Development of Strain-specific PCR Primers Based on a DNA Probe Fu12 for the Identification of *Fusobacterium nucleatum* subsp. *nucleatum* ATCC 25586[T]

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The objective of this study was to assess the strain-specificity of a DNA probe, Fu12, for *Fusobacterium nucleatum* subsp. *nucleatum* ATCC 25586[T] (*F. nucleatum* ATCC 25586[T]), and to develop sets of strain-specific polymerase chain reaction (PCR) primers. Strain-specificity was tested against 16 strains of *F. nucleatum* and 3 strains of distinct *Fusobacterium* species. Southern blot hybridization revealed that the Fu12 reacted exclusively with the *Hind*III-digested genomic DNA of *F. nucleatum* ATCC 25586[T]. The results of PCR revealed that three pairs of PCR primers, based on the nucleotide sequence of Fu12, generated the strain-specific amplicons from *F. nucleatum* ATCC 25586[T]. These results suggest that the DNA probe Fu12 and the three pairs of PCR primers could be useful in the identification of *F. nucleatum* ATCC 25586[T], especially with regard to the determination of the authenticity of the strain.

Key words: DNA probe Fu12, identification, *Fusobacterium nucleatum* ATCC 25586[T], strain-specific PCR primers

Fusobacterium nucleatum is one of the principal bacteria causing periodontitis (Albandar *et al.*, 1997). *F. nucleatum* may also play a role in the development of diseases by providing the necessary anaerobic environment for the growth of pathogens (Diaz *et al.*, 2002). *F. nucleatum* strains have been shown to be capable of coaggregating all of the oral bacterial species thus far tested, thereby acting as a bridge between early and late colonizers during the development of dental plaque on tooth surfaces (Roberts, 2000). *F. nucleatum* was also shown to modulate the secondary immune responses of T-cells to *Actinobacillus actinomycetemcomitans* (Tew *et al.*, 1987).

Many bacterial identification methods have been developed to explore the relationships occurring between infectious diseases and their associated causative agents. Among these, nucleic acid-based methods, including DNA-DNA hybridization, DNA fingerprinting, DNA probes, 16S or 23S rRNA gene sequencing, and PCR have been utilized (Krieg, 2001; Wang and Lee, 2003; Lee *et al.*, 2004). PCR methods, which employ 16S rRNA as a target, have several advantages (Slots *et al.*, 1995; Tran and Rudney, 1999). However, the 16S rDNA

sequences within the same species are too homogeneous for PCR assays to identify bacteria at the subspecies or strain levels. As the homology of genomic DNA sequences in the same species differs between strains, the DNA probe method can be used in the detection and identification of bacteria at the subspecies or strain levels (Krieg, 2001). The advantages of DNA probe methods are a high degree of sensitivity, specificity, and capability of microbial identification at a wide range of levels, from family to strain; therefore, DNA probes have been used extensively in the identification of bacteria at the species level (DiRienzo *et al.*, 1991; Komiya *et al.*, 2000; Kook *et al.*, 2003a).

Recently, we introduced a new method for the rapid screening of bacterial species- or subspecies-specific DNA probes, which is referred to as the inverted dot blot hybridization screening method (Kook *et al.*, 2003b). In that study, we applied this method to the development of bacterial subspecies-specific DNA probes, using *F. nucleatum* as a test species, as this species has been previously subdivided into 4 or 5 subspecies (Dzink *et al.*, 1990; Gharbia and Shah, 1992). In the study where 96 candidate-DNA probes were screened, 4 putative subspecies-specific DNA probes for *F. nucleatum* have been cloned by only 5 inverted dot blot hybridizations and 4 Southern blot hybridizations. In addition, the data

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obtained from the inverted dot blot hybridization (Kook *et al.*, 2003b) and Southern blot analysis (Baek *et al.*, 2003) showed that Fu12, one of the 96 candidate probes described above, could be hybridized only with the genomic DNA of *F. nucleatum* subsp. *nucleatum* ATCC 25586[T] (*F. nucleatum* ATCC 25586[T]). However, in that study, the specificity of the probe Fu12 was assessed using only the type and reference strains of *F. nucleatum*. .

In the present study, we undertook to confirm the strain-specificity of the Fu12 for *F. nucleatum* ATCC 25586, by southern blot analysis of the genomic DNA isolated from clinical strains of *F. nucleatum* cultured from Korean periodontitis patients. In addition, we developed specific PCR primers for *F. nucleatum* ATCC 25586[T] based on the nucleotide sequence of the Fu12.

