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NOTE

Development of *Porphyromonas gingivalis*-Specific Quantitative Real-Time PCR Primers Based on the Nucleotide Sequence of *rpoB*

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Species-specific quantitative real-time PCR (qPCR) primers were developed for the detection of *Porphyromonas gingivalis*. These primers, Pg-F/Pg-R, were designed based on the nucleotide sequences of RNA polymerase β-subunit gene (*rpoB*). Species-specific amplicons were obtained from the tested *P. gingivalis* strains but not in any of the other strains (46 strains of 46 species). The qPCR primers could detect as little as 4 fg of *P. gingivalis* chromosomal DNA. These findings suggest that these qPCR primers are suitable for applications in epidemiological studies.

***Keywords*:** *Porphyromonas gingivalis*, *rpoB*, species-specific, qPCR primers

Periodontitis is a bacteria-infectious oral disease. The major causative factor of the periodontitis is gram negative anaerobic bacteria in subgingival plaque (Darveau *et al*., 1997). Approximately 400 bacterial species exist in human subgingival plaque (Paster *et al*., 2001). Among the bacteria, *Porphyromonas gingivalis* is a bacterium closely related to the initiation and progression of periodontal diseases, and has many virulence factors, such as fimbriae, proteinases, exopolysaccharides, and hemin-binding proteins (Holt *et al*., 1999). Initially, the close relationship between periodontitis and *P. gingivalis* was investigated from the epidemiological studies (Uematsu and Hoshino, 1992; Moore and Moore, 1994). Before molecular biological tools, such as DNA sequencing, synthesis of oligonucleotides, and PCR, were introduced in epidemiological studies, traditional bacterial cultures, and biochemical tests were used to detect *P. gingivalis* (Slots, 1977, 1979; White and Mayrand, 1981). The golden standard method for identifying bacterial species is a comparison of the 16S ribosomal RNA gene (16S rDNA) and DNA-DNA hybridization methods (Krieg, 2001). However, the major traditional methods of bacterial identification used to detect bacteria in subgingival plaque involved a comparison of the biochemical tests and the phenotypes of bacteria like the color response under long wave of ultraviolet light. This is one of the main reasons of the discrepancy between epidemiological studies. To overcome these problems, bacterial detection methods using molecular biological tools were used in periodontitis-related epidemiological studies (Slots *et al*., 1995; Ashimoto *et al*., 1996; Conrads *et al*., 1999; Trans and Rudney, 1999; Lee *et al*., 2003). Of them, conventional PCR methods are used widely (Ashimoto *et al*., 1996; Conrads *et al*., 1999; Trans and Rudney, 1999). Using the conventional PCR method, the bacteria can be detected only qualitatively. Therefore, quantitative real-time PCR (qPCR) methods are applied to use in the detection of bacteria quantitatively.

The 16S rDNA gene has been generally used as a target gene to design a conventional PCR or qPCR for the detection of bacterial species. The nucleotide sequences of 16S rDNA are well conserved among the bacterial species in phylogenetic aspects (Krieg, 2001). However, the homogeneity between some bacterial species, such as *Streptococcus mitis* and *Streptococcus oralis*, is too high to discriminate them at the species-level and PCR primers can not be designed. Recently, DNA-dependent RNA polymerase gene (*rpoB*) has been used instead of 16S rDNA to identify bacteria from the genus to subspecies levels (Kim *et al*., 1999; Drancourt and Raoult, 2002; Ko *et al*., 2002; Khamis *et al*., 2004; Kim *et al*., 2010). In this study, species-specific qPCR primers were developed based on the nucleotide sequence of *rpoB* for the quantitative detection of *P. gingivalis* which is one of the major causative bacteria of periodontal diseases.

The bacterial strains used in this study were obtained from the Type Culture Collection (ATCC, USA), the Culture Collection, University of Göteborg (CCUG, Sweden) and the Korean Collection for Type Cultures (KCTC, Biological Resource Center, Korea) (Table 1).

