Rapid Identification of Mutans Streptococcal Species

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Abstract: Mutans streptococci are considered to be the predominant pathogens in dental caries. Three methods, i.e. dot blot hybridization analysis, PCR analysis and SDS-blue dextran-PAGE, were examined for identifying mutans streptococcal species. In dot blot hybridization, DNA probe derived from the dextranase gene (dexA) of Streptococcus mutans hybridized with different intensities under the condition of low stringency against each species of mutans streptococci although the dexA probe was specific for S. mutans under the condition of high stringency. Oligonucleotide primers for polymerase chain reaction (PCR) were designed on the basis of the dexA DNA sequence. The primers amplified species-specific PCR products in the reference species (15 strains of 5 species) of mutans streptococci. An electrophoretic profile of dextranases from the mutans streptococci on SDS-blue dextran-PAGE also showed species-specific behavior. These results suggest that the three identification methods examined here are useful for distinguishing the species of mutans streptococci and also indicate that PCR analysis is suitable for simple, rapid and reliable identification of mutans streptococcal species.

Key words: Dextranase, Identification, Mutans streptococci, Polymerase chain reaction

Mutans streptococci are considered to be causative agents of dental caries and have been classified into 5 species: Streptococcus cricetus (serotype a), S. rattus (serotype b), S. mutans (serotypes c, e, and f), S. sobrinus (serotypes d and g), and S. downei (serotype h) (11, 24). Several techniques for detection and identification of mutans streptococci have been reported. Differentiation on the basis of colony morphology on selective Mitis-salivarius agar has usually been used as a primarily effective isolation method for mutans streptococci (9). Then, additional tests are required to confirm the identification of those isolates; e.g., a number of biochemical tests have been proposed for distinguishing the species of mutans streptococci (2, 16, 17). As these tests easily lead to erroneous conclusions, immunological (6, 10, 18) and DNA probe (5, 19) methods, which are more sensitive and specific, are utilized as more accurate identification methods, although they are cumbersome, tedious and time consuming. In order to obviate such limitations, PCR seems to be useful. PCR has recently been used to detect and identify a wide range of pathogens (3, 4, 7, 23). Lately, we have reported a simpler, more rapid and accurate PCR method for the detection and identification of S. mutans (15), but it was only specific for the S. mutans species. So far, there have been no reports of PCR identification of other species in mutans streptococci. We have therefore tried to design PCR primers to identify the mutans streptococcal species.

Mutans streptococci produce an extracellular dextranase which is considered to be one of the virulence factors of those bacteria (11, 21). The dextranase gene (dexA) from S. mutans strain Ingbrit (serotype c) was cloned (13) and sequenced (14). In this report, we describe the hybridization analysis with dexA probe and the electrophoretic profiles of dextranase among mutans streptococci for identification of the mutans streptococcal species. We also designed PCR primers on the basis of dexA DNA sequence of S. mutans in order to develop a simple, rapid and reliable identification method of the mutans streptococcal species.

The strains of mutans streptococcal species used in this study are listed in Table 1. These bacteria were grown and maintained anaerobically at 37°C on brain-heart infusion agar (Difco Laboratories, Detroit, Mich., U.S.A.) plates containing 1% yeast extract (Difco).

Chromosomal DNAs were purified as described previously (13). Briefly, the cells were treated with lysozyme, mutanolysin, RNaseA and pronaseE, and then lysed by adding sodium dodecyl sulfate (SDS). Total DNA was purified by centrifugation in CsCl-ethidrumide gradients. All the DNA fractions were

Abbreviations: BCIP, 5-bromo-4-chloro-3-indolyl phosphate; BD, blue dextran 2000; dexA, S. sobrinus dextranase gene; dexA, S. mutans dextranase gene; DIG, digoxigenin; NBT, nitro blue tetrazolium; PCR, polymerase chain reaction; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

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dialyzed against and stored in TE (10 mM Tris-HCl, 1 mM EDTA) buffer, pH 8.0. Dot blot hybridization analysis was performed with DIG high-prime labeling and detection starter kit I (Boehringer Mannheim, Mannheim, Germany) as described by the manufacturer unless otherwise noted. Both hybridization and washing were carried out at 68°C (high stringency) or 55°C (low stringency).

