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Overestimation of *Streptococcus mutans* prevalence by nested PCR detection of the 16S rRNA gene

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This study was carried out in order to compare two PCR-based methods in the detection of *Streptococcus mutans*. The first PCR method was based on primers for the 16S rRNA gene and the second method was based on specific primers that targeted the glucosyltransferase gene (*gtfB*). Each PCR was performed with eight different streptococci from the viridans group, five other streptococci and 17 different non-streptococcal bacterial strains. Direct use of the *S. mutans* 16S rRNA gene-specific primers revealed that *Streptococcus gordonii* and *Streptococcus infantis* were also detected. After amplifying the 16S rRNA gene with universal primers and subsequently performing nested PCR, the *S. mutans*-specific nested primers based on the 16S rRNA gene detected all tested streptococci. There was no cross-reaction of the *gtfB* primers after direct PCR. Our results indicate that direct PCR and nested PCR based on 16S rRNA genes can reveal false-positive results for oral streptococci and lead to an overestimation of the prevalence of *S. mutans* with regards to its role as the most prevalent causative agent of dental caries.

INTRODUCTION

Oral streptococci, e.g. *Streptococcus mutans* and *Streptococcus sobrinus*, are important constituents of dental plaque. Being able to distinguish between them is believed to be useful for the detection and prevention of dental caries. Biochemical tests and immunological and genetic methods have been used to differentiate them (de Soet *et al.*, 1990; Beighton *et al.*, 1991; Cangelosi *et al.*, 1994).

Because of its high specificity and sensitivity, PCR is currently being applied in a wide range of medical diagnostics and research. The occurrence of several gene copies of 16S rRNA in the cell and the key role of this genetic target for bacterial taxonomy has made it an established target for PCR detection of bacteria in all different fields of microbiology.

Other targets often used for the detection of bacteria are virulence factors, which are species-specific and provide an additional detection marker in order to avoid ambiguous PCR results caused by the high similarity of species-specific rRNA gene sequences, e.g. in oral streptococci. Species-specific primers based on the 16S rRNA gene sequences were used for the detection of *S. sobrinus* and *S. mutans* in a direct PCR (Rupf *et al.*, 1999, 2001). The *S. mutans*-specific primers for the 16S rRNA gene were also used in a nested PCR to detect *S. mutans* in dental plaque (Sato *et al.*, 2003; Kato *et al.*, 2004). Nested PCR was performed after amplification of the 16S rRNA gene with universal primers (Sato *et al.*, 1997). Oho *et al.* (2000) developed a PCR detection method to distinguish between *S. mutans* and *S. sobrinus* based on amplification of glucosyltransferase genes, e.g. the *gtfB* gene, which encodes a glucosyltransferase that synthesizes water-insoluble glucan from sucrose (Kuramitsu *et al.*, 1995). This approach was extended by Hoshino *et al.* (2004) to detect the oral streptococci *Streptococcus oralis*, *Streptococcus salivarius*, *Streptococcus sanguinis* and *Streptococcus gordonii*. The aim of this study was to assess the effectiveness of both PCR detection methods in distinguishing *S. mutans* from other oral streptococci. Therefore, we compared the two PCR detection methods described above by using them simultaneously to distinguish *S. mutans* from 12 different streptococcal strains and 17 other bacterial strains.

METHODS

Bacterial strains. *S. mutans* ATCC 25175^T, *S. salivarius* DSM 20067 and *S. oralis* ATCC 35037^T were kindly provided by the Institute of Medical Microbiology and Hygiene of the Albert Ludwigs University, Freiburg, Germany. *S. sobrinus* DSM 20381 was obtained from the DSMZ (Braunschweig, Germany). All bacterial strains were maintained routinely with weekly subculturing on Columbia blood agar (heipha Diagnostika). Long-term storage of these bacteria was at −70 °C in basic growth medium containing 15 % (v/v) glycerol as described by Jones *et al.* (1991).