A. actinomycetemcomitans was grown in tryptic soy broth (TSB, Difco Laboratories, USA) medium supplemented with 0.6% yeast extract, 5% horse serum, 75 μg/ml of bacitracin, and 5 μg/ml of vancomycin (Sigma, USA) at 37℃ in anaerobic atmosphere (10% H_2, 5% CO_2, and 85% N_2). *Haemophilus* spp. and *Streptococcus* spp. were allowed to grow in TSB medium supplemented with 0.5% yeast extract, 0.5 mg/ml of hemin, and 2 μg/ml of vitamin K_1 at 37℃ in an air atmosphere

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Table 1. Bacterial strains used in this study

Species	Strains
Porphyromonas gingivalis	ATCC 33277^T, ATCC 53978, ATCC 49417, ATCC BAA-1703
Porphyromonas endodontalis	ATCC 35406T
Prevotella intermedia	ATCC 25611T
Prevotella nigrescens	ATCC 33563^T
Tannerella forsythia	ATCC 43037^T
Campylobacter rectus	ATCC 33238^T
Eubacterium limosum	KCTC 2487^T
Rothia dentocariosa	KCTC 3204^T
Selenomonas artemidis	KCTC 5742^T
Selenomonas noxia	KCTC 5749^T
Filifactor alocis	ATCC 35896^T
Fusobacterium nucleatum	ATCC 25586^T
Fusobacterium necrophorum	ATCC 25286^T
Fusobacterium periodonticum	ATCC 33693^T
Fusobacterium canifelinum	CCUG 49733^T
Fusobacterium simiae	CCUG 16798^T
Leptotrichia buccalis	CCUG 34316^T
Capnocytophaga gingivalis	ATCC 33624^T
Capnocytophaga ochracea	KCTC 5787^T
Capnocytophaga sputigena	KCTC 5789^T
Propionibacterium propionicum	KCTC 5342^T
Propionibacterium acnes	KCTC 3314^T
Aggregatibacter actinomycetemcomitans	ATCC 33384^T
Haemophilus aphrophilus	ATCC 33389^T
Haemophilus paraphrophilus	ATCC 29242^T
Haemophilus parainfluenzae	ATCC 33392^T
Neisseria sicca	ATCC 29256^T
Neisseria subflava	ATCC 49275^T
Neisseria meningitidis	ATCC 13077^T
Neisseria mucosa	ATCC 19696^T
Actinomyces georgiae	CCUG 32935^T
Actinomyces odontolyticus	CCUG 20536^T
Actinomyces israelii	ATCC 12102^T
Actinomyces meyeri	CCUG 21024^T
Actinomyces naeslundii	CCUG 35333^T
Streptococcus gordonii	CCUG 33482^T
Streptococcus anginosus	ATCC 700231^T
Streptococcus intermedius	KCTC 3268^T
Streptococcus constellatus	ATCC 27823^T
Streptococcus mutans	ATCC 25175^T
Streptococcus sobrinus	ATCC 33478^T
Streptococcus mitis	KCTC 3556^T
Streptococcus oralis	CCUG 13229^T
Streptococcus parasanguinis	CCUG 30417^T
Streptococcus pneumoniae	CCUG 28588^T
Streptococcus sanguinis	CCUG 17826^T
Veillonella parvula	KCTC 5019^T

ATCC, American Type Culture Collection; CCUG, Culture Collection, University of Göteborg; KCTC, Korean Collection for Type Cultures; KCOM, Korean Collection for Oral Microbiology; T, type strain

containing 5% CO_2. The other bacterial species were cultured in TSB 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 anaerobic atmosphere (10% H_2, 5% CO_2, and 85% N_2).

