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Strain-specific PCR Primers for the Detection of *Prevotella intermedia* ATCC 49046

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The aim of this study was to develop *Prevotella intermedia* ATCC 49046-specific PCR primers designed based on the nucleotide sequence of a DNA probe Pig28. The strain-specificity of the PCR primers, Pig28-F1/Pig28-R1, was confirmed with 9 strains of *P. intermedia* and 25 strains (15 species) of *Prevotella* species. The detection limit of the PCR primers was 2 pg of the purified genomic DNA of *P. intermedia* ATCC 49046. These PCR primers were found to be useful for identifying *P. intermedia* ATCC 49046, particularly for determining the authenticity of the strain.

Key words: Detection, *Prevotella intermedia* ATCC 49046, strain-specific PCR primers

Introduction

Prevotella intermedia is closely associated with periodontal diseases and endodontic infections (Milsom *et al.*, 1996; Okamoto *et al.*, 1999; Papapanou *et al.*, 2000). It was reported that there was a close association between the presence of bleeding on probing (BOP) and the presence of *Prevotella intermedia* (Kim *et al*., 2004; Kook *et al.*, 2005). Furthermore, the sites harboring both *T. forsythia* and *P. intermedia* at the baseline had a poorer response to treatment than the sites where these two species were not detected (Kook *et al.*, 2005).

Among the techniques used in bacterial taxonomy, restriction fragment length polymorphism, low frequency restriction fragment analysis, phage and bacteriocin typing, serological techniques, ribotyping, artificial-primed PCR, amplified rDNA restriction analysis, repetitive element sequence-based PCR (rep-PCR), total cellular protein electrophoretic patterns, DNA probe and DNA sequencing methods can be used in the taxonomical resolution of bacteria at the strain-level (Krig, 2001). However, there are few reports on strain-specific identification (Mathsone *et al.*, 1997; Baek *et al.*, 2003; Kim *et al.*, 2005; Shin *et al.*, 2010).

In a previous study, a putative *Prevotella intermedia*-specific DNA probe, Pig28 originating from *P. intermedia* ATCC 49046, was cloned using the inverted dot blot hybridization screening method (Kim *et al.*, 2011). Southern blot analysis was performed to confirm the *Prevotella intermedia*-specificity of the Pig28. Interestingly, the Pig28 DNA probe had strain-specificity to the *Prevotella intermedia* ATCC 49046 (data not shown). *P intermedia* ATCC 49046 has been used for pathogenesis studies related to periodontal diseases (Van Hoogmoed *et al.*, 2008). Therefore, in this study, an attempt was made to develop strain-specific PCR primers based on the nucleotide sequences of the Pig28 DNA probe for the identification of the *P. intermedia* ATCC 49046.

Materials and Methods

Bacterial strains and growth condition

The reference strains of bacteria used in this study are as follows: *P. intermedia* ATCC 49046, *P. intermedia* ATCC 25611^T, *P. nigrescens* ATCC 33563^T, *Prevotella loescheii* ATCC 15930^T, *Prevotella brevis* ATCC 19188^T, *Prevotella ruminicola* ATCC 19189^T, *Prevotella melaninogenica* ATCC 25845^T, *Prevotella bivia* ATCC 29303^T, *Prevotella oralis* ATCC 33269^T, *Prevotella corporis* ATCC 33547^T, *Prevotella*

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buccae ATCC 33574^T, *Prevotella veroralis* ATCC 33779^T, *Prevotella heparinolytica* ATCC 35895^T, *Prevotella oulorum* ATCC 43324^T, *Prevotella dentalis* ATCC 49559^T, *Prevotella enoeca* ATCC 51261^T, *Prevotella pallens* ATCC 700821^T, and *Porphyromonas gingivalis* ATCC 33277^T. These strains were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA). Six *P. intermedia* strains (KCOM 1101, KCOM 2671, KCOM 1104, KCOM 1106, KCOM 1107, and KCOM 1109) and ten *P. nigrescens* strains (KCOM 1032, KCOM 1034, KCOM 1036, KCOM 1040, KCOM 1041, KCOM 1042, KCOM 1044, KCOM 1102, KCOM 1103, and KCOM 1108) were obtained from the Korean Collection for Oral Microbiology (KCOM, Gwangju, Korea). The above strains were cultured in Tryptic Soy broth supplemented with 0.5% yeast extract, 0.05% cysteine HCl-H_2O, 0.5 mg/ml of hemin and 2 μg/ml of vitamin K_1 at 37°C in an anaerobic chamber (Bactron I, Sheldon Manufacturing Inc., Cornelius, OR) under 10% H_2, 5% CO_2 and 85% N_2.