Extraction of DNA and PCR analysis. Bacterial DNA from the strains listed in Table 1 was either provided by GeneScan Europe AG (Freiburg, Germany) or was extracted directly from bacteria grown in brain heart infusion (BHI) according to Oho *et al.* (2000). Bacterial cultures were centrifuged at 12 000 ***g*** for 5 min and the resulting pellet was boiled in 200 µl lysis buffer (10 mM Tris/HCl buffer, 1 mM EDTA, 1 % Triton X-100, pH 8·0) for 10 min. After centrifugation at 12 000 ***g*** for 10 min, the supernatant containing DNA was used for PCR. For PCR detection using nested primers for the 16S rRNA gene, the first round of PCR was carried out by using HotStarTaq Polymerase (Qiagen) with the universal primers 8UA (forward: 5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492 (reverse: 5′-TACGGGTACCTTGTTACGACTT-3′) (Sato *et al.*, 1997). PCR was performed in a MyiQ PCR cycler (Bio-Rad) according to the temperature program described by Kato *et al.* (2004). The resulting PCR products were then amplified with the nested species-specific primers, which were based on the 16S rRNA gene sequence described by Rupf *et al.* (1999, 2001). The nested-primer sequences were: forward primer (sm1), 5′-GGTCAGGAAAGTCTGGAGTAA-AAGGCTA-3′; reverse primer (sm2), 5′-GCGTTAGCTCCGGCA-CTAAGCC-3′. PCR was performed according to the temperature program described by Kato *et al.* (2004) in a MyiQ cycler. The size of the expected PCR product was 282 bp. The same species-specific primers and temperature program were used to detect the bacterial strains directly without prior amplification using the universal primers for 16S rRNA genes.

A second PCR was conducted by using primers specific for the glucosyltransferase gene of *S. mutans* (*gtfB*) described by Oho *et al.* (2000), following the same temperature program and in the same MyiQ cycler. The primer sequences used were as follows: forward primer (*gtfB*-F), 5′-ACTACACTTTCGGGTGGCTTGG-3′; reverse primer (*gtfB*-R), 5′-CAGTATAAGCGCCAGTTTCATC-3′. The size of the expected PCR product was 517 bp.

Table 1. Detection pattern of tested bacterial strains using direct PCR for the 16S rRNA gene, nested 16S rRNA gene PCR and direct glucosyltransferase (*gtfB*) gene PCR

+, Detected; −, not detected.

Bacterial species	*S. mutans*-specific 16S rRNA gene primers		*gftB* primer specific for glucosyltransferase gene
	Direct PCR	Nested PCR	
Streptococcus mutans ATCC 25175^T	+	+	+
Streptococcus salivarius DSM 20067	−	+	−
Streptococcus gordonii DSM 20568	+	+	−
Streptococcus infantis DSM 12492^T	+	+	−
Streptococcus pyogenes DSM 2072	−	+	−
Streptococcus pneumoniae DSM 20566^T	−	+	−
Streptococcus thermophilus DSM 20617^T	−	+	−
Streptococcus ratti DSM 20564^T	−	+	−
Streptococcus canis DSM 20715^T	−	+	−
Streptococcus downei DSM 5635^T	−	+	−
Streptococcus sobrinus DSM 20381	−	+	−
Streptococcus suis DSM 9682^T	−	+	−
Streptococcus oralis ATCC 35037^T	−	+	−
Staphylococcus epidermidis DSM 20042	−	+	−
Staphylococcus simulans DSM 20322^T	−	−	−
Staphylococcus pasteuri DSM 10656^T	−	−	−
Staphylococcus aureus DSM 3463	−	−	−
Lactobacillus acidophilus DSM 20079^T	−	−	−
Lactobacillus oris DSM 4864^T	−	−	−
Lactobacillus pentosus DSM 20314^T	−	−	−
Lactobacillus brevis DSM 20054^T	−	−	−
Pseudomonas aeruginosa DSM 3227	−	−	−
Pseudomonas putida DSM 7162	−	−	−
Burkholderia cepacia DSM 50181	−	−	−
Aeromonas caviae DSM 7323^T	−	−	−
Acinetobacter johnsonii DSM 6963^T	−	−	−
Listeria grayi DSM 20596	−	−	−
Erwinia mallotivora DSM 4565^T	−	−	−
Enterococcus faecium DSM 20477^T	−	−	−
Propionibacterium avidum DSM 4901^T	−	−	−

PCR products were analysed electrophoretically in 1·5 % agarose gel in Tris/borate/EDTA running buffer (TBE), stained with ethidium bromide and visualized with UV light in a Gel Doc EQ universal hood (Bio-Rad). All PCRs were conducted in duplicate.

RESULTS AND DISCUSSION

Fig. 1 shows representative electrophoresis results of nested PCR products using the *S. mutans*-specific 16S rRNA gene primers (lanes 1–4) and direct PCR products using the *S. mutans*-specific glucosyltransferase gene (*gtfB*) primers (lanes 6–9). In the nested PCR, the *S. mutans*-specific primers based on the 16S rRNA gene not only delivered PCR products for *S. mutans* (lane 4), but also for *S. oralis* (lane 1) and *S. salivarius* (lane 3). *Enterococcus faecium* was not detected (lane 2). In the performed direct PCR, the *gtfB* primers were only specific for *S. mutans*.

