IDENTIFICATION OF BACTERIA

PCR for detection and identification of *Streptococcus sobrinus*

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Oligonucleotide primers were designed based upon a comparison of the dextranase gene (*dex*) sequences from *Streptococcus sobrinus* and *S. mutans*. The primers amplified a 1610-bp long DNA fragment on the *dex* gene by a PCR. The pair of primers was specific to *S. sobrinus* as the other members of the mutans streptococci – *S. mutans*, *S. downei*, *S. cricetus*, *S. rattus*, *S. macacae* and *S. ferus* – gave no PCR products. Other gram-positive oral bacteria (15 strains of 10 species of cocci and 18 strains of 12 species of rods) and gram-negative oral bacteria (3 strains of 3 species of cocci and 31 strains of 22 species of rods) also gave negative results in the PCR. The PCR procedure was able to detect as little as 100 fg of purified chromosomal DNA or as few as 9 cfu of *S. sobrinus* NIDR6715. Seven clinical isolates of *S. sobrinus* were also positive in the *dex* PCR. This laboratory developed the *S. mutans*-specific PCR (*dexA* PCR) method with the primers specific for a portion of the dextranase gene of *S. mutans* Ingbritt. Primers for the *dex* and *dexA* PCR methods detected two species exclusively from the mutans streptococci. Furthermore, these two species were effectively differentiated by the species-specific amplicons with different lengths. The application of the PCR method to human dental plaque showed that the prevalence of *S. sobrinus* (83%) in oral cavities was higher than currently supposed (0–50%). These results suggest that the described PCR method is suitable for the specific detection and identification of human cariogenic bacteria, *S. sobrinus* and *S. mutans*.

Introduction

The mutans group of oral streptococci consists of seven species: *Streptococcus cricetus*, *S. rattus*, *S. mutans*, *S. sobrinus*, *S. downei*, *S. macacae* and *S. ferus* [1]. Among the group, *S. mutans* and *S. sobrinus* are most frequently isolated from human dental plaque and closely associated with human dental caries [2, 3]. Prevalence of *S. sobrinus* and *S. mutans* in the human oral cavity has been reported by epidemiological studies in which the isolation frequency of *S. mutans* from dental plaque is much higher than that of *S. sobrinus* [3–5]. The primary differentiation of these cariogenic species is usually based on the colonial morphology on mitis-salivarius (MS) or MS-bacitracin (MSB) agar [5–9]; the isolated colonies are then identified by biochemical, immunological and genetic tests [2, 10–12]. However, this procedure is sometimes inaccurate, and also time-consuming and laborious.

New tools such as DNA probes and PCR primers may be required to overcome these limitations. Smorawinska and Kuramitsu [13] and Colby *et al.* [14] developed DNA probes which hybridised the *gtfB*, *ftf* and *wapA* genes of *S. mutans*, and Ono *et al.* [15] and Colby *et al.* [14] designed PCR primers for *spaP* and *wapA* genes, respectively. On the other hand, work at this laboratory focused on the dextranase genes (*dex*) of cariogenic species for detection and identification. Dextranase is an enzyme that hydrolyses glucans in a plaque matrix and is believed to be involved in the pathogenesis of dental caries [16, 17]. The enzymes produced by *S. mutans* and *S. sobrinus* have been well studied to some extent, [16–22] and so far the DNA probe and PCR primers have been designed for the *S. mutans dex* gene to establish the species-specific DNA probe and PCR methods for detecting and identifying *S. mutans* [23, 24].

In this study, PCR primers were designed to amplify the *dex* gene of *S. sobrinus* to establish a simple, rapid and specific method for the detection and identification of *S. sobrinus* by PCR. Direct detection of *S. sobrinus*

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and *S. mutans* in human dental plaque by the *S. sobrinus*- and *S. mutans*-specific PCR methods was also tried and the prevalence of these cariogenic species in oral cavities was examined.

Materials and methods

Bacterial strains

All bacterial strains used in this study, which were maintained and grown as described previously [24], are listed in Table 1. *Escherichia coli* strain JM109 was grown on LB agar plates.