Two forward (F1 and F4) primers and one reverse (RV8) primer were designed based on the dexA DNA sequence (14). The sequences of F1, F4 and RV8 were 5'-TAT TAC AGC TAC TGT TGA GG-3' (positions 729 to 748), 5'-TAT GCT GCT ATT GGA GGT TC-3' (positions 973 to 992) and 5'-CAG TTT TCA TAG CTT GAG CC-3' (positions 1450 to 1471), respectively. The PCR reaction mixture (50 µl) contained 50 pmol of each primer, 200 µM each of the deoxyribonucleotides, 2.5 U Taq DNA polymerase, reaction buffer (Takara Shuzo Co., Ltd., Kyoto, Japan) and 10 µl of template solution. The PCR procedure consisted of initial denaturation of 94°C for 3 min, followed by 94°C for 1 min, 55°C for 1 min and 72°C for 1 min (30 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 electrophoresed on 1% agarose gels and stained with ethidium bromide.

Active staining of dextranase on SDS-polyacrylamide gel electrophoresis (PAGE) containing blue dextran (BD) was carried out as described previously (12). High molecular-weight markers (SDS-6H; Sigma Chemical Co., St. Louis, Mo., U.S.A.) were used as molecular mass standards for SDS-BD-PAGE. Crude enzymes of mutans streptococcal strains were prepared using 70% ammonium sulfate precipitation of the culture supernatants, followed by dialysis in 20 mM phosphate buffer.

<table>
<thead>
<tr>
<th>Species</th>
<th>Strains (serotype)</th>
<th>Length (kbp) of PCR products&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>S. cricetus</em></td>
<td>E49 (a)</td>
<td>1.27</td>
</tr>
<tr>
<td></td>
<td>HS6 (a)</td>
<td>1.30</td>
</tr>
<tr>
<td><em>S. rattus</em></td>
<td>BHT (b)</td>
<td>1.27</td>
</tr>
<tr>
<td></td>
<td>FA1 (b)</td>
<td>1.30</td>
</tr>
<tr>
<td><em>S. mutans</em></td>
<td>ATCC 25175 (c)</td>
<td>0.72</td>
</tr>
<tr>
<td></td>
<td>ATCC 33535 (c)</td>
<td>0.48</td>
</tr>
<tr>
<td></td>
<td>GS5 (c)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Ingbrtt (c)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>JCM 5175 (c)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>LM7 (e)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>MT703 (e)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>OMZ 175 (f)</td>
<td></td>
</tr>
<tr>
<td><em>S. sobrinus</em></td>
<td>OMZ 176 (d)</td>
<td>0.46</td>
</tr>
<tr>
<td></td>
<td>NIDR 6715 (g)</td>
<td>0.44</td>
</tr>
<tr>
<td><em>S. downei</em></td>
<td>NCTC 11391 (h)</td>
<td>0.91</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.87</td>
</tr>
</tbody>
</table>

<sup>a</sup> The length of the PCR products were measured from the electrophoretic profiles.

<sup>b</sup> F1-RV8 and F4-RV8 are the primer pairs used for PCR.
pH 6.0.

With dot blot hybridization to chromosomal DNA from mutans streptococci, the distribution of dexA-related sequences among the reference strains was examined (Fig. 1). Chromosomal DNAs from the strains were probed with dexA (2.4 kb PstI fragment, ref. 14) at low stringency to detect any homologies among the strains. Strong hybridization to the chromosomal DNAs from S. mutans (serotypes c, e and f) was observed. S. cricetus (serotype a) and S. rattus (serotype b) also harbored weak homologous sequences. Very weak homologies with S. sobrinus (serotypes d and g) and S. downei (serotype h) were detected. A 57.8% DNA sequence homology between S. mutans dexA and S. sobrinus dextranase (dex) genes (14) suggests that the homologies of S. cricetus and S. rattus against dexA were higher than that of S. sobrinus. Only S. mutans (serotypes c, e and f) was strongly hybridized at high stringency, and others were not (data not shown). The highest DNA homology in serotypes c, e and f of S. mutans is consistent with the close genetic relationships among these serotypes (8). The conservation of dexA-related genes among mutans streptococci would explain the importance of the dextranase of these species in the mouth.

