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Detection of cariogenic bacterial genes by microchip electrophoresis

Koji Karasawa[a], Hidetoshi Arakawa[a,*], Takeshi Igarashi[b], Nobuichi Goto[b], Masako Maeda[a]

[a] *Department of Analytical Chemistry, School of Pharmaceutical Sciences, Showa University, 1-5-8 Hatanodai, Shinagawa, Tokyo 142-8555, Japan*
[b] *Department of Oral Microbiology, School of Dentistry, Showa University, Shinagawa, Tokyo 142-8555, Japan*

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Abstract

Allele-specific PCR primers were designed, based on the dextranase (*dex*) gene, to identify *Streptococcus mutans* and *Streptococcus sobrinus* in dental plaque; subsequently, PCR products were detected via microchip electrophoresis (ME). In order to amplify the *dex* gene fragment of *S. mutans* and *S. sobrinus*, the following two PCR methods were established. Duplex allele-specific PCR primers were designed on a region of low DNA homology; furthermore, 211 and 126-bp fragments were amplified for *S. mutans* and *S. sobrinus*, respectively. Common PCR primer for single allele-specific PCR was designed so as to sandwich a region exhibiting high homology and amplify PCR product of different DNA size due to deletion of small DNA fragment in two *dex* genes. *S. mutans* and *S. sobrinus* were amplified, leading to the generation of 202 and 226-bp products, respectively. Analysis of DNA base size by ME in order to achieve efficient separation employed a polymer mixture consisting of hydroxypropyl methylcellulose (HPMC) and polyethylene oxide (PEO). In the presence of a polymer mixture of 0.125% PEO/0.6% HPMC, two PCR products were obtained, displaying degree of separation of 226 bp/202 bp of 2.67 (Rs). Reproducibility (CV%, $n = 7$) was 0.3%; additionally, separation time was approximately 85 s. This method was applied to the detection of *S. mutans* and *S. sobrinus* in dental plaque. Detection of the *dex* genes of *S. mutans* and *S. sobrinus* characterized by quickness, precision and high sensitivity was possible.

Keywords: Microchip electrophoresis; Dextranase gene; *Streptococcus mutans*; Dental caries

1. Introduction

Dental caries is widespread in humans; moreover, caries is accompanied by pain. Maintenance of tooth health, which is important to the health of the entire body, enhances the quality of life. Removal of dental plaque by tooth brushing following meals is effective in the prevention of caries. Bacteria comprise 75% of dental plaque. One gram of dental plaque contains 2×10^{11} bacterial cells [1,2]. *Streptococcus mutans* and *Streptococcus sobrinus*, which are the primary cariogenic species, play a role in the generation of caries; consequently, these bacteria have been vigorously examined. Previous data clearly demonstrated that cariogenic ability is promoted when two species co-exist [3,4]. Therefore, identification and determination of the habitation ratio of these species, as a diagnostic method for caries prevention in a clinical setting, are essential.

Currently, identification of *S. mutans* and *S. sobrinus* is conducted with biochemical and immunological techniques, or via genetic analysis involving allele-specific PCR, restriction enzyme fragment length polymorphism (RFLP) and hybridization methodologies, etc. However, these approaches generally employ slab gel electrophoresis; as a result, considerable time and skill are required for proper utility of these distinct procedures. In addition, results are occasionally unsatisfactory. Furthermore, application of these techniques in a clinical setting presents problems. Recently, a DNA analysis method, microchip electrophoresis (ME), was introduced in an attempt to circumvent the aforementioned difficulties [5–11].

* Corresponding author. Tel.: +81 3 3784 8194; fax: +81 3 3784 8247.
E-mail address: arakawa@pharm.showa-u.ac.jp (H. Arakawa).

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Manz et al. described microchip electrophoresis in 1992, which involved electrophoresis on a small glass chip [12,13]. ME affords high separation ability and high sensitivity characteristic of capillary electrophoresis (CE); furthermore, time required for separation is only a few seconds, and many samples are simultaneously separable. Consequently, ME may provide the next generation of analytical instrumentation, enabling a series of chemical-analysis operations consisting of pretreatment, reaction, separation and detection.

In this investigation, the dextranase (*dex*) gene of *S. mutans* and *S. sobrinus* was selected as a marker gene with respect to dental caries prevention diagnosis. We developed allele-specific PCR; moreover, ME functioned as the detection method. A DNA analysis approach, characterized by rapid throughput and high sensitivity, was examined.