Oligonucleotide primers and PCR procedure

Oligonucleotide primers, SOF14 and SOR1623, were designed to amplify a 1610-bp fragment on the *dex* DNA sequence of *S. sobrinus* UAB66 (GenBank

accession no. M96978) [22]. The nucleotide sequences of SOF14 and SOR1623 were 5'-TGC TAT CTT TCC CTA GCA TG-3' (positions 134–153) and 5'-GGT ATT CGG TTT GAC TGC-3' (positions 1743–1726), respectively. PCR was performed as described previously [24]. Briefly, the reaction mixture (50 µl) was denatured at 95°C for 3 min followed by a series of amplification: denaturation at 95°C for 1 min, annealing at 55°C for 1 min and extension at 72°C for 1 min. The series was repeated for 26 cycles. The final cycle comprised 94°C for 1 min, 55°C for 1 min and 72°C for 5 min. The PCR products were subjected to electrophoresis on a 1.0% or 1.5% agarose gel and stained with ethidium bromide.

DNA preparation

Chromosomal DNAs of mutans streptococci, *S. sanguis*, *S. gordonii*, and *S. salivarius*, were purified as

Table 1. Bacterial strains used in this study

Gram-positive cocci	
Enterococcus faecalis 4532D	<i>S. sobrinus</i> ATCC33478
Gemella morbillorum ATCC 27824	<i>S. downei</i> NCTC 11391
Streptococcus cricetus E49	<i>S. ferus</i> ATCC 33477
<i>S. cricetus</i> HS6	<i>S. macacae</i> ATCC 35911
<i>S. rattus</i> BHT	<i>S. sanguis</i> ATCC 10556
<i>S. rattus</i> FA1	<i>S. gordonii</i> ATCC 10558
<i>S. mutans</i> ATCC 25175	<i>S. salivarius</i> ATCC7073
<i>S. mutans</i> ATCC 33535	<i>S. salivarius</i> KT12
<i>S. mutans</i> GS5	<i>S. mitis</i> ATCC 903
<i>S. mutans</i> Ingbritt	<i>S. mitis</i> ATCC 9811
<i>S. mutans</i> JCM 5175	<i>S. mitis</i> IID39
<i>S. mutans</i> LM7	<i>S. anginosus</i> ATCC 9895
<i>S. mutans</i> MT703	<i>S. bovis</i> ATCC 9809
<i>S. mutans</i> OMZ175	<i>S. pyogenes</i> ATCC 10389
<i>S. sobrinus</i> OMZ176	<i>S. pyogenes</i> ATCC 12348
<i>S. sobrinus</i> NIDR6715	Streptococcus sp. ATCC 12388
<i>S. sobrinus</i> ATCC27607	Streptococcus sp. ATCC 12394
Gram-positive rods	
Actinomyces viscosus ATCC 19246	<i>L. casei</i> JCM 1134
Act. viscosus ATCC 15987	Propionibacterium acnes ATCC 6919
Act. naeslundii ATCC 12140	<i>P. acnes</i> ATCC 11827
Act. israelii ATCC 12102	<i>P. acnes</i> ATCC 11828
Act. israelii ATCC 12836	<i>P. granulosum</i> ATCC 11829
Act. odontolyticus ATCC 12929	<i>P. propionicum</i> ATCC 29324
Corynebacterium matruchotii ATCC 14266	<i>P. propionicum</i> ATCC 29326
Lactobacillus acidophilus JCM 1132	Rothia dentocariosa ATCC 14189
<i>L. alactosus</i> JCM 1133	<i>R. dentocariosa</i> ATCC 14190
Gram-negative cocci	
Veillonella atypica ATCC 17744	<i>V. dispar</i> ATCC 17748
<i>V. parvula</i> ATCC 17745	
Gram-negative rods	
Actinobacillus actinomycetemcomitans ATCC 29522	Por. endodontalis ATCC 35406
<i>A. actinomycetemcomitans</i> ATCC 29523	Por. gingivalis ATCC 33277
<i>A. actinomycetemcomitans</i> ATCC 29524	Por. gingivalis 381
<i>A. actinomycetemcomitans</i> ATCC 33384	Prevotella. denticola ATCC 33185
<i>A. actinomycetemcomitans</i> Y4	Pr. intermedia ATCC 15032
Bacteroides gracilis ATCC 33236	Pr. intermedia ATCC 25261
<i>B. macacae</i> ATCC 33141	Pr. intermedia ATCC 25611
Campylobacter curvus ATCC 35224	Pr. intermedia VIP 4197
<i>C. rectus</i> ATCC 33238	Pr. loescheii ATCC 15930
<i>C. showae</i> ATCC 51146	Pr. melaninogenica ATCC 25845
Capnocytophaga gingivalis ATCC 33624	Pr. nigrescens ATCC 33563
Cap. ochracea ATCC 27872	Pr. oralis ATCC 33269
Cap. sputigena ATCC 33612	Pr. oralis Ig4a
Eikenella corrodens ATCC 23834	Selenomonas flueggeii ATCC 43531
Fusobacterium nucleatum ATCC 25586	Sel. infelix ATCC 43532
Porphyromonas asaccharolytica ATCC 25260	