Two sets of primers (F1-RV8 and F4-RV8) were used for PCR amplification against the reference strains of mutans streptococci. The PCR effectively differentiated the species (Fig. 2): species-specific PCR products were observed. The lengths of the PCR products are shown in Table 1. Although S. mutans and S. sobrinus contain several serotypes in each species (Table 1), the length of the PCR products was identical among serotypes in the same species. Comparing the two sets of primers, i.e. F1-RV8 and F4-RV8, the F1-RV8 pair is recommended for differentiating S. mutans and S. sobrinus because the products (0.48 and 0.44 kb) of these two species amplified by F4-RV8 primers were very close in length and not easily distinguishable on agarose gel (Fig. 2B). Unfortunately, S. cricetus and S. rattus could not be differentiated by their PCR products (Fig. 2) although it was easy to distinguish these two species from the others. In addition, the PCR products of S. cricetus and S. rattus had the same restriction patterns for BamHI, HindIII, EcoRI, PstI and SacI (data not shown). Accordingly, biochemical testing would be required for differentiation: S. rattus produces ammonium from arginine and is resistant to bacitracin, which are not characteristics of S. cricetus (16). S. sanguis ATCC 10556 and S. gordonii

![Fig. 2](image-url)
ATCC 10558 did not give any PCR products amplified by these primers. In order to examine whether the primers amplified the dextranase gene of each strain, dexA-hybridization analysis against the PCR products shown in Fig. 2 was tested at both high and low stringencies. Only the PCR products from S. mutans (serotypes c, e, and f) were detected by the dexA probe at not only high but also low stringencies (data not shown), implying that the PCR products (0.72 and 0.48 kbp) were derived from dexA sequence in S. mutans species. On the other hand, the PCR products from other four species did not hybridize with the dexA probe. In S. sobrinus, a homology analysis between dex sequence and the PCR primers (F1, F4, and RV8) showed that F1-RV8 and F4-RV8 primers were not able to amplify either 0.46 or 0.44 kbp PCR products on dex DNA sequence. These results suggest that the PCR products were not derived from the dex sequence of S. sobrinus. Although gene cloning and sequence analyses of dextranases from S. cricetus, S. rattus and S. downei have not been reported yet, similar speculation is also thought in these species. However, a single, clear and species-specific PCR product was amplified by F1-RV8 and F4-RV8 primers as shown in Fig. 2, which would suggest that the sequences highly homologous with these primers existed on their chromosomal DNAs. Further analysis is needed to clarify the sources of the PCR products. Amplification of the species-specific PCR products indicates that the present PCR method is useful for identification of the species in mutans streptococci.

It has been reported that mutans streptococci produce extracellular dextranase (20, 22). However, the electrophoretic patterns of the enzymes on SDS-BD-PAGE have not been shown yet, except those of S. mutans (serotype c) and S. sobrinus (serotype g) (1, 12). Therefore, we tried to analyze the active staining patterns of dextranase from reference strains. As shown in Fig. 3, analysis of the dextranases on SDS-BD-PAGE showed species-specific electrophoretic patterns, which were parallel to those of PCR products (Fig. 2). S. cricetus, S. rattus and serotypes c, e and f of S. mutans exhibited a single active band, and the molecular sizes were about 116 kDa for S. cricetus and S. rattus and about 100 kDa for S. mutans. S. sobrinus (serotypes d and g) and S. downei showed ladder active bands with much larger molecular sizes (about 225 to 155 kDa). The largest enzyme (220 kDa) of S. downei was slightly smaller than that (225 kDa) of S. sobrinus, and the differences in molecular size were easily distinguished on the gel. It should be noted that several minor bands appeared in all strains when incubation for renaturation was prolonged. This electrophoretic profile of dextranase was comparable to the results of both DNA hybridization and PCR analysis, and is helpful for species-identification in mutans streptococci.

Since mutans streptococci are believed to be involved in the pathogenesis of dental caries, a number of identification methods have been developed to distinguish these species in clinical specimens. Although biochemical tests have been widely used for identification (2, 16, 17), they have often given erroneous conclusions. An immunological method using polyclonal and monoclonal antibodies is more reliable than a biochemical test, but reagents are not widely available (6, 10, 18). In order to obviate such limitations, DNA probes have recently been designed for a DNA hybridization test on the basis of sequence of species-specific proteins or ribosomal RNA, which are considerably more convenient than the above tests (5, 19). However, these tests would take longer and still be troublesome. Therefore, a simpler, more rapid and reliable identification method has been required. We newly report here three identification methods for the mutans streptococcal species: dexA-hybridization analysis, PCR analysis and electrophoretic profile of dextranase. In particular, the PCR method would satisfy the above requirements and could potentially replace conventional identification methods such as biochemical and immunological tests for identifying mutans streptococcal species.

References