2. Experiment

2.1. Reagents and equipment

The microchip was an *i*-chip (Hitachi Chemical Co., Ltd). Channels were 100 μm in width and 30 μm in depth; effective length was 3 cm. Hydroxypropyl methylcellulose (HPMC) (MW 4000) (Sigma) and polyethylene oxide (PEO) (MW 8,000,000) (Aldrich) were used in this experiment. PCR reagent containing *Taq* DNA polymerase was coupled with the Gene Amp PCR kit (Takara Shuzo Co.). A DNA marker (*φ*X174/*Hae*III digest: 72, 118, 194, 234, 271, 281, 310, 603, 872, 1078 and 1353 bp) (Wako Co.) and Hitachi SV1100 microchip electrophoresis equipment (Kosumoai) were employed.

2.2. Preparation of electrophoresis solution

The mixed polymer consisting of hydroxypropyl methylcellulose (0.6%, w/v) and polyethylene oxide (0.125%, w/v) was dissolved in 0.09 mol/L Tris-borate buffer (pH 8.4) containing 2 mmol/L EDTA. Ethidium bromide was dissolved in the mixed polymer, producing a concentration of 0.05 μL/mL (10 mg/mL).

2.3. Preparation of template DNA

The *dex* genes of *S. mutans* and *S. sobrinus* consist of 2550 and 4011 base pairs, respectively. DNA extraction of reference strains and clinical isolates was effected via the addition of lysozyme, mutanolysin, RNase A and pronase E to the cells of *S. mutans* and *S. sobrinus*. All reagents were dissolved in 10% sodium dodecyl sulfate (SDS). The extract was mixed with CsCl–ethidium bromide; subsequently, centrifugal separation was performed. Isolated DNA was dissolved in 10 mmol/L Tris-HCl 1 mmol/L EDTA buffer solution (pH 8.0). Isolates were stored at −40 °C until use (14). DNA extraction of bacterial plaque samples (dental plaque) was conducted following protein degradation by lysozyme, mutanolysin and proteinase K [14,15]. That is, after phenol–chloroform extraction, DNA was refined by ethanol precipitation. Isolated DNA samples were dried, followed by dissolution in 10 mmol/L Tris-HCl 1 mmol/L EDTA buffer solution (pH 8.0). Samples were stored at −40 °C until use.

2.4. DNA amplification by allele-specific PCR

Allele-specific PCR of *dex* gene of *S. mutans* and *S. sobrinus* was conducted. Briefly, the reaction mixture (50 μL) consisted of purified DNA (1 μL; 20 ng/μL), 10× buffer (5 μL), dNTP (dATP, dCTP, dGTP and dTTP) (4 μL) and each primer (1 μL) (20 pmol), *Taq* DNA polymerase (0.5 μL) (2.5 U) and H_2O. Moreover, thermal cycling (PERKIN ELMER Co.; Gene Amp PCR system 9600) was performed at 94 °C for 5 min as initial denaturation, followed by 30 cycles of denaturation at 94 °C for 1 min, annealing at 45 °C for 1 min and extension at 72 °C for 1 min. Finally, incomplete PCR products were extended for 7 min at 72 °C.

2.5. Electrophoresis conditions

Hitachi SV1100 microchip electrophoresis instrumentation (KOSUMOAI), equipped with fluorescence and confocal detection methodology, were employed in the present investigation. Sample injection was effected at 300 V; separation was performed at 750 V (return voltage: 130 V). Polymethylmethacrylate (PMMA) plastic chips (8.5 cm × 5 cm) for the microchip capillary electrophoresis were produced by injection molding [16]. Furthermore, width and depth of the channel were 100 and 30 μm, respectively (Fig. 1). In order to perform electrophoresis on a chip, the polymer solution was filled to three reservoirs (1–3) of a chip, and the sample introduction channel and the separation channel. Subsequently, sample (10 μL) was put into sample reservoir (4), the microchip was placed in the apparatus and electrophore-

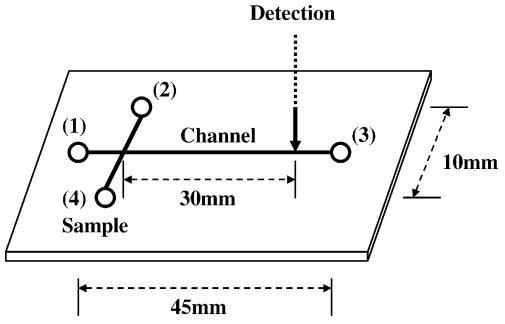

Fig. 1. A diagram of microchip: there is a reservoir by four all. Between reservoir (2) and reservoir (4) is the sample introduction channel. Between reservoir (1) and reservoir (3) is the separation channel. Reservoir (4) is sample reservoir. Effect length of channel is 30 mm.