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

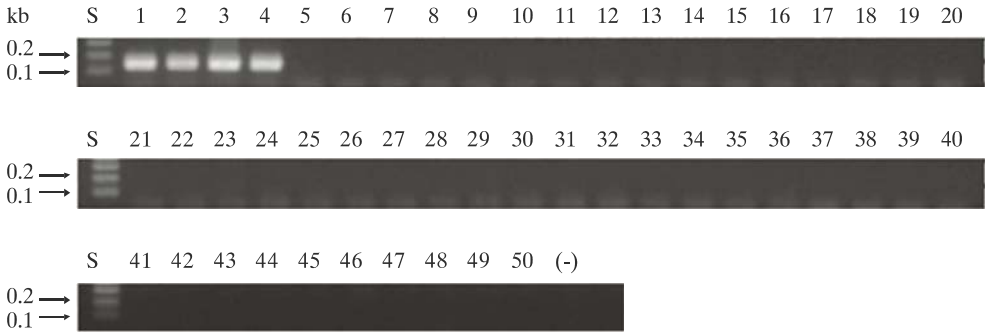

Fig. 1. Specificity test of the PCR primers, Pg-F/Pg-R, with 4 ng of each bacterial genomic DNA. The PCR reaction products were electrophoresed on 1.5% agarose gel. Lanes: S, size marker (100 bp ladder); 1, *P. gingivalis* ATCC 33277^T; 2, *P. gingivalis* ATCC 53978; 3, *P. gingivalis* ATCC 49417; 4, *P. gingivalis* ATCC BAA-1703; 5, *P. endodontalis* ATCC 35406^T; 6, *P. intermedia* ATCC 25611^T; 7, *P. nigrescens* ATCC 33563^T; 8, *T. forsythia* ATCC 43037^T; 9, *C. rectus* ATCC 33238^T; 10, *E. limosum* KCTC 2487^T; 11, *R. dentocariosa* KCTC 3204^T; 12, *S. artemidis* KCTC 5742^T; 13, *S. noxia* KCTC 5749^T; 14, *F. alocis* ATCC 35896^T; 15, *F. nucleatum* ATCC 25586^T; 16, *F. necrophorum* ATCC 25286^T; 17, *F. periodonticum* ATCC 33693^T; 18, *F. canifelinum* CCUG 49733^T; 19, *F. simiae* CCUG 16798^T; 20, *L. buccalis* CCUG 34316^T; 21, *C. gingivalis* ATCC 33624^T; 22, *C. ochracea* KCTC 5787^T; 23, *C. sputigena* KCTC 5789^T; 24, *P. propionicum* KCTC 5342^T; 25, *P. acnes* KCTC 3314^T; 26, *A. actinomycetemcomitans* ATCC 33384^T; 27, *H. aphrophilus* ATCC 33389^T; 28, *H. paraphrophilus* ATCC 29242^T; 29, *H. parainfluenzae* ATCC 33392^T; 30, *N. sicca* ATCC 29256^T; 31, *N. subflava* ATCC 49275^T; 32, *N. meningitidis* ATCC 13077^T; 33, *N. mucosa* ATCC 19696^T; 34, *A. georgiae* CCUG 32935^T; 35, *A. odontolyticus* CCUG 20536^T; 36, *A. israelii* ATCC 12102^T; 37, *A. meyeri* CCUG 21024^T; 38, *A. naeslundii* CCUG 35333^T; 39, *S. gordonii* CCUG 33482^T; 40, *S. anginosus* ATCC 700231^T; 41, *S. intermidius* KCTC 3268^T; 42, *S. constellatus* ATCC 27823^T; 43, *S. mutans* ATCC 25175^T; 44, *S. sobrinus* ATCC 33478^T; 45, *S. mitis* KCTC 3556^T; 46, *S. oralis* CCUG 13229^T; 47, *S. parasanguinis* CCUG 30417^T; 48, *S. pneumoniae* CCUG 28588^T; 49, *S. sanguinis* CCUG 17826^T; 50, *V. parvula* KCTC 5019^T; (-), negative control (deionized distilled water).

A pair of qPCR primers was designed based on the nucleotide sequences of *rpoB* of *P. gingivalis* (GenBank accession no. NC_010729) using the program PRIMERSECT (DNASTAR Inc., USA). The primer sequences were as follows: forward primer (Pg-F), 5′-GGA AGA GAA GAC CGT AGC ACA AGG A-3′; and reverse primer (Pg-R), 5′-GAG TAG GCG AAA CGT CCA TCA GGT C-3′. The expected product length for *P. gingivalis* was 143 bp.

The specificity test of the qPCR primers (Pg-F/Pg-R) for *P. gingivalis* was conducted using conventional PCR. PCR was performed using an *AccuPower*® PCR PreMix (Bioneer, Korea) containing 5 nmole of each deoxynucleoside triphosphate, 0.8 μmole of KCl, 0.2 μmole of Tris-HCl (pH 9.0), 0.03 μmole of $MgCl_2$, and 1 unit of *Taq* DNA polymerase.