Genomic DNA preparation

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

DNA sequencing and PCR primer design

The nucleotide sequence of the Pig28 DNA probe, which was determined using a chain termination method, was submitted to GenBank under accession no. HQ615932. The strain-specific PCR primers for *P. intermedia* ATCC 49046 were designed using the PRIMERSECT program (Version 8.0, DNASTAR Inc., Madison, WI) based on the nucleotide sequence of the Pig28 DNA probe. The nucleotide sequences of the PCR primers were as follows: forward primer (Pig28-F1), 5'-AGG TGC GCC TCC CTG TTA CG-3' and reverse primer (Pig28-R1), 5'-TTT ATT TCA CCG GGG ATT ACC TCT T-3'. The expected product lengths of *P. intermedia* were 502 bp. The PCR primers were synthesized by Bioneer.

Specificity and Sensitivity test

PCR was carried out to validate the primer specificity and determine the detection limits of the PCR primers. The PCR specificity was evaluated by testing 17 reference strains of *Prevotella* spp., 6 clinical isolates of *P. intermedia,* and 10 clinical isolates of *P. nigrescens* (4 ng aliquots of genomic DNA). The PCR specificity was evaluated by testing 6 strains of *P. intermedia* and 10 strains of *P. nigrescens* (2 ng aliquots of genomic DNA). For the PCR sensitivity test, the lower limit of detection was defined as the smallest amount of bacterial genomic DNA detectable by PCR, and was determined by a serial dilution (2 ng to 2 fg in 10-fold dilution intervals) of the genomic DNA from *P. intermedia* ATCC 49046. PCR was carried out using an *AccuPower*® PCR PreMix (Bioneer) containing 5 nmole each of deoxynucleoside triphosphate, 0.8 μmoles of KCl, 0.2 μmoles of Tris-HCl (pH 9.0), 0.03 μmoles of $MgCl_2$ and 1 unit of *Taq* DNA polymerase. The bacterial genomic DNA and 20 pmoles of each primer were added to a PCR PreMix tube. PCR was carried out at a final volume of 20 μl. PCR was run for 32 cycles on a Peltier thermal cycler (Model PTC-200 DNA engine™, MJ Research Inc., Watertown, MA) under the following conditions: denaturation at 94°C for 30 sec, primer annealing at 65°C for 30 sec and extension at 72°C for 30 sec. The final cycle included an additional 5 min extension time at 72°C. A 4 μl aliquot of the reaction mixture was then analyzed by 1.5% agarose gel electrophoresis in a Tris-acetate buffer (0.04 M Tris-acetate, 0.001 M EDTA, pH 8.0) at 100V for 30 min. The amplification products were stained with ethidium bromide and visualized by UV transillumination.

Results

The Pig28 DNA probe is composed of 1,651 nucleotides. The genome of *Prevotella intermedia* 17 was sequenced by the Institute of Genomic Research (TIGR). First, the homology of the nucleotide sequences of the Pig28 was searched using blastn program supported by The Institute of Genomic Research (TIGR) (http://blast.jcvi.org/cmr-blast/). The homology search data showed that there was no matched gene in the genome of *P. intermedia* 17 corresponding to the nucleotide sequences of the Pig28 probe. Next, the homology was searched using the blastn and blastx programs in the NCBI website (http://blast.ncbi.nlm.nih.gov/Blast.cgi). According to the blastx analysis data, the encoded amino acid in Pig28 DNA protein is unknown.

The strain-specificity of the PCR primers, Pig28-F1/Pig28-R1, was tested with 9 strains of *P. intermedia* and 25 strains (15 species) of *Prevotella* genus (Figs.1 and 2). On gel electrophoresis, the PCR product of the PCR primers showed DNA bands for the genomic DNA of *P. intermedia* ATCC 49046 with the expected size of 502 bp (Figs. 1 and 2). However, the PCR amplicons did not occur when the genomic DNAs of other *P. intermedia* strains as well as *Prevotella* spp.

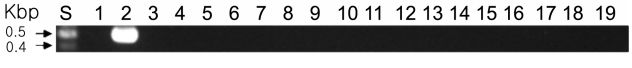

Fig. 1. Specificity test of the Pig28-F1/Pig28-R1 primers using 2 ng of each bacterial of type strains of *Prevotella* species. The PCR reaction products were electrophoresed on 1.5% agarose gel. Lanes: S, size marker (100 bp ladder); 1, (-) negative control (water); 2, *P. intermedia* ATCC 49046; 3, *P. intermedia* ATCC 25611^T; 4, *P. nigrescens* ATCC 33563^T; 5, *P. loescheii* ATCC 15930^T; 6, *P. brevis* ATCC 19188^T; 7, *P. ruminicola* ATCC 19189^T; 8, *P. melaninogenica* ATCC 25845^T; 9, *P. bivia* ATCC 29303^T; 10, *P. oralis* ATCC 33269^T; 11, *P. corporis* ATCC 33547^T; 12, *P. buccae* ATCC 33574^T; 13, *P. verovalis* ATCC 33779^T; 14, *P. heparinolytica* ATCC 35895^T; 15, *P. oulorum* ATCC 43324^T; 16, *P. dentalis* ATCC 49559^T; 17, *P. enoeca* ATCC 51261^T; 18, *P. pallens* ATCC 700821^T.