sis was conducted. First, voltage (300 V/cm) was impressed to reservoir (2) and a sample was introduced toward reservoir (2) from sample reservoir. Voltage (167V /cm) was impressed to reservoir (3) after introduction of fixed time. A very small quantity of the sample in the intersection part of the sample introduction channel and the separation channel migrated toward reservoir (3). At this time, the voltage (130 V/cm) was impressed also between sample reservoir and reservoir (2). Consequently, the excessive sample influx into the separation channel from the sample introduction channel is protected. This voltage is given in the return voltage. Fluorescence detection of the sample is carried out at 3 cm from an intersection part; effective length for separation is 3 cm.

3. Results and discussion

Polymerase chain reaction (PCR) is a widely applied method for DNA diagnosis, which permits amplification of target DNA fragments by 100,000× in a short period. The *dex* genes of *S. mutans* and *S. sobrinus* were evaluated in this investigation. The present study examined development of optimal PCR conditions for the *dex* gene of *S. mutans* and *S. sobrinus*, as well as rapid, highly accurate separation of PCR products by ME.

3.1. Allele-specific PCR

Allele-specific PCR of *S. mutans* and *S. sobrinus* was performed employing two methods. Duplex allele-specific PCR was conducted utilizing two kinds of primer sets for *S. mutans* and *S. sobrinus*; thus, the two *dex* genes were exposed to different primers. In contrast, PCR involved a single common primer set; thus, the common PCR primer for PCR was designed to sandwich a region displaying high homology as the primer and as amplify PCR product of different DNA size due to a deletion of small DNA fragment in two *dex* genes. Amplification of the *dex* genes of *S. mutans* and *S. sobrinus* was attempted via these two techniques. In duplex allele-specific PCR, sequences of the sense (MF) and antisense (MR) primers for amplification of *S. mutans dex* were 5′-GACCTTAAGGTGCAAGAGAA-3′ and 5′-GGTTCTGTGCAGTTTCTAGC-3′, respectively. Sense (SF) and antisense (SR) primers for amplification of *S. sobrinus dex* were 5′-GGGTTATCCGAGCAACAAGT-3′ and 5′-GGTTGACATCTTCACCCTGA-3′, respectively. Respective sizes of the PCR products of these two primers were 211 bp between 88 and 298 of the *S. mutans* gene, and 126 bp between 416 and 541 of the *S. sobrinus* gene.

In single allele-specific PCR, common sense (CF) and antisense (CR) primers for amplification of *S. sobrinus* and *S. mutans dex* genes were 5′-CTGTTTTGCCAGAGAC-GGAATAC-3′ and 5′-GTATCACCCTGCCAACCATCAAA-3′, respectively. Respective sizes of the PCR products of the common primer were 202 bp between 956 and 1157 for the *S. mutans dex* gene and 226 bp between 1139 and 1364 for the *S. sobrinus dex* gene. Fig. 2 illustrates a frame format of the amplification aspect of *S. mutans* and *S. sobrinus*.

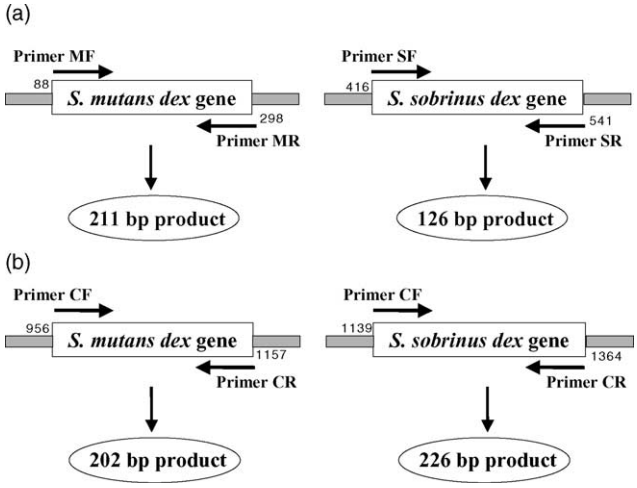

Fig. 2. Duplex allele-specific PCR (a) and single allele-specific PCR (b) for *dex* genes of *S. mutans* and *S. sobrinus*. (a) Respective sizes of the PCR products were 211 bp for the *S. mutans* gene and 126 bp for the *S. sobrinus* gene. (b) Respective sizes of the PCR products were 202 bp for the *S. mutans* gene and 226 bp for the *S. sobrinus* gene.

