

NEW DIAGNOSTIC METHODS

Detection of *Streptococcus mutans* by PCR amplification of *spaP* gene

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Summary. Synthetic oligonucleotide primers were used in the polymerase chain reaction (PCR) to amplify a sequence of the *spaP* gene, which encodes the surface protein antigen I/II of *Streptococcus mutans*. A DNA fragment of c. 192 bp was amplified from lysed *S. mutans* cells or isolated DNA. With *S. mutans* cells, the lower limit of detection was 4–40 cfu. With these primers, 13 reference and 50 clinical strains of *S. mutans* were identified. Amplification of the 192-bp product was not demonstrated when 41 strains of other streptococcal and non-streptococcal species were tested. The *spaP* gene PCR has potential for the rapid diagnosis of *S. mutans* infections.

Introduction

Viridans streptococci are the most common cause of bacterial endocarditis.^{1–3} *Streptococcus mutans*, a member of the viridans group of streptococci, is recognised as part of the normal oral flora of man, and is an aetiological agent in smooth-surface dental caries.^{4,5} In experimental animals, induction of bacteraemia has been demonstrated following dental extraction^{6,7} and, generally, diagnosis depends on the isolation of the bacterium from the infection site or blood. DNA probes to *gtfB* and *fif* genes for glucosyltransferase and fructosyltransferase, respectively, have been reported for the detection of *S. mutans*,⁸ but these probes are not sensitive.

PCR procedures for the amplification of streptococcal and enterococcal genes have been reported,^{9,10} but there has been no report of PCR of a specific gene fragment for the identification of *S. mutans*. *S. mutans* strains produce surface protein antigen I/II (Ag I/II) and this may facilitate their ability to colonise sites.^{11,12} The gene encoding Ag I/II, *spaP* or *pac*, has been cloned from two strains of serotype *c* and one strain of serotype *f* of *S. mutans*,^{13–15} and although similar antigens have been found in other streptococci, it is likely that species-specific regions exist in the *spaP* gene.^{16–18} The purpose of this study was to utilise the unique DNA sequence for Ag I/II to amplify the *spaP* gene by PCR.

Materials and methods

Bacterial strains and culture conditions

The 13 reference strains of *S. mutans* used in this study are listed in table I. Fifty clinical strains were isolated from the dental plaque of students (18–20 years old) and identified by biochemical tests including API-20 Strep system and API-Zym (bioMérieux, La Balme des Grottes, France) and immunological reactions between whole cells and antiserum or monoclonal antibodies.¹⁹ Other oral streptococci and non-streptococcal strains are listed in table II. All strains were grown in brain heart infusion broth (BHI). *S. mutans* strain B56 was grown at 37°C for 24 h in BHI supplemented with sucrose 5% for injection into mice.

DNA preparation

Chromosomal DNA was purified from the Ingbritt strain of *S. mutans* by the method reported by Okahashi *et al.*¹³

Oligonucleotide probes

Primers were selected on the basis of the *spaP* gene from *S. mutans* strain NG5.¹⁷ The sequences of the two primers were 5'-AACGACCGCTCTTCAGCAG-ATACC-3' (sense primer) and 5'-AGAAAGAAC-ATCTCTAATTTCTTG-3' (anti-sense primer).