Materials and Methods

Bacterial strains and growth condition

The type strains and reference strains of the bacteria used in this study were as follows: *Fusobacterium nucleatum* subsp. *nucleatum* ATCC 25586[T], *F. nucleatum* subsp. *nucleatum* ATCC 23726, *F. nucleatum* subsp. *fusiforme* ATCC 51190[T], *F. nucleatum* subsp. *polymorphum* ATCC 10953[T], *F. nucleatum* subsp. *vincentii* ATCC 49256[T], *F. nucleatum* subsp. *animalis* ATCC 51191[T], *F. periodonticum* ATCC 33693[T], and *F. necrophorum* ATCC 25286[T]. All strains were obtained from the American Type Culture Collection (ATCC, University Boulevard, USA). Three strains of *F. nucleatum* subsp. *nucleatum* (ChDC F311, ChDC F130, and ChDC PV-F42), six strains of *F. nucleatum* subsp. *polymorphum* (ChDC PV-F48, ChDC PV-F55, ChDC F113, ChDC F119, ChDC F219, and ChDC F286), one strain of *F. nucleatum* subsp. *animalis* (ChDC PV-F58), and one strain of *F. periodonticum* (ChDC F251) were isolated from the dental plaques of 15 Korean periodontitis patients; all strains were identified at the species or subspecies level using a 16S rDNA sequencing method. All of these strains were grown in Schaedler broth (Difco, USA) at 37°C, in an anaerobic chamber (Bactron I, Sheldon Manufacturing, USA) under an atmospheric condition of 10% H_2, 10% CO_2 and 80% N_2.

Bacterial genomic DNA preparation

Whole bacterial genomic DNAs were isolated according to the method described by Lippke *et al.* (1987) with minor modifications (Kook *et al.*, 2003b). DNA concentrations were determined at 260 and 280 nm by UV-spectrophotometry (Ultrospec 2000, Pharmacia Biotech., UK).

DNA labeling

Twenty ng of purified Fu12 DNA fragments were labeled with Digoxigenin-11-dUTP (DIG) using the DIG-High Prime system (Roche, Germany) according to the manufacturer's instructions. The DIG-labeled DNAs were puri-

fied with an *Accu*Prep™ PCR Purification kit (Bioneer, Korea), according to the manufacturer's instructions.

Southern blot hybridization

Southern blot analysis was performed in order to verify the specificity of the Fu12 DNA fragments for *F. nucleatum* ATCC 25586[T]. Purified bacterial genomic DNAs were digested with *Hin*dIII overnight at 37°C, electrophoresed on 0.8% agarose gel in TAE buffer at 30 V for 4 h, and transferred to nylon membrane by vacuum transfer (Bio-Rad, USA), in accordance with the manufacturer's instructions. Membranes were heated at 120°C for 30 min, and prehybridization and hybridization were analyzed using the DIG hybridization system (Roche, Germany). The membranes were prehybridized for 2 h at 42°C in a hybridization solution (50% deionized formamide, 5×SSC [0.75 M sodium chloride plus 0.075 M sodium citrate], 2% blocking reagent [Roche, Germany], 0.1% *N*-lauroylsarcosine, 0.02% SDS) and hybridized overnight at 42°C in the hybridization solution plus the DIG-labeled Fu12 DNA fragments (20 ng/ml in hybridization solution). Thereafter, the membranes were washed twice in 2×wash buffer (2×SSC, 0.1% SDS) at room temperature and twice in 0.5×wash buffer (0.5×SSC) at 68°C.

Chemiluminescence detection

The DIG-labeled nucleic acids were detected with a chemiluminescent detection system using the DIG Luminescent Detection Kit (Roche, USA). The membranes were washed twice for 5 min with 2 × Wash solution (2 × SSC, 0.1% SDS) at room temperature, then twice for 15 min with 0.5 × Wash solution (0.5 × SSC, 0.1% SDS), at 65°C. Detection was performed as follows: the membranes were equilibrated in washing buffer (100 mM maleic acid, 150 mM NaCl; pH7.5; 0.3% Tween® 20) for 1 min, blocked with blocking solution (1% blocking reagent in 100 mM maleic acid, 150 mM NaCl; pH 7.5) for 1 h, and incubated in DIG-alkaline phosphatase conjugate (150 mU/ml in blocking buffer) for 30 min. The membranes were then washed twice for 15 min in washing buffer, equilibrated for 2 min in detection buffer (0.1 M Tris-HCl, pH 9.5; 0.1 M NaCl), incubated for 5 min in CSPD® [25 mM disodium 3 - < - 4 - methoxysporo{1.2 - dioxetane - 3,2'- (5'-chloro) tricyclo[3.3.1.1[3,7]]decan}- 4 - yl > phenyl phosphate] substrate solution diluted to a 1:100 concentration in detection buffer. The excess liquid was allowed to drip off the membranes, before they were sealed in a hybridization bag, and incubated for 15 min at 37°C. The membranes were exposed to the Lumi-Film Chemiluminescent Detection Film (Roche, Germany) at room temperature for 2 h.

