K., Kojima, S., Matsuyama, T. and Morishima, T. (1999) Quantitative analysis of Epstein-Barr virus load by using a real-time PCR assay. J. Clin. Microbiol. 37, 132–136.
[18] Ono, T., Hirota, K., Nemoto, K., Fernandez, E.J., Ota, F. and Fukui, K. (1994) Detection of *Streptococcus mutans* by PCR amplification of *spaP* gene. J. Med. Microbiol. 41, 231–235.
[19] Igarashi, T., Yamamoto, A. and Goto, N. (1996) Rapid identification of mutans streptococcal species. Microbiol. Immunol. 40, 867–871.
[20] Rupf, S., Kneist, S., Merte, K. and Eschrich, K. (1999) Quantitative determination of *Streptococcus mutans* by using competitive polymerase chain reaction. Eur. J. Oral Sci. 107, 75–81.
[21] Oho, T., Yamashita, Y., Shimazaki, Y., Kushiyama, M. and Koga, T. (2000) Simple and rapid detection of *Streptococcus mutans* and *Streptococcus sobrinus* in human saliva by polymerase chain reaction. Oral Microbiol. Immunol. 15, 258–262.
[22] Thompson, J.D., Gibson, T.J., Plewniak, F., Jeanmougin, F. and Higgins, D.G. (1997) The CLUSTAL X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Res. 24, 4876–4882.
[23] Whiley, R.A. and Beighton, D. (1998) Current classification of the oral streptococci. Oral Microbiol. Immunol. 13, 195–216.
[24] Abo, H., Matsumura, T., Kodama, T., Ohta, H., Fukui, K., Kato, K. and Kagawa, H. (1991) Peptide sequences for sucrose splitting and glucan binding within *Streptococcus sobrinus* glucosyltransferase (water-insoluble glucan synthetase). J. Bacteriol. 173, 989–996.
[25] Okahashi, N., Sasakawa, C., Okada, N., Yamada, M., Yoshikawa, M., Tokuda, M., Takahashi, I. and Koga, T. (1990) Construction of a *Not*I restruction map of the *Streptococcus mutans* genome. J. Gen. Microbiol. 136, 2217–2223.
[26] Roe, B.A., Tian, R.Y., Jia, H.G., Qian, Y.D., Lin, S.P., Li, S., Kenton, S., Lai, H., White, J.D., McLaughlin, R.E., McShan, M., Ajdic, D. and Ferretti, J. (2002) Streptococcus mutans strain UA159 Genome Sequencing, on line [http://www.genome.ou.edu/smutans.html].
[27] Skeidsvoll, J. and Ueland, P.M. (1995) Analysis of double-stranded DNA by capillary electrophoresis with laser-induced fluorescence detection using the monomeric dye SYBR Green I. Anal. Biochem. 231, 359–365.
[28] Lick, S. and Heller, K.J. (1998) Quantification by PCR of yoghurt starters in a model yoghurt produced with a genetically modified *Streptococcus thermophilus*. Milchwissenschaft 53, 671–675.
[29] Beighton, D., Russell, R.R.B. and Hayday, H. (1981) The isolation and characterization of *Streptococcus mutans* serotype h from dental plaque of monkeys (*Macaca fascicularis*). J. Gen. Microbiol. 124, 271–279.
[30] Whiley, R.A., Russell, R.R.B., Hardie, J.M. and Beighton, D. (1988) *Streptococcus downei* sp. nov. for strains previously described as *Streptococcus mutans* serotype h. Int. J. Syst. Bacteriol. 38, 25–29.
[31] Hamada, S., Masuda, N., Ooshima, T., Sobue, S. and Kotani, S. (1976) Epidemiological survey of *Streptococcus mutans* among Japanese children. Identification and serological typing on the isolated strains. Jpn. J. Microbiol. 20, 33–44.
[32] Colby, S.M., McLaughlin, R.E., Ferretti, J.J. and Russell, R.B.B. (1999) Effect of inactivation of gtf genes on adherence of *Streptococcus downei*. Oral Microbiol. Immunol. 14, 27–32.
[33] de Soet, J.J., van Loveren, C., Lammens, A.J., Pavicic, M.J., Homburg, C.H., ten Cate, J.M. and de Graaff, J. (1991) Differences in cariogenicity between fresh isolates of *Streptococcus sobrinus* and *Streptococcus mutans*. Caries Res. 25, 116–122.
[34] Hirose, H., Hirose, K., Isogai, E., Miura, H. and Ueda, I. (1993) Close association between *Streptococcus sobrinus* in the saliva of young children and smooth-surface caries increment. Caries Res. 27, 292–297.
[35] Hein, I., Klein, D., Lehner, A., Bubert, A., Brandl, E. and Wanger, M. (2001) Detection and quantification of the *iap* gene of *Listeria monocytogenes* and *Listeria innocua* by a new real-time quantitative PCR assay. Res. Microbiol. 152, 37–46.
[36] Hamada, S., Torii, M., Kotani, S., Masuda, N., Ooshima, T., Yokogawa, K and Kawata, S. (1978) Lysis of *Streptococcus mutans* cells with mutanolysin, a lytic enzyme prepared from a culture liquor of *Streptomyces globisporus* 1829. Arch. Oral Biol. 23, 543–549.