Preparation of bacteria for PCR

Bacterial cells were harvested by centrifugation. The

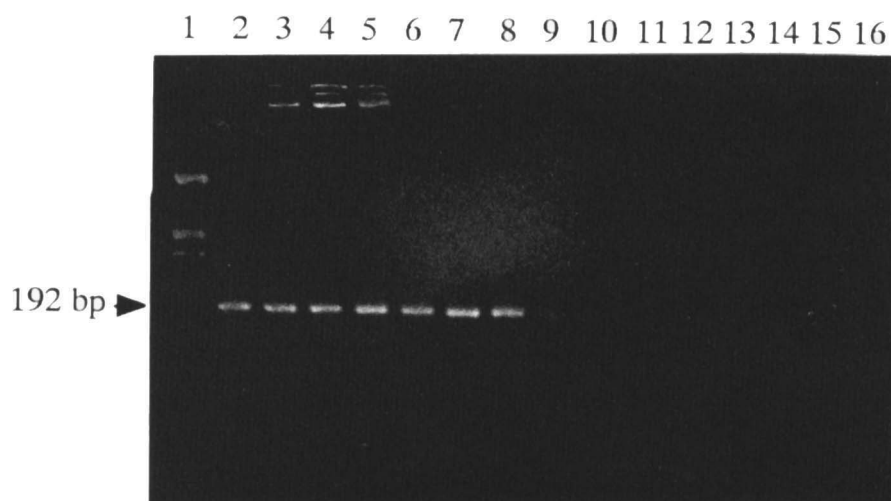


Fig. 1. PCR amplification of *spaP* gene from cell lysates of *S. mutans*, *S. sobrinus* and *S. sanguis*. Lane 1, molecular mass markers of pUC119 *Hinf*I digests; 2–8, *S. mutans* Ingbritt, MT6R, NCTC10449, LM7, MT703R, SE11, OMZ175, respectively; 9–14, *S. sobrinus* MT615R, OMZ176, B13, OMZ65, 6715, K1R, respectively; 15–16, *S. sanguis* ST6, ATCC10556. ►, 192-bp products.

cell pellet was resuspended in 100 ml of sucrose 20% w/v in STE (10 mM Tris-HCl, pH 8.0; 100 mM NaCl; 1 mM EDTA, pH 8.0) containing lysozyme (6 × crystallised, Seikagaku Kogyo Co., Tokyo) 0.5 mg/ml and *N*-acetylmuramidase (Seikagaku Kogyo Co.) 0.1 mg/ml. After incubation at 37°C for 60 min, samples were centrifuged and the pellets were resuspended in 100 ml of lysis buffer consisting of 50 mM KCl, 10 mM Tris-HCl, pH 8.3, gelatin 0.1 mg/ml, Nonidet P-40, 0.45% and Tween 20, 0.45%, together with 80 mg of proteinase K. After incubation at 60°C for 60 min, the samples were heated at 100°C for 5 min to inactivate proteinase K, residual cellular proteinases or nucleases, and to denature the target DNA.

For PCR amplification of *S. mutans* cultured *in vitro* in simulated clinical specimens, 2–(2 × 10⁴) cfu of *S. mutans* cells were added to 10 ml of culture-negative blood and diluted in 1 ml of TE buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA). Pellets of cells were made by centrifugation at 13000 *g* for 15 min, washed three times with the same buffer, and resuspended in the lysis buffer described above. Blood samples from mice challenged with *S. mutans* were treated in the same manner.

PCR amplification

Amplification was performed in a thermal cycler (IWAKI TSR-300, Tokyo) with a recombinant Taq DNA polymerase (Wako Junyaku, Osaka). The reaction mixture consisted of 1–4 ml of lysate, 2 ml of 10 × PCR amplification buffer (100 mM Tris-HCl, 500 mM KCl, 15 mM MgCl₂, Triton X-100, 1%), 0.5 ml of sense and anti-sense primers (20 mM, Daiichi Kikai, Tokushima, Japan) and 0.5 ml of Taq polymerase (1.25 u/ml; Wako Junyaku, Osaka) and was adjusted to 20 ml by the addition of distilled water. Mineral oil was also added to the mixtures to prevent evaporation. In all, 37 cycles were run under the following conditions: DNA denaturation at 94°C for 30 s, primer annealing at 48°C for 1 min, and DNA

extension at 74°C for 30 s. After the final cycle the reaction was terminated at 74°C for 2 min.

Agarose gel electrophoresis

The PCR amplification products were analysed by agarose gel electrophoresis in agarose (NuSieve 3:1, FMC, USA) 2.5% gels with ethidium bromide 0.5 mg/ml in TAE (0.04 M Tris-acetate, 1 mM EDTA, pH 8.0).

Enzyme immunoassay (EIA)

EIA was done according to procedures described previously.²⁰ Monoclonal antibodies, MAb498 and MAb516 against Ag I/II were used.¹⁹

Preparation of blood samples

Three male ddY mice weighing 30–40 g were given 0.1 ml of *S. mutans* strain B56 cell suspension in phosphate-buffered saline (5 × 10⁷ cfu) by injection into the tail vein. After injection, blood samples were collected at 5 min, 30 min, 24 h and 72 h.

Results

The primer set synthesised for this study allowed the PCR amplification of a gene fragment of *S. mutans* strains after 37 cycles. Results obtained by PCR with reference strains of *S. mutans*, *S. sobrinus* and *S. sanguis* and reactivity with two MAbs specific for Ag I/II are shown in fig. 1. Only the *S. mutans* strains gave an amplification product (table I and fig. 1); this appeared as a single DNA band of *c.* 192 bp. In contrast to MAb498, which reacted with both *S. mutans* and *S. sobrinus*, the PCR primer set resulted in a PCR product from *S. mutans* cell lysates only. MAb516 specific for *S. mutans* serotype *c* strains did not react with MT6R (serotype *c*), whereas DNA from