reported previously [20]. Chromosomal DNA from the other gram-positive bacteria and clinical isolates of *S. sobrinus* and *S. mutans* was extracted by the chloroform method of Gillespie *et al.* [24, 25]. Chromosomal DNA from gram-negative bacteria was purified by the method of Marmur [26]. Chromosomal DNA was extracted from dental plaque by the SDS-lysis method as described previously [24]. Plasmid DNA was isolated by a Wizard Miniprep Purification Kit as indicated by the manufacturer (Promega, Madison, WI, USA).

Nucleotide sequence analysis

PCR products were cloned into a pT7Blue T-vector (Novagen, Madison, WI, USA) and sequenced with an ABI Prime Cycle Sequencing Kit and a model 373S automated DNA sequencer (Perkin Elmer Applied Biosystems, Foster City, CA, USA) as described previously [20]. Nucleotide and deduced amino-acid sequences were analysed with Genetyx-Mac software (version 10.1, Software Development, Tokyo, Japan).

Isolation of *S. sobrinus* clinical isolates from human dental plaque

Clinical isolates of *S. sobrinus* were screened and isolated as described previously [24]. Briefly, supragingival plaque was collected from human subjects and suspended in phosphate-buffered saline and cultured on MS plates. *S. sobrinus* was initially screened on the basis of colonial morphology on the MS plates and then characterised biochemically with API STREP 20 (bioMérieux, La Balme des Grottes, France). Isolates YK13 and YK42 were characterised as *S. sobrinus* in this laboratory, and isolates MKS4A, MKS4B, MKS13A, MKS27A and MKS100A, which were characterised by biochemical and immunological tests, were kindly provided by Professor K. Fukushima (School of Dentistry at Matsudo, Nihon University, Chiba, Japan). *S. mutans* isolates from dental plaque

which were obtained previously [24] were also used in this study.

Results

Specificity of oligonucleotide primers

Oligonucleotide primers (SOF14 and SOR1623) were designed on the nucleotide sequence of the *dex* gene of *S. sobrinus* on the basis of a sequence comparison between *S. sobrinus* and *S. mutans* dextranase genes. To examine the specificity of the primers to detect the *S. sobrinus dex* gene, PCR was performed with all the strains shown in Table 1. Of 19 strains of the mutans streptococcal group, only four strains of *S. sobrinus* gave a PCR product that corresponded to the 1610-bp DNA fragment as predicted from the sequence data (Fig. 1, lanes 5–8); none of the other mutans streptococcal species was positive (Fig. 1). Amplified DNA was detected in no other gram-positive species (15 strains of 10 species of cocci and 18 strains of 12 species of bacilli) or gram-negative species (3 strains of 3 species of cocci and 31 strains of 22 species of bacilli). To confirm that the PCR-amplified fragment was derived from the *dex* gene of *S. sobrinus*, the nucleotide sequence of the fragment was determined after cloning into the pT7Blue T-vector. The resultant sequence was the same as reported previously [22]. These results indicate that the PCR method used was specific to *S. sobrinus*.