Two nanogram of bacterial genomic DNA and 20 pmoles of each primer were added to a PCR PreMix tube. PCR was carried out in a final volume of 20 μl using a Peltier thermal cycler (Model PTC-200 DNA Engine™, MJ Research Inc., USA) under the following conditions: initial denaturation at 95°C for 10 min; and 30 cycles of a denaturation at 95°C for 30 sec, primer annealing and extension at 72°C for 30 sec; followed by final extension at 72°C for 5 min. A 6 μl of PCR product was analyzed by 1.5% agarose gel electrophoresis in Tris-acetate buffer (0.04 M Tris-acetate, 0.001 M EDTA, pH 8.0) at 100 V for 30 min. The amplification products were stained with ethidium bromide and visualized using a UV transilluminator.

qPCR was performed with an *AccuPower*® *GreenStar*™ qPCR PreMix using *Exicycler*™ 96 Real-Time Quantitative Thermal Block (Bioneer). Each PCR was performed in a total volume of 50 μl containing 3 μl each of the forward and reverse primers (final concentration, 500 nM each), 5 μl of genomic DNA and the appropriate dose of sterilized DNase-RNase-free water in PreMix PCR tubes. The qPCR condition was initial denaturation at 95°C for 10 min, 40 cycles of a denaturation at 95°C for 10 sec, primer annealing and extension at 72°C for 30 sec, and final cooling at 25°C for 1 min. The reaction specificities were confirmed by melting curve analysis with a progressive increase in temperature from 65°C to 94°C at a 1°C/sec transition rate and continuous fluorescence acquisition.

The specificity of the qPCR primers for detecting *P. gingivalis* was tested by conventional PCR using 4 strains of *P. gingivalis* and 46 type strains of other oral bacterial species including *P. endodontalis* which is the mostly closely related species with *P. gingivalis* at the genetic level. The data showed the PCR product was amplified from the genomic DNA of *P. gingivalis* (Fig. 1). The melting curve analysis showed that non-specific PCR products were not amplified (data not shown). According to the recommendation for determining the optimal annealing temperature (OAT) for PCR (Shin *et al*., 2010), gradient PCR was performed with the genomic DNAs of *P. gingivalis* ATCC 33277^T and *P. endodontalis* ATCC 35406^T (data not shown). According to the PrimerSelect program, the recommended OAT was 55.7°C but according to the gradient PCR data, the Pg-F/Pg-R primers can detect *P. gingivalis* up to 72°C while maintaining the signal intensity of the amplicons. In this study, 72°C was chosen as an annealing temperature to reduce the non-specific amplification and save qPCR time by simultaneously performing the annealing and extension step in a single step.

The sensitivity of the Pg-F/Pg-R primers was investigated by qPCR to determine the detection limit of the genomic DNA of *P. gingivalis* with 10-fold serial diluted genomic DNA of *P. gingivalis* ATCC 33277^T ranging from 4 ng to 4 fg. The