Table I. Reactivity of reference strains of mutans streptococci with monoclonal antibodies and PCR positivity of a *spaP* gene fragment

Species	Strains	EIA reactivity with*		PCR with <i>spaP</i>
		MAB498	MAB516	
<i>S. mutans</i>	Ingbritt	+	+	+
	MT6R	+	—	+
	K302	+	+	+
	T282	+	+	+
	B56	+	+	+
	NCTC10449	+	+	+
	LM7	+	—	+
	P4	+	—	+
	MT703R	+	—	+
	SE11	+	—	+
	MT557	—	—	+
	OMZ175	+	—	+
	161	—	—	+
<i>S. sobrinus</i>	OMZ176	+	—	—
	MT615R	+	—	—
	B13	+	—	—
	OMZ65	+	—	—
	6715	+	—	—
	K1R	+	—	—
	TH21	+	—	—
	SL1	+	—	—
<i>S. cricetus</i>	HS1	—	—	—
<i>S. rattus</i>	FA1	—	—	—
<i>S. downei</i>	MF25	—	—	—

* Reactivity of NCTC10449 was taken as 100 % and the positive values were > 50 %.

this strain was successfully amplified. DNA from strains MT557 and 161, which did not react with MAB 498, were also amplified. Therefore, the primer set can be used for PCR amplification of the *spaP* gene 192-bp fragment even when Ag I/II is not expressed on the cell surface.

Sensitivity

The minimum level for detection of *S. mutans* bacterial cells or isolated DNA by PCR product was

Table II. Reactions of reference strains of other oral streptococcal and various non-streptococcal bacteria by PCR for amplification of a *spaP* gene fragment

Species	Strains	PCR result
<i>S. sanguis</i>	ATCC10556	—
	KPE2	—
	ST6	—
<i>S. salivarius</i>	M36	—
<i>S. oralis</i>	NCTC8618	—
	ATCC9811	—
<i>S. gordonii</i>	ATCC10557	—
	M5	—
	Charlis	—
<i>S. ferus</i>	NCTC10231	—
	8S1	—
	HD3	—
<i>S. pyogenes</i>	A374	—
<i>Staphylococcus aureus</i>	209P	—
<i>Lactobacillus plantarum</i>	ATCC8014	—
	ATCC4646	—
<i>Actinobacillus</i>	Y4	—
<i>actinomycetemcomitans</i>		
<i>Eikenella corrodens</i>	1073	—
<i>Porphyromonas gingivalis</i>	381	—
<i>Propionibacterium acnes</i>	11827	—
<i>Candida albicans</i>	IFO1060	—

examined. A cell suspension of *S. mutans* Ingbritt was diluted serially in saline to $4-(4 \times 10^6)$ cfu/ml. The detection limits ranged from 4 to 40 cfu (fig. 2A). DNA from *S. mutans* Ingbritt was isolated, serially diluted in distilled water, and used as a template. The results showed that 0.3 pg of *S. mutans* DNA in the reaction mixture was the minimum needed to obtain a detectable PCR product (fig. 2B).

Specificity

Thirteen reference strains of *S. mutans* and 24 reference strains of other streptococci were used as templates for the primers with 1×10^4 bacterial cells in

