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Identification and Detection of *Streptococcus anginosus* Using Species-Specific 16S rDNA Primers

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This study was undertaken to develop PCR primers for the identification and detection of *Streptococcus anginosus* using species-specific forward and reverse primers. These primers targeted the variable regions of the 16S ribosomal RNA coding gene (rDNA). The primer specificity was tested against 12 *S. anginosus* strains and 6 different species (10 strains) of oral bacteria. The primer sensitivity was determined by testing serial dilutions of the purified genomic DNA of *S. anginosus* ATCC 33397[T]. The data showed that species-specific amplicons were obtained from all the *S. anginosus* strains tested, but not in the six other species. The PCR could detect as little as 0.4 pg of the chromosomal DNA from *S. anginosus*. This suggests that the PCR primers are highly sensitive and applicable to the detection and identification of *S. anginosus*.

Keywords: Identification, PCR primer, *Streptococcus anginosus*, 16S rDNA

Introduction

In order to assess the epidemiology of the oral pathogens, as well as the diagnosis, treatment, and prognosis of oral infectious diseases, it is important to first identify the principal putative pathogens involved. Several putative periodontal pathogens have already been detected in previous studies using DNA probe methods (Kook *et al.*, 2003) or PCR (Ashimoto *et al.*, 1996; Kim *et al.*, 2004; Slots *et al.*, 1995). The advent of PCR has resulted in substantial savings of time, cost, and experimental effort compared with other bacterial identification methods, including cell culture, the DNA probe method, 16S ribosomal RNA coding gene (rDNA) sequencing, ribotyping, *etc.* The advantages of PCR have guaranteed its wide use in diagnosis as well as the identification of bacterial species (Lee *et al.*, 2004; Wang and Lee, 2003). 16S rDNA can be used effectively in PCR assays because 16S rDNA is ubiquitous in all prokaryotic organisms, and a comparative analysis of 16S rDNA has demonstrated that variable sequence regions are interspersed with highly conserved regions (Woese, 1987).

In a study of the genus *Streptococcus* based on the sequence comparisons of the 16S rDNA, a total of six species groups, the anginosus, mitis, mutans, salivalius, bovis, and pyogenic groups, were demonstrated (Kawamura *et al.*, 1995). Among *Streptococcus* spp., the anginosus group is known to have a high abscess forming ability. A recent study reported that the anginosus group included 3 species (*S. anignosus*, *S. intermedius*, and *S. constellatus*) and 2 subspecies (*S. constellatus* subsp. *constellatus* and *S. constellatus* subsp. *pharyngis*) (Takao *et al.*, 2004). Using checkerboard DNA-DNA hybridization, Siqueira *et al.*, (2002) found an anginosus group in 19.2 and 44.4% of chronic pulpitis lesions and acute periapical abscess, respectively. *S. anginosus* belongs to the anginosus group, which has been found in 13 and 44%

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of oral and esophagus cancers, respectively (Morita *et al.*, 2003). The checkerboard DNA-DNA hybridization uses the whole genomic DNA as probe, and there might be some cross-reaction with other bacterial species making it less sensitive than PCR. *S. anginosus* is known to be related to oral infectious diseases such as pulpal and periapical abscess.

Recently, a set of PCR primers based on the 16S rDNA was developed, and used to identify *S. anginosus* (Takao *et al.*, 2004). Considering the fact that a mutation occurs more often in prokaryotic cells than in eukaryotic cells during DNA replication, a false positive or negative reaction could be better screened through the development of more species-specific PCR primers. Therefore, the aim of this study was to develop a new set of PCR primers that could be used to detect and identify *S. anginosus*.

Materials and Methods

Bacterial strains and growth condition

The type strains and reference strains of the bacteria used in this study are as follows: *S. anginosus* ATCC 33397^T, *S. intermedius* KCTC 3268^T, *S. constellatus* subsp. *constellatus* ATCC 27823^T, *Streptococcus mutans* ATCC 25175^T, *Streptococcus thermophins* KCTC 3658^T, *S. mitis* KCTC 3556^T, and *S. sanguinis* KCTC 3634^T. All the strains were obtained from either the American Type Culture Collection (ATCC, University Boulevand, USA) or the Korean Collection for Type Cultures (KCTC, Korea). Eleven *S. anginosus* strains (ChDC YA1, ChDC YA2, ChDC YA3, ChDC YA4, ChDC YA5, ChDC YA7, ChDC YA8, ChDC YA9, ChDC YA10, ChDC YA11, and ChDC YA12), one *S. intermedius* (ChDC B717), and three *S. constellatus* subsp. *constellatus* (ChDC B707, ChDC B280 and ChDC B290) were isolated from the dental plaque of Korean dental patients. All the *S. anginosus* isolates were identified by 16S rDNA cloning and sequencing. The clinical isolates of *S. intermedius* and *S. constellatus* subsp. *constellatus* were identified by the 16S rDNA sequence comparison and the species-specific PCR technique reported by Takao *et al.*, (2004). All the streptococcal strains were grown in Todd Hewitt broth (Difco Diagnostics, USA) in a 37°C incubator.