Nucleotide sequencing of the DNA probe Fu12 and primer design

We performed nucleotide sequencing using the following

primers: ChDC-GEM-F (5'-TTC CCA GTC ACG ACG TTG TAA AA-3') and ChDC-GEM-R (5'-GTG TGG AAT TGT GAG CGG ATA AC-3'), a Big Dye Terminator Cycle Sequencing Kit (Applied Biosystems, USA), and an ABI PRISM 310 Genetic Analyzer (Applied Biosystems, USA). Since the entire *F. nucleatum* ATCC 25586^T nucleotide sequence has been known, only the partial sequences of both the 5'-end and 3'-end were determined in this study. These sequences were then compared with the similar sequences of the reference organisms, provided by BLAST (a genome database of the National Center for Biotechnology Information).

The strain specific PCR primers for *F. nucleatum* ATCC 25586^T were designed using the PRIMERSECT program (DNASTAR Inc., USA), predicated on the nucleotide sequence of the Fu12 DNA probe. The nucleotide sequences of the three selected primers are listed in Table 1.

Specificity and sensitivity of PCR

PCR was performed in order to validate primer specific-

ity, and to determine the detection limits of the PCR primers. The PCR specificity was evaluated by testing sixteen *F. nucleatum*, two *F. periodonticum*, and one *F. necrophorum* strains (4 ng aliquots of nucleic acid). For the PCR sensitivity test, the lower limit of detection was defined as the smallest amount of bacterial genomic DNA detectable by PCR. This was determined by the serial dilution of the genomic DNA from *F. nucleatum* ATCC 25586^T. These dilutions ranged from 4 ng to 4 fg, in 10-fold dilution intervals.

PCR was conducted using an *AccuPower*® PCR PreMix (Bioneer, Korea), which contained 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 then added to a PCR PreMix tube. PCR was conducted at a final volume of 20 µl. The PCR was run for 32 cycles on a Peltier thermal cycler (Model PTC-200 DNA engine™, MJ Research, U.S.A) under the following conditions: denaturation at

Table 1. PCR primers designed in this study

Name	Oligonucleotide sequence (5' → 3')	Base position* (nt)	Size of Amplicons (bp)	Annealing temperature (°C)
Fu12-F1	ATA CCG GGA ATA AAG ACA	1,092,490 - 1,092,504	393	57
Fu12-R1	TAC AAC CCA ATC CAT AAG T	1,092,882 - 1,092,864		
Fu12-F2	CCT GCA GGA ACA ATA AGA C	1,090,536 - 1,090,554	328	57
Fu12-R2	TGA AAG GCA AGG TGA AG	1,090,863 - 1,090,847		
Fu12-F3	AAA GTA GGG ATA ATT GAA GAT GA	1,091,059 - 1,091,081	535	60
Fu12-R3	GAT GTT GAA AAG GGC AGA C	1,091,593 - 1,091,575		

*Base positions of primers are from nucleotide sequence [GenBank accession no. NC_003454] of genomic DNA derived from *F. nucleatum* subsp. *nucleatum* ATCC 25586^T.
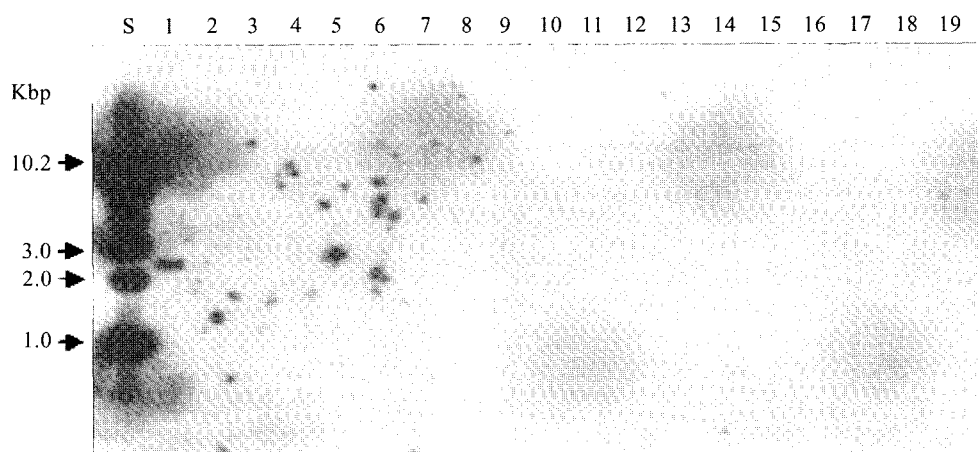