Duplex allele-specific PCR is specific for the *dex* genes of *S. mutans* and *S. sobrinus*. In order to set primers against a low homology domain, Mutans primer (MF, MR) and Sobrinus primer (SF, SR) were developed for *S. mutans* and *S. sobrinus*, respectively. To obtain the optimal PCR products with these primers, annealing temperature was evaluated between 35 and 55 °C. Consequently, at all temperatures, high singularity and amplification efficiency were apparent; moreover, highest amplification occurred around 55 °C. However, the annealing temperature was set at 45 °C in consideration of the conditions of single allele-specific PCR. This situation was established so as to permit the simultaneous performance of single and duplex allele-specific PCR on a single instrument. The 211 and 126-bp species from *S. mutans* and *S. sobrinus*, respectively, were amplified via this approach.

On the other hand, single allele-specific PCR sets primers against high homology domains in the *dex* genes of *S. mutans* and *S. sobrinus*. *S. mutans* and *S. sobrinus* contain a domain exhibiting homology of 57.8% of that of DNA sequences. In particular, the 793–1193 domain of *S. mutans* and the 1070–1400 domain of *S. sobrinus* demonstrate high homology and deletion of several bases. This arrangement can lead to products characterized by distinct DNA base size by a single PCR primer. In order to obtain *S. mutans* (202 bp) and *S. sobrinus* (226 bp), PCR primers between 956–1157 and 1139–1364, respectively, were generated in the current investigation. Annealing temperature for PCR conditions was evaluated in a similar manner. Consequently, 45 °C was selected as the optimal annealing condition; this condition resulted in equivalent, specific amplification of both genes.

3.2. Separation examination of φX174/HaeIII digest

The PCR products of the *dex* genes of *S. mutans* and *S. sobrinus* are approximately 200 bp. Therefore, the separation efficiency of DNA fragments between 200 and 300 bp must be greater in order to obtain an accurate determination. Indication of separation condition was examined employing separation degrees of 271 and 281 bp in the φX174/*Hae*III digest. The type and concentration of polymer and the electrophoresis voltage were examined with efficient separation of 271 bp/281 bp fragments as an index. The peak resolution is evaluated by resolution of separation (Rs). Rs is calculated with following formula: $Rs = 1.18 \times (t_{R2} - t_{R1})/(W_{0.5h1} + W_{0.5h2})$ (t_{R2}, t_{R1} = retention time; $W_{0.5h1}$, $W_{0.5h2}$ = the peak width in the middle point of peak height). In this study, the optimum condition was set so that the separation time and the Rs are less than 100 s and 1.5 or more, respectively. Separation was performed at 750 V (return voltage: 130 V).

Generally as a polymer for separation of DNA, linear polyacrylamide and cellulose derivatives serve primarily as a molecular sieve medium. Hydroxypropyl methylcellulose (MW 4000) and polyethylene oxide (MW 8,000,000) were evaluated in this study; subsequently, mixture of these two polymers was assessed.

First, each polymer, PEO and HPMC, was investigated. Polymer concentrations of HPMC and PEO were 0.1, 0.2, 0.3, 0.6 and 0.8%; and 0.25, 0.5, 0.75, 1 and 1.25%, respectively. As a result, peaks of DNA fragments overlapped in the presence of low-concentration polymer; furthermore, 11 DNA fragments were not clearly separable. Moreover, although the degree of separation improved with high-concentration polymer, electrophoresis time became long at 2 min or more. Next, mixed polymer concentrations were examined, namely, 1%/0.04%, 1%/0.06%, 1%/0.12%, 0.5%/0.1%, 0.125%/0.6%, 0.3125%/0.6% (PEO%/HPMC%); degree of separation was determined. Consequently, enhanced separation of 271 bp/281 bp fragments was obtained with mixtures of 0.125% HPMC/0.6% PEO and 0.3125% HPMC/0.6% PEO (1.5). Utility of two polymers possessing distinct meshes-of-a-net structure led to improved separation ability. And it is considered that a mixture of deferent polymers forms optimal meshed net for the separation of 100–300 bp. On the basis of these results, the 0.125% HPMC/0.6% PEO polymer mixture was selected as the optimal condition for separation of small DNA fragments.