Table 1 shows the results of the different PCR detection methods. The *S. mutans* primers specific for the 16S rRNA gene also detected *S. gordonii* and *S. infantis* when used directly without prior amplification of the 16S rRNA gene. After amplification of the 16S rRNA gene with universal primers, all of the streptococcal strains, as well as *Staphylococcus epidermidis*, were detected in the nested PCR when using the *S. mutans*-specific primers. No other non-streptococcal strains tested were detected in the nested 16S rRNA gene PCR. The primers specific for the glucosyltransferase gene detected only *S. mutans*. No cross-reactions were observed to any of the other tested streptococcal or non-streptococcal strains in the direct PCR with *gtfB* gene-specific primers.

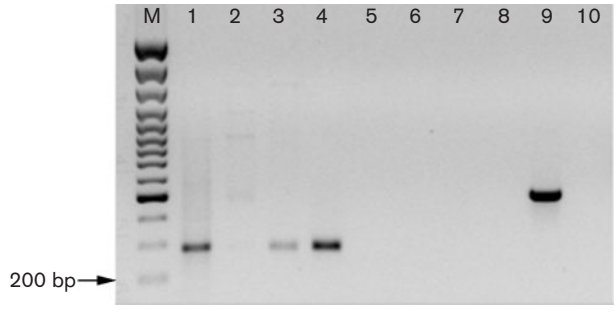

Fig. 1. Representative results of the electrophoresis of PCR products using *S. mutans*-specific nested 16S rRNA gene primers (lanes 1–4) and *S. mutans*-specific glucosyltransferase gene (*gtfB*) primers (lanes 6–9). M, 100 bp molecular mass marker with a first visible band of 200 bp; lane 1, PCR product of *S. oralis* (282 bp); lane 2, PCR product of *E. faecium*; lane 3, PCR product of *S. salivarius* (282 bp); lane 4, PCR product of *S. mutans* (282 bp); lane 5, blank control with water; lane 6, PCR product of *S. oralis*; lane 7, PCR product of *S. sobrinus*; lane 8, PCR product of *S. salivarius*; lane 9, 517 bp fragment obtained from PCR of *S. mutans* with *gtfB* primers; lane 10, blank control with water.

Table 2. Alignment of the 16S rRNA gene primer sites of streptococcal strains and *Staphylococcus epidermidis*

Homologous nucleotides are in bold type.

Bacterial species	GenBank accession no.	Alignment of forward-primer site	Similarity (%)*	Alignment of reverse-primer site	Similarity (%)*
Streptococcus mutans	AE015003	GGTCAGGAAAGTCTGGAGTAAAAGGCT	100·0	GGCTTAGTGCCGGAGCTAACGC	100·0
Streptococcus salivarius	AF459433	**GGTTTGATAAGTCTGAAGTTAAAGGCT**	77·8	**GATTCAGTGCCGCAGCTAACGC**	81·8
Streptococcus gordonii	AF003931	**GGTTAGATAAGTCTGAAGTTAAAGGCT**	81·5	**GGCTTAGTGCCGCAGCTAACGC**	95·4
Streptococcus infantis	AY485603	**GGTTAGATAAGTCTGAAGTTAAAGGCT**	81·5	**GGTTTAGTGCCGAAGCTAACGC**	90·9
Streptococcus pyogenes	AF076028	**GGTTTTTTTAAGTCTGAAGTTAAAGGCA**	70·5	**GGCTTAGTGCCGGAGCTAACGC**	100·0
Streptococcus pneumoniae	AY525794	**GGTTAGATAAGTCTGAAGTTAAAGGCT**	81·5	**GGTTTAGTGCCGTAGCTAACGC**	90·9
Streptococcus thermophilus	AB200871	**GGTTTGATAAGTCTGAAGTTAAAGGCT**	77·8	**GATTCAGTGCCGCAGCTAACGC**	81·8
Streptococcus ratti	AJ420201	**GGTTTTGTAAGTCTGAAGTCAAAGGCA**	66·7	**GGCTTAGTGCCGGAGCTAACGC**	100·0
Streptococcus canis	AB002483	**GGTTCTTTAAGTCTGAAGTTAAAGGCA**	70·4	**GGCTTAGTGCCGGCTAAGACGC**	77·3
Streptococcus downei	AY188350	**GGTTTAGTAAGTCTGAAGTTAAAGGCA**	74·1	**GACTTAGTGCCGCAGCTAACGC**	90·9
Streptococcus sobrinus	AY691533	**GGTTTAGTAAGTCTGAAGTTAAAGGCA**	74·1	**GACTTAGTGCCGACGCTAACGC**	81·8
Streptococcus suis	AF009509	**GGTTCTGTAAGTCTGAAGTTAAAGGCA**	81·5	**GGCTTAGTGCCGTAGCTAACGC**	95·4
Streptococcus oralis	AY281080	**GGTTAGATAAGTCTGAAGTTAAAGGCT**	81·5	**GGTTTAGTGCCGCAGCTAACGC**	90·9
Staphylococcus epidermidis	AJ880759	**GGTTTTTTTAAGTCTGATGTGAAAGCCC**	63·0	CCCTTAGTGCTGCAGCTAACGC	81·8

*Similarity to *S. mutans* primer site.