Bacterial genomic DNA preparation

The bacterial genomes were prepared using a G-spin^{TM} Genomic DNA Extraction kit (iNtRON Co., Seoul, Korea) according to the manufacturer's instructions. The DNA concentrations were determined by measuring OD at 260 and 280 nm using UV-spectrophotometry (Ultrospec 2000, Pharmacia Biotech., UK).

Primer Design

The oligonucleotide forward and reverse primers, which were specific to *S. anginosus*, were designed using the PRIMERSECT program (DNASTAR Inc., USA). The 16S rDNA sequences of *S. anginosus*, in the GenBank database, were used as the DNA templates in PRIMERSECT. The strains and GenBank accession numbers are as follows: *S. anginosus* ATCC 33397^T (AF104678), 367 (AF145239), 1012 (AF104679), 1204 (AF145240), ChDC YA1 (AY691534), ChDC YA2 (AY986761), ChDC YA3 (AY691535), ChDC YA4 (AY691536), ChDC YA5 (AY986762), ChDC YA7 (AY691538), ChDC YA8 (AY986763), ChDC YA9 (AY691539), ChDC YA10 (AY691540), ChDC YA11 (AY691541), ChDC YA12 (AY986764), and ChDC YA13 (AY986765). The specificity of the prospective primers was tested using the program, PROBE Match, which was obtained from the Ribosomal Database Project II (http://rdp.cme.msu.edu/html/analyses.html). No sequences that were completely homologous to the *S. anginosus* forward and reverse primers were found in the 16S rDNAs for the other oral bacterial species in these databases. The nucleotide sequences of the three selected primers are as follows: forward primer (ChDC-Ang-F7), 5'-ACA GTT TAT ACC GTA GCT TGC TAC ACC AT-3' (77-105 nts) and reverse primer (ChDC-Ang-R7), 5'-CGT AGT TAG CCG TCC CTT TCT GG-3' (538-516 nts). The base positions of the primers were from *S. anginosus* ATCC 33397^T. The expected product size was 461 bp.

Specificity and Sensitivity of the PCR

PCR was performed to validate the primer specificity and to determine the sensitivity of the PCR primers. The specificity of PCR was evaluated by examining 6 different *Streptococcus* species (10 strains) as well as 12 *S. anginosus* strains (1 type strain and 11 clinical isolates) (aliquots of 4 ng of nucleic acid). To test the sensitivity of PCR, the detection limit was defined as the smallest amount of bacterial genomic DNA that could be detected by PCR. In addition, the sensitivity test was also performed using Takao's PCR primers (16S-ANG-U and 16S-ANG-D) in order to compare this with the sensitivity of the PCR primers (ChDC-Aa-F and ChDC-Aa-R). This was determined by a serial dilution of a genomic DNA mixture from *S. anginosus* ATCC 33397^T. The sensitivities ranged from 4 ng to 4 fg using 10-fold dilutions.

The PCR was performed using an *AccuPower*® PCR PreMix (Bioneer Corp.) containing 5 nmole of each deoxynucleoside triphosphate, 0.8 μmole KCl, 0.2 μmole Tris-HCl (pH 9.0), 0.03 μmole $MgCl_2$, and 1 unit of *Taq* DNA polymerase. The bacterial genomic DNA and 20 pmoles of each primer were then added to a PCR PreMix tube. The PCR was carried out in a final volume of 20 μl. The PCR reaction was run for 30 cycles on a Peltier thermal cycler (Model PTC-200 DNA engine^{TM}, MJ Research Inc., USA) under the following conditions: denaturation at 98°C for 1 sec, primer annealing at 65°C for 30 sec, and extension at 72°C for 30 sec. The final cycle included an additional extension time of 10 min at 72°C. A 2 μl aliquot of the reaction mixture was then analyzed by 1.5% agarose gel electro-

phoresis in a Tris-acetate buffer (0.04 M Tris-acetate, 0.001 M EDTA, [pH8.0]) at 100 V for 30 min. The amplification products were stained with ethidium bromide and visualized using UV transilluminator.

Results and Discussion

The optimal annealing temperature was 65°C for the ChDC-Ang-F7 and ChDC-Ang-R7 primers (data not shown). However, the *S. anginosus* genomic DNA was detectable up to 68°C (data not shown). When the species specificity of the ChDC-Ang-F7 and ChDC-Ang-R7 primers was investigated, the 461 bp PCR amplicon or product was amplified only when the *S. anginosus* type strain and clinical isolates were used as the genomic DNA template. In contrast, no PCR product was observed when other streptocococcal strains were used as the genomic DNA template (Fig. 1).

The minimum amount of DNA at which ChDC-Ang-F7 and ChDC-Ang-R7 primers could detect the *S. anginosus* genomic DNA was 0.4 pg (Fig. 2A). Recently, species-specific PCR primers (16S-ANG-U and 16S-ANG-D) for *S. anginosus* were also developed based on the nucleotide sequence of 16S rDNA (Takao *et al.*, 2004). In the study, the size of the amplicons was 105 bp. The comparison test showed that the sensitivity of the Takao's primers was similar to that of the primers developed in this study (Fig. 2B). Therefore, it is believed that the ChDC-Ang-F7 and ChDC-Ang-R7 primers can be also used to both identify and detect *S. anginosus*.