Subsequently, electrophoresis voltage was examined. Separation electrophoresis voltage was changed from 550 to 900 V in cases involving 0.125% HPMC/0.6% PEO. The degree of separation increased at low voltage; however, electrophoresis time increased as well. For this reason, 750 V, at which separation can be achieved within 100 s with degree of

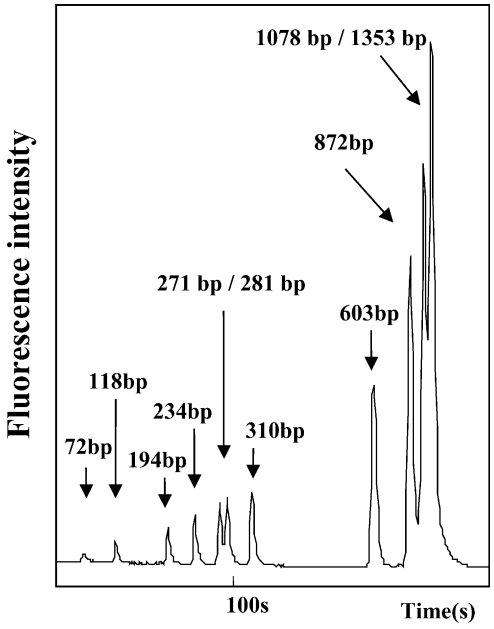

Fig. 3. Separation of φX174/*Hae*III digest by ME.

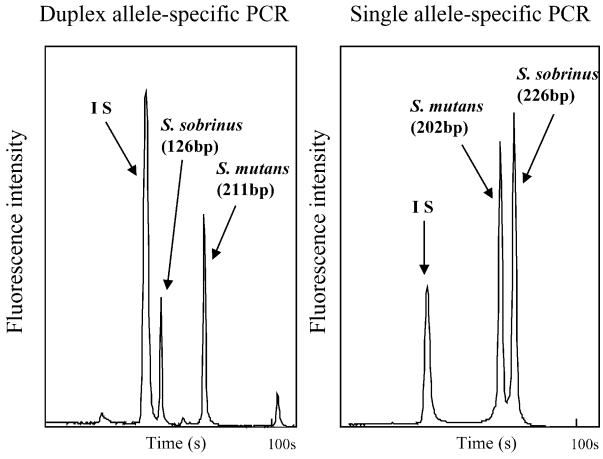

Fig. 4. Separations of *dex* gene obtained by allele-specific PCR. In duplex allele-specific PCR, average electrophoresis time of *S. mutans* and *S. sobrinus* was 84.0 and 72.1 s, respectively. In single allele-specific PCR, average migration time of *S. mutans* and *S. sobrinus* was 84.8 and 88.4 s, respectively.

Table 1
Specificities of single or duplex allele-specific PCR to streptococcal species

Oral streptococci	*dex* gene/ inhabitant	Single allele-specific PCR	Duplex allele-specific PCR
(1) *S. mutans*	(+)/human	○	○
(2) *S. sobrinus*	(+)/human	○	○
(3) *S. rattus*	(+)/rat	○	×
(4) *S. cricetus*	(+)/mouse	○	×
(5) *S. downei*	(+)/monkey	○	×
(6) *S. macacae*	(+)/monkey	○	×
(7) *S. salivarius*	(+)/human	× ※	×
(8) *S. bovis*	(−)	×	×
(9) *S. oralis*	(−)	×	×
(10) *S. mitis*	(−)	×	×
(11) *S. gordonii*	(−)	×	×
(12) *S. faecalis*	(−)	×	×
(13) *S. sanguis*	(−)	×	×

(○): *dex* gene was detected; (×): *dex* gene was not detected. (※) *dex* gene could not be amplified.

separation of 1.9 of 271 bp/281 bp, was used for the following experiments. Fig. 3 presents the microchip electropherogram of φX174/*Hae*III digest with separation conditions (mixed polymer of 0.6% HPMC and 0.125% PEO, and electrophoresis voltage of 750 V).

3.3. Separation of PCR products

PCR products of the *dex* genes of *S. mutans* and *S. sobrinus* were analyzed under the electrophoresis conditions consisting of 0.6% HPMC/0.125% PEO and 750 V. First, PCR products obtained by duplex allele-specific PCR were analyzed. Consequently, the PCR products of *S. mutans* and *S. sobrinus* appeared as two single peaks. Analysis of the PCR products of an *S. mutans*/*S. sobrinus* mixture sample revealed degree of separation of 126 bp/211 bp of 7.15; furthermore, this approach clearly distinguished *S. mutans* from *S. sobrinus*. Average electrophoresis time (n = 7) was 72.1 s for *S. sobrinus* and 84.0 s for *S. mutans*. Reproducibility values (CV%) of migration time for *S. sobrinus* and *S. mutans* were 1.67 and 1.27%, respectively. These results displayed high precision. Reproducibility readings (CV%) of peak height were 20.74 and 18.95%, respectively.