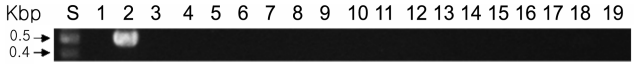

Fig. 2. Specificity test of the Pig28-F1/Pig28-R1 primers using 2 ng of each bacterial of the clinical isolates of *P. intermedia* and *P. nigrescens*. The PCR reaction products were electrophoresed on 1.5% agarose gel. Lanes: S, size marker (100 bp ladder); 1, (-) negative control; 2, *P. intermedia* ATCC 49046; 3, *P. intermedia* KCOM 1101; 4, *P. intermedia* KCOM 2671; 5, *P. intermedia* KCOM 1104; 6, *P. intermedia* KCOM 1106; 7, *P. intermedia* KCOM 1107; 8, *P. intermedia* KCOM 1109; 9, *P. nigrescens* KCOM 1032; 10, *P. nigrescens* KCOM 1034; 11, *P. nigrescens* KCOM 1036; 12, *P. nigrescens* KCOM 1040; 13, *P. nigrescens* KCOM 1041; 14, *P. nigrescens* KCOM 1042; 15, *P. nigrescens* KCOM 1044; 16, *P. nigrescens* KCOM 1102; 17, *P. nigrescens* KCOM 1103; 18, *P. nigrescens* KCOM 1108.

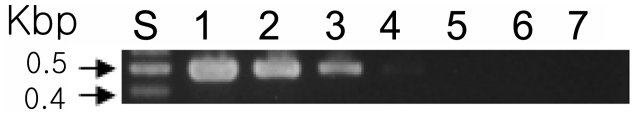

Fig. 3. Sensitivity test of the Pig28-F1/Pig28-R1 primers. PCR was performed with the PCR primers and the purified genomic DNA of *P. intermedia* ATCC 49046 serially diluted 10-fold from 2 ng to 2 fg. The PCR reaction products were electrophoresed on 1.5% agarose gel. Lanes: S, Size marker (100 bp DNA ladder); 1 through 7, purified genomic DNA serially diluted 10-fold, from 2 ng to 2fg.

strains were tested. Under optimal conditions, PCR with the PCR primers could detect up to 2.0 pg of the purified genomic DNA of *P. intermedia* ATCC 49046 (Fig. 3).

Discussion

While conducting studies on bacteria-host interaction, sometimes it is needed to confirm the authority of the bacterial strain. In this case, we tested it by morphology of the strain grown on the agar plate or by the gram staining. However, these methods are not enough to identify the bacteria at the strain level. The first report on the strain-specific identification was strain-specific DNA probe cloned using the rep-PCR method (Mathsone *et al.*, 1997). Recently, strain-specific PCR primers were developed based on the nucleotide sequences of the strain-specific DNA probes (Kim *et al.*, 2005; Shin *et al.*, 2010) which were cloned by an inverted dot blot hybridization screening method and Southern blot analysis (Kook *et al.*, 2003; Baek *et al.*, 2003). According to the Southern blot analysis, Pig28 DNA probe was reacted strongly with the genomic DNA of *P. intermedia* ATCC 49046 (data not shown). The Pig28-F1/Pig28-R1 primers were designed from the strain-specific DNA probe, Pig28. In addition, the strain-specificity of the DNA probe Pig28 was confirmed against the genomic DNAs of 7 strains of *P. intermedia* and 6 strains of *P. nigrescens* by Southern blot analysis (data not shown). These results suggest that the bacterial genomes have strain-specific regions and the stain-specific PCR primers can be designed base on the nucleotide sequence of the regions.

The detection limit of the Pig28-F1/Pig28-R1 primers was 2 pg of the purified genomic DNA of *P. intermedia* ATCC 49046. Considering that the genome size of the *P. intermedia* 17 is about 2.7 Mb (http://cmr.jcvi.org/cgi-bin/CMR/GenomePage.cgi?org=gpi), the primers can detect the about 676 genomes of *P. intermedia.* However, the results of the sensitivity test of the PCR obtained using cell lysate were 10-fold lower than that observed when using the purified genomic DNA (data not shown). This might be due to the inhibition of PCR by the cellular components of the lysate, or by detergents present in the lysis buffer. These results show that the Pig28-F1/Pig28-R1 primers have strain-specificity for *P. intermedia* ATCC 49046.

The optimal annealing temperature of the primers was calculated to be 55.6°C using the PrimerSelect program. However, the real annealing temperature was determined by gradient PCR as 65°C (data not shown) to increase the specificity. In our experience, the actual annealing temperature of the bacterial species or strain-specific PCR primers is 8.6°C to 12°C higher than the OAT recommended in the PrimerSelect Program (Park *et al.*, 2010; Shin *et al.*, 2010). Therefore, it is important that gradient PCR be performed to determine the OAT of the PCR primers designed using the PrimerSelect program.

In summary, the PCR primers, Pig28-F1/Pig28-R1, have strain-specificity for *P. intermedia* ATCC 49046. These primers can be used to determine the authenticity of the strain rapidly and accurately.

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