Table 2 shows the alignment of 16S rRNA genes (sites of forward and reverse primers) from all streptococcal strains tested, as well as of *Staphylococcus epidermidis*, in addition to the GenBank accession numbers (http://www.ncbi.nlm.nih.gov/gquery/gquery.fcgi). The degrees of similarity for the forward primer were between 63 % for *Staphylococcus epidermidis* and 81·5 % for five of the tested streptococci, whereas the values for the reverse primer were between 77·3 % for *Streptococcus canis* and 100 % for *Streptococcus pyogenes* and *Streptococcus ratti*.

Both PCR methods are simple and rapid and both of them were applied to saliva and native plaque substances to distinguish between *S. mutans* and *S. sobrinus*, the most frequently detected cariogenic oral streptococci (Oho *et al.*, 2000; Sato *et al.*, 2003; Kato *et al.*, 2004). In the present study, the *S. mutans*-specific primers used in nested 16S rRNA gene PCR were not specific for *S. mutans*, but also detected 12 other tested streptococcal strains, including important oral streptococci. Using these primers in direct 16S rRNA gene PCR also revealed a specificity for *S. infantis* and *S. gordonii*. This indicates that this PCR method could give false-positive results and consequently lead to false conclusions of a higher prevalence of *S. mutans*. Prior amplification of the 16S rRNA gene enhanced false-positive results and decreased the specificity of nested primers. These false-positive results could not be caused by contamination of *Taq* polymerase as described by Corless *et al.* (2000), as the negative control, as well as the PCR of 16 other bacterial strains tested, showed no positive results with the nested 16S rRNA gene primers. The higher sensitivity of nested in comparison to direct 16S rRNA gene PCR was reported by Sato *et al.* (2003). The authors found a detection limit of 100 fg for *S. mutans* DNA. The primers for *gtfB* were more specific and did not give false-positive results for any of the other bacterial strains tested. The detection limit of *gtfB* gene PCR was reported to be 3·2 pg *S. mutans* DNA (Hoshino *et al.*, 2004). It is known that higher sensitivity is an advantage of nested PCR in the detection of genetic targets; however, the usefulness of nested PCR has to be correlated with the required level of specificity, which can be demonstrated by using the proper control micro-organisms. The enhancement of sensitivity of detection using the nested 16S rRNA gene PCR led to lower overall specificity, destroying the advantage of nested PCR.

In the study by Kato *et al.* (2004), *S. mutans* and *S. sobrinus* were detected in different sections of impregnated plaque materials by using the same nested, 16S rRNA gene-specific primers and the same temperature program as were applied in this study. *S. mutans* was found to have a wider habitat in the different plaque layers. In each layer, *S. mutans* only or both species were detected. Rupf *et al.* (1999) also used the same annealing temperature for the detection of *S. mutans* with these primers. The high similarity in the primer sites of 16S rRNA gene sequences in the strains of oral streptococci that were tested underlines the possibility of false-positive results and the difficulty of controlling specificity by changing the annealing temperature, particularly when using these primers in the nested 16S rRNA gene PCR. The PCR primers that were based on detection of the *gtfB* gene would be a more proper PCR tool for the confirmation of *S. mutans*. This is due to the fact that 16S rRNA gene sequencing has been difficult to use in distinguishing oral streptococci, because some species have 16S rRNA gene sequences that differ at only a few bases (Paster *et al.*, 1998, 2001). Taking oral bacteria that cannot be cultured from native dental plaque into consideration, care is essentially required when using 16S rRNA genes to detect oral streptococci by PCR. Examples of such bacterial strains related closely to oral streptococci are members of the genera *Abiotrophia* and *Granulicatella*. We would recommend using more than one specific PCR method for the detection of *S. mutans*. Future studies on the usefulness of direct *gtfB* PCR and the possibilities of developing a nested PCR method based on the *gtfB* gene should be assessed. Results of simple PCR detection of the 16S rRNA gene sequences need to be confirmed with a second PCR method. A combination of more than one molecular method, e.g. arbitrarily primed PCR (Napimoga *et al.*, 2004; Barone *et al.*, 2005), PCR and the molecular analysis of genes other than 16S rRNA (Igarashi *et al.*, 2001; Teng *et al.*, 2002; Chen *et al.*, 2005) and chequerboard hybridization (Paster *et al.*, 1998), is needed to avoid overestimating the prevalence and role of *S. mutans* in dental plaque.

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