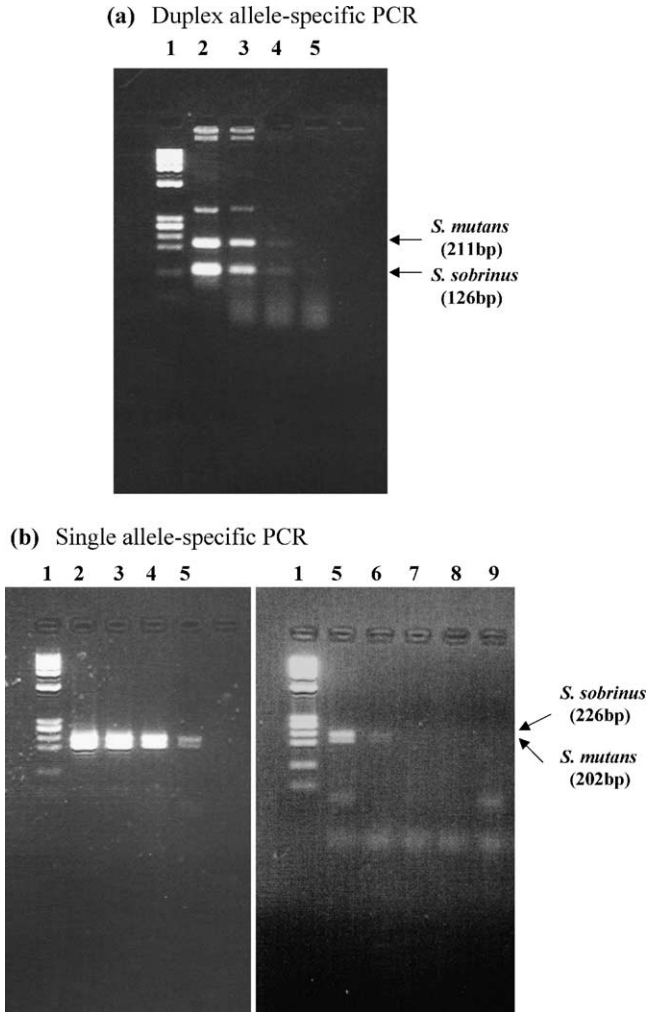

Fig. 5. Detection limit of template DNA by agarose gel electrophoresis. (a) Duplex allele-specific PCR of *dex* genes; lane 1: φX174/*Hae*III digest, lane 2: 20 ng/μL, lane 3: 2 ng/μL, lane 4: 2×10^{-1} ng/μL, lane 5: 2×10^{-2} ng/μL (the amount of template DNAs). (b) Single allele-specific PCR of *dex* genes; lane 1: φX174/*Hae*III digest, lane 2: 20 ng/μL, lane 3: 2 ng/μL, lane 4: 2×10^{-1} ng/μL, lane 5: 2×10^{-2} ng/μL, lane 6: 2×10^{-3} ng/μL, lane 7: 2×10^{-4} ng/μL, lane 8: 2×10^{-5} ng/μL, lane 9: 2×10^{-6} ng/μL.

PCR products obtained by single allele-specific PCR were analyzed in a similar manner by microchip electrophoresis under identical conditions. Consequently, *S. mutans* and *S. sobrinus* appeared as two single peaks, analysis of the PCR products of an *S. mutans*/*S. sobrinus* mixture sample revealed degree of separation of 226 bp/202 bp of 2.67; furthermore, this approach clearly distinguished *S. mutans* from *S. sobrinus*. Average migration time (n = 7) of *S. mutans* and *S. sobrinus* was 84.8 and 88.4 s, respectively. Reproducibility readings (CV%) of migration time were 0.32% for *S. sobrinus* and 0.29% for *S. mutans*; high precision was attainable. Reproducibility values (CV%) of peak height were 11.80 and 10.40%, respectively. Fig. 4 depicts results obtained by ME.

Additionally, in order to perform exact qualitative and quantitative analyses, fluorescein was used as an internal standard; moreover, migration time and peak height were corrected. Fluorescein (2×10^{-7} mol/L) was mixed with the sample (1:1). Thus, reproducibility of migration time and peak height were enhanced by this correlation. But, correct determination of the quantity of the cariogenic bacteria existence ratio requires accuracy less than several CV percent; compensation by only fluorescein has limitation in quantitative analysis. A sample injection method utilizing pressure and development of polymers of lower viscosity should be examined in order to improve reproducibility.