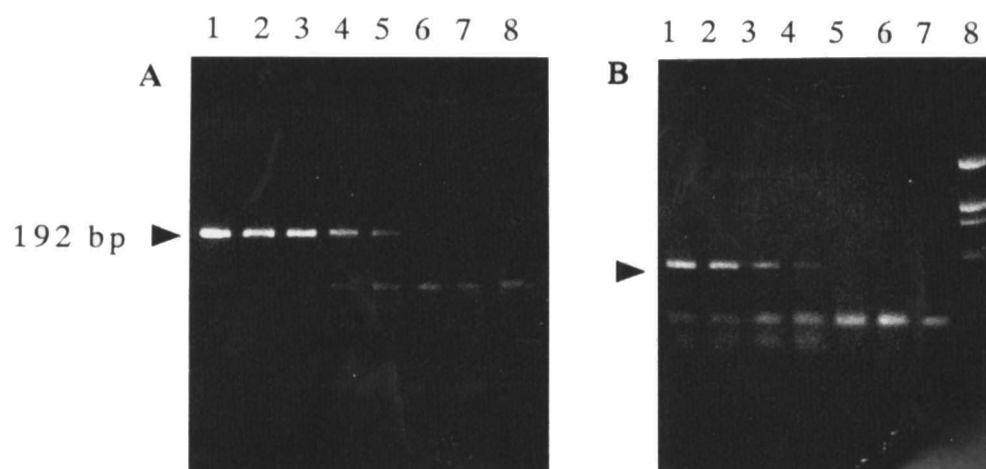


Fig. 2. PCR products amplified from serial dilutions of **A**, *S. mutans* Ingbritt and *S. sobrinus* SL1 cells and **B**, purified *S. mutans* DNA. **A**: lanes 1–7, cfu of *S. mutans* Ingbritt are 4×10^6 , 4×10^4 , 4×10^3 , 4×10^2 , 4×10 , 4, 0, respectively; 8, *S. sobrinus* SL1 4×10^6 cfu. **B**: lanes 1–7, the DNA contents are 300, 30, 3, 0.3, 0.03, 0.003 pg, respectively; 8, molecular mass markers are pUC119 *Hinf* I digests. ►, 192-bp products. The bands appearing at c. 50 bp are primer-dimers.

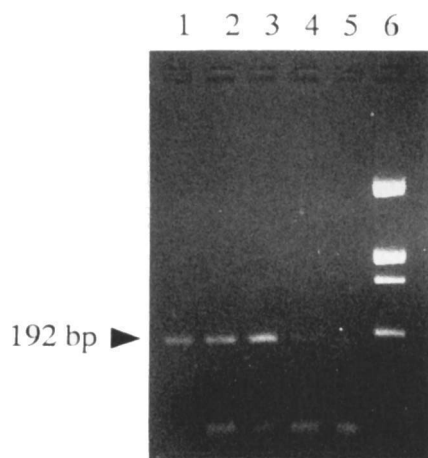


Fig. 3. PCR products generated with *S. mutans* Ingbritt cells in culture-negative whole blood. Lanes: 1–5, cfu are 2×10^4 , 2×10^2 , 2, 0, respectively; 6, molecular mass markers. ►, 192-bp PCR products.

the PCR mixture. All strains of *S. mutans* yielded amplification product, unlike the other streptococci examined (tables I, II and fig. 1). The specificity of the primer set was also examined by testing 50 isolates of *S. mutans* and nine of *S. sobrinus*. With all the *S. mutans* isolates, a PCR product of *c.* 192 bp was generated. None of the other species tested gave an amplification product (table II), demonstrating that the 192-bp gene fragment amplified by PCR with the primer set was specific for *S. mutans* strains.

PCR of blood samples

Culture-negative whole blood was tested with and without the addition of *S. mutans* strain B56 cells cultured *in vitro* (fig. 3). Samples containing 200 cells of *S. mutans* were positive by PCR. In experimental mice challenged with *S. mutans* cells, PCR products were generated from bacteria in the blood (fig. 3) when the viable cells were 19.2% and 3.3% of the original cell number at 30 min and 24 h after injection (data not shown). After 3 days, a positive PCR result

was also obtained when the viable cell count was < 100 cfu/ml.

Discussion

Results show that the detection of *S. mutans* by PCR with the *spaP* gene is more sensitive than when using the *gtfB* and *fif* gene probes;⁸ the sensitivity of the latter is 10^4 cfu less. Furthermore, the present method is simpler and more suitable for routine use than the methods employing Southern hybridisation⁸ for the rapid and accurate diagnosis of *S. mutans* infections.

Viridans streptococci are the most frequently isolated bacteria from cases of bacterial endocarditis resulting from dental extraction and it has been reported that *S. sanguis* and *S. mutans* are the most frequent isolates from the bloodstream of endocarditis patients.^{3,21} However, there is a need for studies with up-to-date protocols to identify the causative species and to enable more precise studies of the pathogenic mechanisms involved in endocarditis. Also, because *S. mutans* is resistant to host clearance mechanisms, having been detected in blood up to 3 days after the injection of these streptococci, it would be useful to clarify the mechanism of colonisation of the surfaces of heart valves and kidneys.

Recently, it has been reported that *S. sanguis*, *S. oralis* and *S. gordonii* have been isolated frequently from cases of endocarditis,²² but the identity of the causative organisms remains unclear because identification was based on biochemical methods and, therefore, difficult to compare with previous reports. The PCR identification strategy used in this study could more accurately identify strains of *S. mutans* than in previous studies and also heralds the development of a PCR method for the identification of *S. sanguis* and other species of oral viridans streptococci.

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Fig. 4. PCR products generated by direct testing of blood from mice infected with *S. mutans*. Lanes 1–3, before bacterial injection, 4–6, 5 min; 7–8, 30 min; 9–12, 24 h; 13–15, 72 h after injection, respectively; 16, *S. mutans* Ingbritt 2×10^4 cfu. ►, 192-bp PCR products.

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