Sensitivity of the PCR

The sensitivity of this PCR method was tested with known amounts of *S. sobrinus* NIDR6715 DNA as a template. Chromosomal DNA serially diluted from 1 ng to 1 fg was used as templates in the PCR. The detection limit was 100 fg of template DNA (Fig. 2a, lane 5). The detection sensitivity was also examined with serially diluted *S. sobrinus* NIDR6715 cells. The PCR gave a detectable amount of DNA fragment from

Fig. 1. PCR amplification of the *dex* DNA sequence on chromosomal DNAs from strains of mutans streptococci. Lane 1, *S. cricetus* HS6; 2, *S. rattus* FA1; 3, *S. mutans* IFO13955; 4, *S. mutans* JCM5175; 5, *S. sobrinus* OMZ176; 6, *S. sobrinus* NIDR6715; 7, *S. sobrinus* ATCC27607; 8, *S. sobrinus* ATCC33487; 9, *S. downei* NCTC11391; 10, *S. ferus* ATCC33477; 11, *S. macacae* ATCC35911; 12, *S. sanguis* ATCC10556; 13, *S. gordonii* ATCC10558; 14, *S. salivarius* ATCC7073; M, λ DNA digested with *Hind*III.

Fig. 2. Sensitivity of the *dex* PCR with (a) purified chromosomal DNA from *S. sobrinus* NIDR6715 and (b) the lysed cells of *S. sobrinus* NIDR6715. (a) Amount of purified DNA in lanes: 1, 1 ng; 2, 100 pg; 3, 10 pg; 4, 1 pg; 5, 100 fg; 6, 10 fg; 7, 1 fg; M, λ DNA digested with *Hind*III. (b) Number of lysed cells in lanes: 1, 9×10^3 ; 2, 9×10^2 ; 3, 9×10^1 ; 4, 9; 5, 4; 6, 2; 7, 1; M, λ DNA digested with *Hind*III.

the DNA sample prepared by the SDS-lysis method [24] from the cells of as few as 9 cfu (Fig. 2b, lane 4).

Evaluation of the PCR with clinical isolates and human dental plaque

PCR primer pairs of *S. sobrinus* (this study) and *S. mutans* [24] were used for the detection of these two species. The primer pairs specifically amplified species-specific amplicons of different sizes (1610 and 1272 bp, respectively) from dextranase genes of *S. sobrinus* and *S. mutans* (Fig. 3, lane 8) and PCR-amplification by the primer pairs yielded the species-specific amplicons from each clinical isolate of the species tested (Fig. 3, lanes 1–7 and 9–15). These results indicate that the primer pairs of *S. sobrinus* and *S. mutans* amplified the species-specific amplicons with different lengths, indicating that the present PCR method is useful for detection and identification of the human cariogenic species, *S. sobrinus* and *S. mutans*.

To investigate the prevalence of *S. sobrinus* and *S. mutans* in the human oral cavity, direct detection of these two species was applied to dental plaque samples. A representative example of detection by PCR is shown

in Fig. 4. Two different detection patterns were observed: one was positive for *S. mutans* but negative for *S. sobrinus* (Fig. 4, lanes 1 and 4) and the other was positive for both species (Fig. 4, lanes 2, 3, 5–7). To exclude the possibility of non-specific amplification products, the PCR products (1.61 kb for *S. sobrinus* and 1.27 kb for *S. mutans*) obtained from dental plaque were sequenced after cloning into the pT7Blue T-vector; care was taken to ensure that the PCR products were derived from the dextranase gene sequences of *S. sobrinus* and *S. mutans* (data not shown). This analysis was extended to dental plaque samples from 23 individuals. As shown in Table 2, an *S. mutans*-specific amplicon was detected in the dental plaque samples from all 23 individuals: the prevalence of *S. mutans* was 100%. In contrast, the amplicon specific to *S. sobrinus* was obtained from 19 of 23 individuals: the prevalence of *S. sobrinus* was 83%, which was higher than previously reported (0–50%) [3–5].

Discussion

An earlier study designed oligonucleotide primers for the *dex* gene of *S. mutans* Ingbritt, which amplified a 1272-bp fragment by PCR. The method was species-

Fig. 3. PCR amplification of DNA of oral isolates of *S. sobrinus* and *S. mutans*. Chromosomal DNA was amplified with the primers for either *S. sobrinus* or *S. mutans*. Source of DNA: lanes 1–7, isolates of *S. sobrinus* from different individuals; 9–15, isolates of *S. mutans* from different individuals; M, λ DNA digested with *Hind*III; 8, positive control containing the PCR products from *S. sobrinus* ATCC27607 and *S. mutans* IFO13955.

Fig. 4. Direct detection of *S. mutans* and *S. sobrinus* in dental plaque by PCR amplification. Lanes 1–7, dental plaque samples from different individuals were amplified with species-specific primers; M, *S. mutans*-specific primers; S, *S. sobrinus*-specific primers; 8, PCR products of (M) *S. mutans* IFO13955 and (S) *S. sobrinus* ATCC27607.