3.4. Specificity of allele-specific PCR

S. mutans and *S. sobrinus*, which possess the gene for dextranase, inhabit the human oral cavity. Specificity against

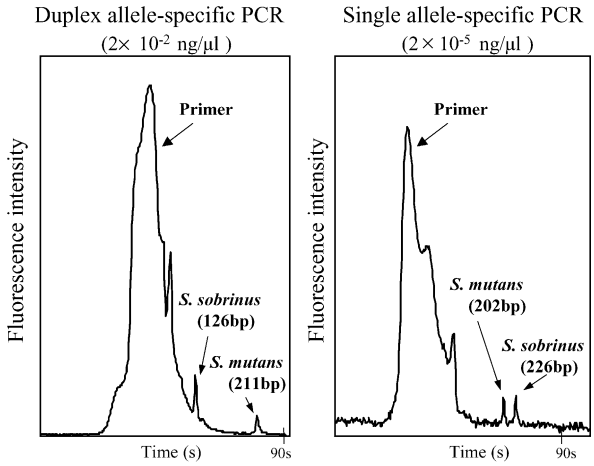

Fig. 6. Electropherograms of detection limit of template DNA by ME Duplex allele-specific PCR product and single allele-specific PCR product used 2×10^{-2} ng/μL and 2×10^{-5} ng/μL as a template DNA, respectively.

streptococcal species of *dex* (+) and *dex* (−) indigenous to human, rat, mouse and monkey was examined. Consequently, duplex allele-specific PCR products of *S. mutans* and *S. sobrinus* were detected exclusively. On the other hand, single allele-specific PCR employing primers set at the homology regions of a *dex* gene were observed for all other species of *dex* (+), with the exception of *S. salivarius*. Therefore, single allele-specific PCR is an effective method for detection of mutans streptococci (Table 1 (1–6)). These findings demonstrated that duplex allele-specific PCR exhibits very high specificity for *S. mutans* and *S. sobrinus*, whereas single allele-specific PCR is specific for bacteria with the *dex* gene, with the exception of *S. salivarius*. Table 1 presents results of streptococcal species used in this study and their specificities.

3.5. Detection limit of dex gene by microchip electrophoresis

Detection limit of the *dex* gene of *S. mutans* and *S. sobrinus* determined by ME was examined; the methods depend on PCR cycle number and template DNA amount. As a comparison, detection by agarose gel electrophoresis was conducted utilizing the same sample. At initial examination of PCR cycle number, agarose electrophoresis detected single allele-specific PCR product at 20 cycles and duplex allele-specific PCR product at 15 cycles. Subsequently, detection of PCR products by ME revealed that both single and duplex allele-specific PCR products were detectable at 10 cycles. It is possible to reduce the PCR cycle number and to decrease operation time with ME.