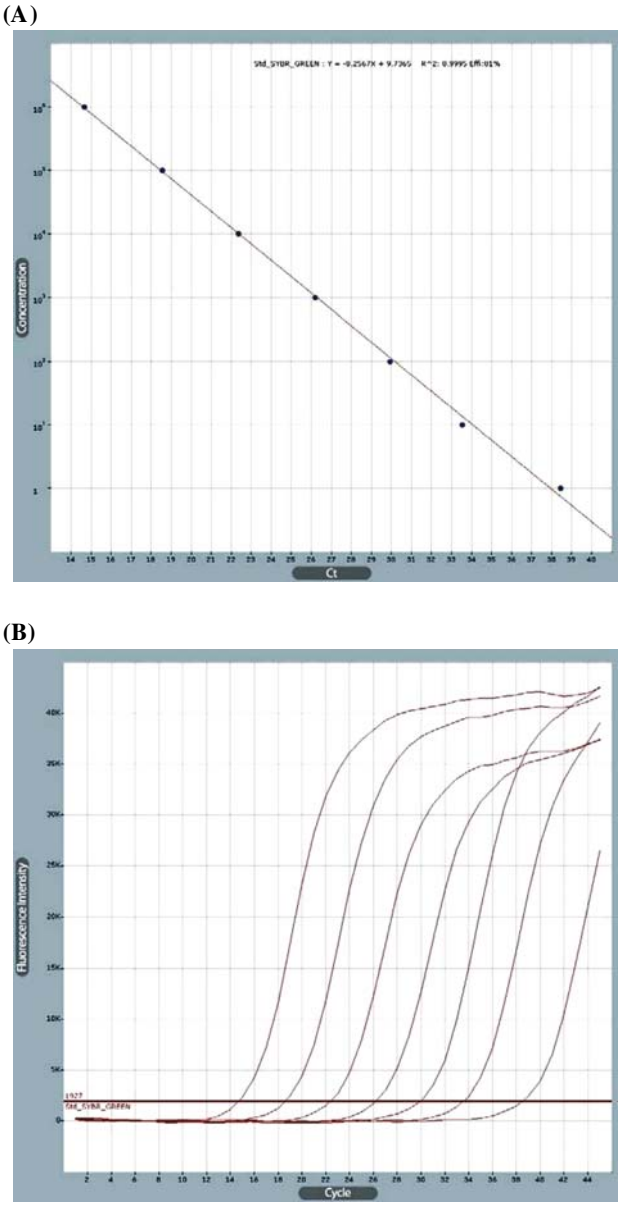

Fig. 2. (A) Standard curve and (B) minimal limit of detection obtained by qPCR using the Pg-F/Pg-R primers from 10-fold serial dilutions of the genomic DNA of *P. gingivalis* ATCC 33277^T ranging from 4 ng to 4 fg. In the standard curve, the regression equation was as follows: Y=-0.2567X+9.7365. The R^2 value was 0.9995.

results showed that the Pg-F/Pg-R primers could detect up to 4 fg of genomic DNA of *P. gingivalis* ATCC 33277^T (Fig. 2 and Table 2). Considering that the genome size of the *P. gingivalis* ATCC 33277^T is 2.35 Mb (http://www.ncbi.nlm.nih.gov/nuccore/188993864?report=genbank), the Pg-F/Pg-R qPCR primers can detect the 1.6 genome of *P. gingivalis* (Table 2). Recently, *P. gingivalis*-specific qPCR primers designed based on the nucleotide sequences of 3-deoxy-D-*manno*-oct-2-ulosonic acid transferase (*waaA*) were introduced (Hyvärinen *et al*., 2009). According to the study, the detection limit of the qPCR primers was 40 fg of *P. gingivalis* using the TaqMan probe method. The TaqMan probe method is slightly more accurate than the SYBR Green method. However, to conduct the qPCR using TaqMan probe method, a bacterial specific probe with a binding site located between the forward and reverse primers is needed. In addition, the probe needs to be labeled with the reporter and quencher dyes, such as 6-carboxylfluorescein and 6-carboxytetramethylrhodamine. This means that the TaqMan method is a higher cost experiment. Although the SYBR Green method has relatively lower specificity than the TaqMan method, the SYBR Green method can be reasonably used in molecular epidemiological studies of periodontal diseases. The efficiency of these two methods was evaluated by detecting *P. gingivalis* (Maeda *et al*., 2003). In this study, the qPCR primers and probe was designed based on the nucleotide sequences of the 16S rDNA. There was no significant difference in the specificity, precision, and sensitivity between the TaqMan and SYBR Green chemistry (Maeda *et al*., 2003). The detection limit of the qPCR for the detection of *P. gingivalis* was 10 cells in that study (Maeda *et al*., 2003). These results show that the Pg-F/Pg-R primers can be used to detect *P. gingivalis* with high specific and sensitivity by qPCR.

The nucleotide sequence of *rpoB* of *Escherichia coli* was reported to be composed of 4,026 bp with 9 conserved regions and 8 variable regions according to a comparison of the *rpoB* of bacterial species (Severinow *et al*., 1996). In addition, *rpoB* is one of the housekeeping genes and is well conserved within the strains of species in phylogenetic aspects just like a 16S rDNA. In the future, the database of the nucleotide sequences of the *rpoB* of most or all bacterial species, the *rpoB* can be a substitute 16S rDNA as a golden standard for the classification of bacteria.

In summary, the Pg-F/Pg-R primers designed based on the nucleotide sequence of *rpoB* have specificity for detecting *P. gingivalis* and detect as little as 4 fg of *P. gingivalis* chromosomal DNA. These results suggest that these *P. gingivalis*-specific

Table 2. Determination of the C_T value for a dilution series of 4 ng of genomic DNA of *P. gingivalis* ATCC 33277^T

Genomic DNA amount	Cell number corresponding to genomic DNA amount	C_T
4 ng	1.6×10^6	14.66
400 pg	1.6×10^5	18.57
40 pg	1.6×10^4	22.35
4 pg	1.6×10^3	26.19
400 fg	1.6×10^2	29.93
40 fg	1.6×10^1	33.54
4 fg	1.6×10^0	38.46
0	0	ND

qPCR primers are suitable for applications in epidemiological studies.

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