Fig. 1. Southern blot analysis to test the specificity of the probe Fu12. The *Hind*III-digested bacterial genomic DNAs were electrophoresed and transferred to a nylon membrane and hybridized with a DIG-labeled Fu12 DNA fragment. *Hind*III-digested genomic DNAs (lanes 1-23) were from: 1) *F. nucleatum* subsp. *nucleatum* ATCC 25586^T, 2) *F. nucleatum* subsp. *nucleatum* ATCC 23726, 3) *F. nucleatum* subsp. *polymorphum* ATCC 10953^T, 4) *F. nucleatum* subsp. *vincentii* ATCC 49256^T, 5) *F. nucleatum* subsp. *fusiforme* ATCC 51190^T, 6) *F. nucleatum* subsp. *animalis* ATCC 51191^T, 7) *F. periodonticum* ATCC 33693^T, 8) *F. necrophorum* ATCC 25286^T, 9) *F. nucleatum* subsp. *nucleatum* ChDC F311, 10) *F. nucleatum* subsp. *nucleatum* ChDC F130, 11) *F. nucleatum* subsp. *nucleatum* ChDC PV-F42, 12) *F. nucleatum* subsp. *polymorphum* ChDC PV-F48, 13) *F. nucleatum* subsp. *polymorphum* ChDC PV-F55, 14) *F. nucleatum* subsp. *polymorphum* ChDC F113, 15) *F. nucleatum* subsp. *polymorphum* ChDC F119, 16) *F. nucleatum* subsp. *polymorphum* ChDC F219, 17) *F. nucleatum* subsp. *polymorphum* ChDC F286, 18) *F. nucleatum* subsp. *animalis* ChDC PV-F58, and 19) *F. periodonticum* ChDC F251. Lane S, 1 kb DNA ladders.

94°C for 1 min, primer annealing at 57°C or 60°C for 30 sec, and extension at 72°C for 1 min. The final cycle included an additional 10 min of extension time at 72°C. A 2 μl aliquot of the reaction mixture was then analyzed with 1.5% agarose gel electrophoresis in a Tris-acetate buffer (0.04 M Tris-acetate, 0.001 M EDTA, [pH8.0]) at 100V for 30 min. The amplification products were stained with ethidium bromide, and visualized by UV transillumination.

Results and Discussion

The Southern blot analysis revealed that the Fu12 DNA probe reacted only with the HindIII-digested genomic DNA of F. nucleatum ATCC 25586^T (Fig. 1). The observed signal band was 2.4 kbp in size, and this size was consistent with the nucleotide sequence of the 2,444 bp Fu12 probe. We also determined the partial nucleotide sequences (about 400 bp) of both the 5'- and 3'-end of the Fu12 in this study (data not shown). The remainder of the Fu12 sequence was acquired from the GenBank database (accession no. NC_003454; 1,090,445 - 1,092,888). The Fu12 fragment was believed to encode for an open reading frame (ORF) of the DNA repair gene and two partial ORFs of the hypothetical proteins, FN0449 and NIFS (data not shown). This suggests that the Fu12 fragment constitutes a good candidate for a strain-specific probe for F. nucleatum ATCC

25586^T, and may also prove to be useful in the confirmation of the strain.