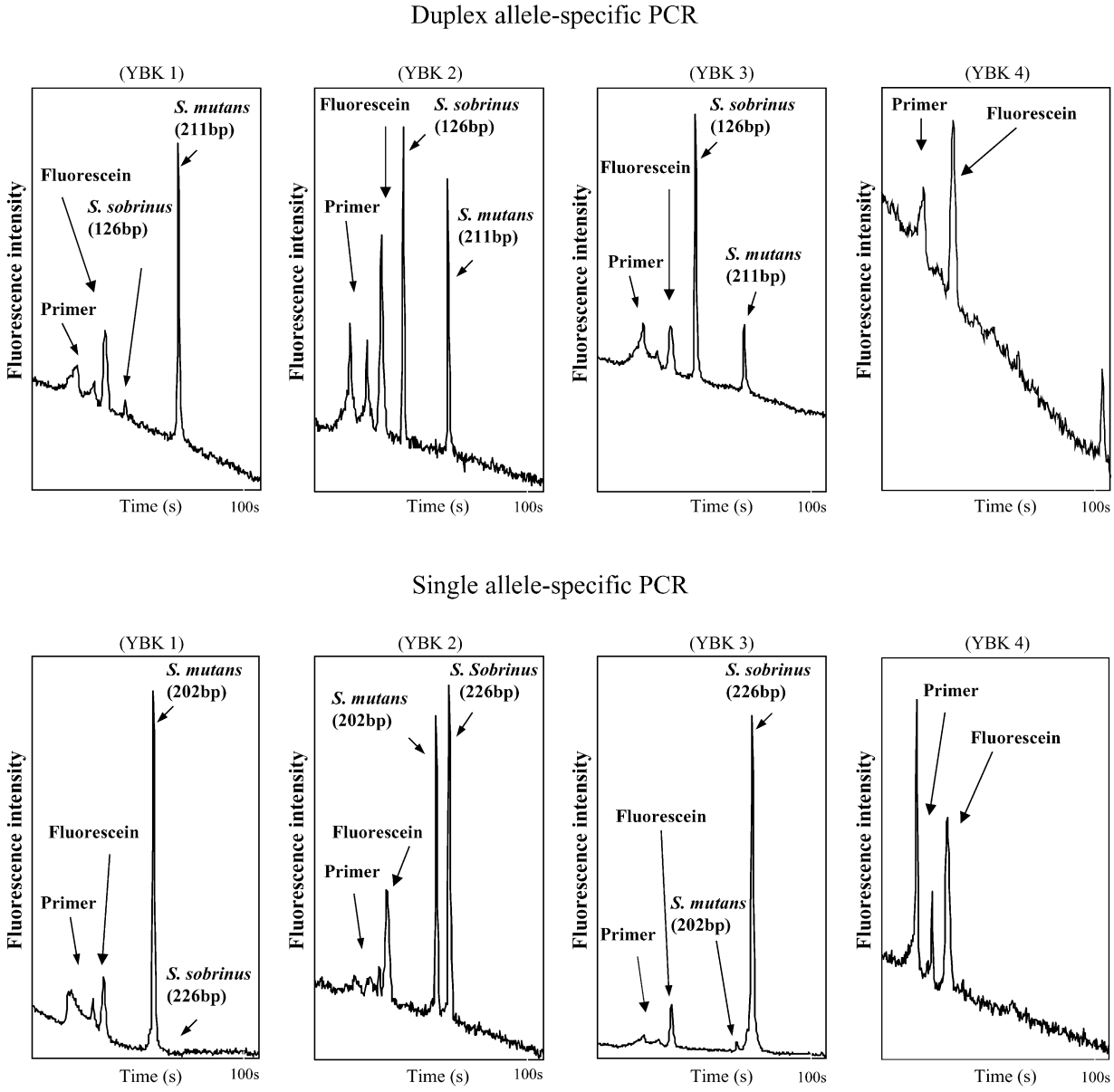

Fig. 7. Analysis of PCR products obtained from dental plaque four samples using duplex allele-specific PCR and single allele-specific PCR are analyzed by proposed method.

The amount of template DNA was examined subsequently. To determine the detection limit of the *dex* gene of *S. mutans* and *S. sobrinus*, the amount of template DNAs was diluted to 20, 2, 2×10^{-1}, and 2×10^{-2}, 2×10^{-3}, 2×10^{-4}, 2×10^{-5} and 2×10^{-6} ng/μL, respectively. These templates were amplified by PCR. Consequently, agarose gel electrophoresis afforded detection of the duplex allele-specific PCR product of 2×10^{-1} ng/μL and of the single allele-specific PCR product of 2×10^{-3} ng/μL (Fig. 5). On the other hand, ME demonstrates detection levels of 2×10^{-5} ng/μL with single allele-specific PCR products and of 2×10^{-2} ng/μL with duplex allele-specific PCR product (Fig. 6). These results confirmed that ME exhibits sensitivity 10∼100× greater than that of agarose gel electrophoresis.

3.6. Applications to dental plaque

In this study, allele-specific PCR analysis was employed to amplify DNA extracted from dental plaque. Dental plaque was collected with a toothpick from back and front faces of the tooth. The plaque, which was suspended into a 1.5 mL tube containing 100 μL of H_2O, was thoroughly mixed; subsequently, DNA was extracted. Next, extracted DNA was amplified by PCR and detected by ME.

Examples of ME of four samples (YBK1–YBK4) characterized by single and duplex allele-specific PCR products are presented in Fig. 7.

S. mutans was detected at high ratio in YBK1; identical levels of *S. sobrinus* and *S. mutans* were detected in YBK2. Moreover, *S. sobrinus* was detected at high ratio in YBK3, and nothing was detected in YBK4. Furthermore, seven samples were determined by the proposed method. Based on these findings, we concluded that this technique is suitable for detection of cariogenic bacterial genes in dental plaque. Moreover, this very interesting result demonstrated that the ratio of habitation species differs between individuals. Simpler, rapid operation is required in the clinic; for example, utility of mixed polymers (0.125% PEO/0.6% HPMC) with this 12-lane microchip could detect simultaneously 12 samples in less than 100 s (data not shown). As a result, specific, rapid detection of the *dex* gene of *S. mutans* and *S. sobrinus* was possible. Based on the aforementioned data, analysis of *S. mutans* and *S. sobrinus* by microchip electrophoresis appears to afford a suitable method for diagnosis of cariogenic bacteria in dental plaque.

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