Better epidemiological results can be obtained when a primer with a high sensitivity and specificity is used. PCR primers were developed based on the nucleotide sequence of the leukotoxin gene (Tonjum and Haas, 1993) and on the 16S rRNA gene (Ashimoto *et al.*, 1996; Kim *et al.*, 2004) for the detection and isolation of *Actinobacillus actinomycetemcomitans*, which is one of the important causative agents of localized juvenile periodontitis as well as rapid progressive periodontitis. In this laboratory, two or three PCR primers sets were routinely used to identify the bacteria grown on the agar plates at the species level. In addition, and the resulting PCR data generally coincided with the 16S rDNA

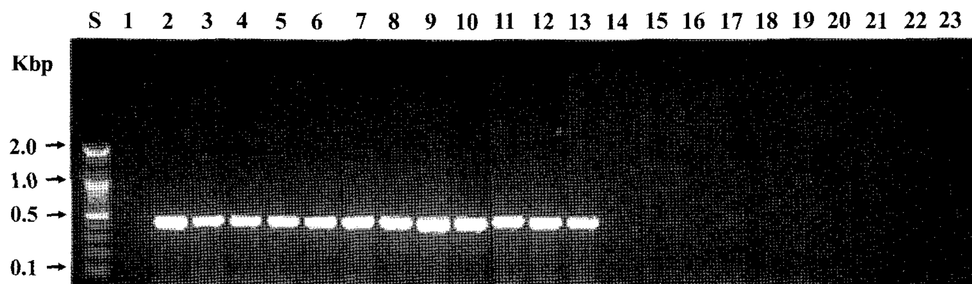

Fig. 1. Specificity test of PCR with the ChDC-Ang-F7 and ChDC-Ang-R7 primers and purified genomic DNA from *S. anginosus* ATCC 33397^T. Four ng of each of bacterial genomic DNA was used as the PCR template. The PCR reactions were electrophoresed in 1.5% agarose gel. Lanes: S, 100 base pair DNA ladder (Bioneer Corp.); 1, (-) control (sterilized DDW); 2, *S. anginous* ATCC 33397^T; 3-13, *S. anginous* ChDC YA1, ChDC YA2, ChDC YA3, ChDC YA4, ChDC YA5, ChDC YA7, ChDC YA8, ChDC YA9, ChDC YA10, ChDC YA11, and ChDC YA12, respectively; 14, *S. intermedius* KCTC 3268^T; 15, *S. intermedius* ChDC B717; 16, *S. constellatus* subsp. *constellatus* ATCC 27823^T; 17, *S. constellatus* subsp. *constellatus* ChDC B707; 18, *S. constellatus* subsp. *constellatus* ChDC B280; 19, *S. constellatus* subsp. *constellatus* ChDC B290; 20, *S. mitis* KCTC 3556^T; 21, *S. sanguinis* KCTC 3634^T.

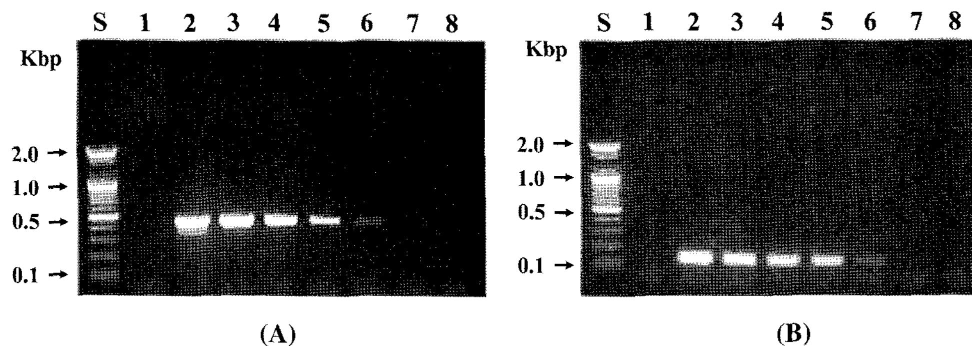

Fig. 2. Comparison of the sensitivity of (**A**) the primer set (ChDC-Ang-F7 and ChDC-Ang-R7), with (**B**) Takao's primer set (16S-ANG-U and 16S-ANG-D), with the primers and the purified genomic DNA from *S. anginosus* ATCC 33397^T. The PCR reactions were electrophoresed in a 1.5% agarose gel. Lanes: S, 100 base pair DNA ladder (Bioneer Corp.); 1, sterilized deionized water (negative control); 2 through 8, purified genomic DNA serially diluted 10-fold from 4 ng to 4 fg.

cloning and sequencing data. Therefore, the PCR primers developed in this study will be a valuable addition to the list of primers already available for the *S. anginosus*.

In conclusion, high specificity and sensitivity for the identification and detection of *S. anginosus* might be obtained using the primers developed in this study (ChDC-Ang-F7 and ChDC-Ang-R7) as well as the primers developed by Takao *et al.* (2004) (16S-ANG-U and 16S-ANG-D).

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