Table 2. PCR detection of cariogenic bacteria from human dental plaque

Species	Number tested	Number positive by PCR (%)
<i>S. mutans</i>	23	23 (100)
<i>S. sobrinus</i>	23	19 (83)

specific, and could detect and identify *S. mutans* directly from dental plaque [24]. The present study attempted to establish a PCR method for another human cariogenic bacteria, *S. sobrinus*, under the same conditions as those for *S. mutans*. Oligonucleotide primers were designed for the *dex* gene of *S. sobrinus* UAB66 based on the comparison of the *dex* gene sequences of *S. sobrinus* and *S. mutans* [20, 22]. Two sets of primer pairs were prepared: one (SOF14 and SOR1623) was designed to amplify a 1610-bp fragment (*Materials and methods*) and the other (SOF14 and SOR762) was expected to amplify a 748-bp fragment (data not shown). The sequence of SOR762 was 5'-ATT GTC AGC GTC ACG CAC G-3' (positions 882–864). Each primer pair gave a single amplicon specifically. Although the primer pair SOF14 and SOR1623 could detect 100 fg of *S. sobrinus* DNA (Fig. 2a), the other primer pair (SOF14 and SOR762) could detect only DNA of > 10 pg (data not shown). Therefore, the primer pair SOF14 and SOR1623 was used in this study.

Some authors have shown that *S. mutans* is more prevalent than *S. sobrinus* in the oral cavity [3–5]. It should be noted that their estimation depended on colony morphology and *S. sobrinus* was screened by bacitracin susceptibility [7, 9, 27]. Therefore, it is conceivable that the actual incidence of *S. sobrinus* may be higher than currently supposed. To understand more fully the relationship between caries incidence and the actual carrier rates of *S. sobrinus* and *S.*

mutans, it would be necessary to evaluate the presence of these species in the oral cavity more directly without a cumbersome culture step. To resolve this issue, establishment of a PCR-based method, which is more rapid, more sensitive and simpler, would be suitable. In addition to the *S. mutans*-specific PCR primers which were previously established [24], PCR primers specific to *S. sobrinus* were introduced in this study. Not only could the two primer pairs detect their target DNAs with equal sensitivity and specificity, but they gave amplicons of different lengths which could be distinguished easily from each other ([24] and this study). The PCR method was further evaluated with clinical isolates of *S. sobrinus* and *S. mutans*; the isolates were effectively differentiated by the PCR (Fig. 3). Whereas the conventional procedures to detect cariogenic bacteria from dental plaque usually take a long time (*c.* 1 week), use of the PCR method described here shortens the time: it is possible to determine the presence of *S. sobrinus* and *S. mutans* in the oral cavity within 1 day (6–7 h) because the bacterial culture and other cumbersome procedures are omitted.

Although this PCR procedure directly detected *S. mutans* and *S. sobrinus* in dental plaque, it may also be useful as a quantitative PCR to estimate the approximate numbers of these two species. In the quantitative PCR, DNA was prepared from known numbers of *S. mutans* (or *S. sobrinus*) cells, and the approximate numbers of the two species in dental plaque were determined by comparing the intensities of the species-specific amplicons from dental plaque sample with those from the standard DNA (data not shown). The ratio of two species in dental plaque shown by the quantitative PCR could be helpful to understand the relationship between caries incidence and cariogenic species prevalent in dental plaque.

It is of interest to understand the prevalence of *S. sobrinus* and *S. mutans* in the oral cavity. The PCR

results shown in Table 2 revealed higher prevalence (83%) of *S. sobrinus* than currently supposed (0–50%) [3–5]. It is speculated that the difference depends on the detection method used: the detection method with PCR was performed directly on dental plaque without the culture step and biochemical tests; the conventional method needs several steps such as selective culture on an MSB plate followed by several confirmation tests. It has been reported that MSB inhibits the growth of *S. sobrinus* to a greater extent than *S. mutans* [7, 11]. If this is true, *S. sobrinus* may consist of strains with different susceptibility to bacitracin. Further studies are needed to clarify this question, and the PCR-based detection method described here would be helpful for subsequent research.

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