Matheson et al. developed a simple technique for the development of strain-specific DNA probes, which could be used in the monitoring of the movement and survival of bacteria in natural and laboratory ecosystems (Matheson et al., 1997). Their method involved the amplification of genomic DNA by repetitive sequence-based PCR (rep-PCR), using primers specific for repetitive extragenic palindromic (REP) elements, followed by the cloning of these amplified fragments. The cloned fragments were then screened to identify those that were strain-specific, and these fragments were subsequently used as probes for the total genomic DNA isolated from the microbial communities, and subjected to rep-PCR. This method is simpler than the random cloning method that uses restriction enzymes. However, in order to screen the specificity of the randomly cloned DNA fragments, which requires the same number of hybridization as the number of DNA fragments. Therefore, this method is rather laborious, expensive, and time-consuming. Recently, a simpler rapid screeing method for bacterial species- or subspecies-specific DNA probes has been introduced (Kook et al., 2003b). Consequently, if the two methods, namely the random cloning method using rep-PCR and the rapid screening method, are used in combination, it should become easier and quicker to develop strain-specific DNA probes.
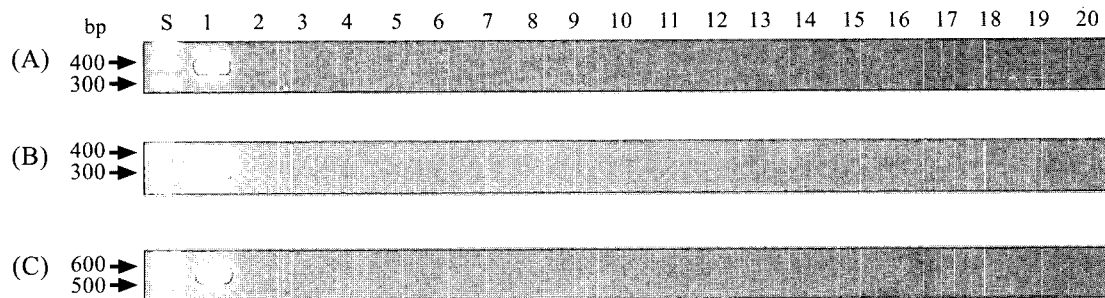

Fig. 2. Specificity test of primers (A) Fu12-F1 and Fu12-R1, (B) Fu12-F2 and Fu12-R2, and (C) Fu12-F3 and Fu12-R3 for F. nucleatum subsp. nucleatum ATCC 25586^T at the strain level. Four ng of each bacterial genomic DNA were used as PCR templates. PCR products were electrophoresed in 1.5% agarose gel. The lanes, S through 19, are identical to those described in Fig. 1. Lane 20 was the negative control.(distilled water).
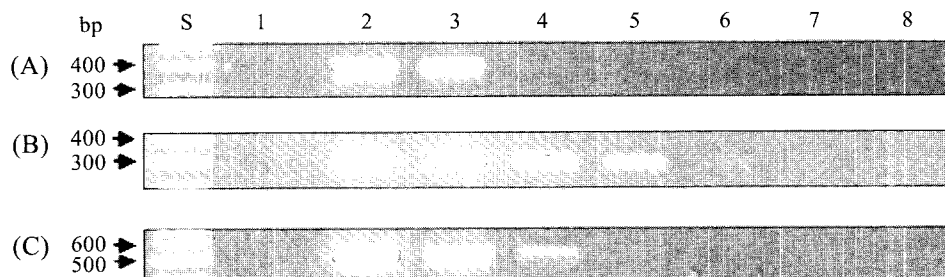

Fig. 3. Sensitivity test of PCR performed with primers (A) Fu12-F1 and Fu12-R1, (B) Fu12-F2 and Fu12-R2, and (C) Fu12-F3 and Fu12-R3, and the purified genomic DNA of F. nucleatum subsp. nucleatum ATCC 25586^T. The PCR products were electrophoresed in 1.5% agarose gel. Lanes: S, 100 base pair DNA ladder (Bioneer, Korea); 2 through 6, purified genomic DNA serially diluted 10-fold, from 4 ng to 4 fg. Lane 1 was the negative control (distilled water).

The data obtained from the specificity test showed that the three pairs of PCR primers, Ful2-F1 and Ful2-R1, Ful2-F2 and Ful2-R2, and Ful2-F3 and Ful2-R3, all of which were based on the nucleotide sequence of Ful2, were strain-specific for *F. nucleatum* ATCC 25586^T (Fig. 2). The optimal annealing temperatures of the three primers, as determined by gradient PCR, were as follows: 57°C, 57°C, and 60°C, respectively (Table 1). For the purposes of this study, the optimal annealing temperature was defined as the lowest temperature at which the PCR products could only be amplified from the genomic DNA of *F. nucleatum* ATCC 25586^T. The optimal annealing temperatures for the three PCR primer sets, as suggested by the PrimerSelect program, were 46.4°C, 46.3°C, and 48.1°C, respectively. However, in practice, the optimal annealing temperatures were determined to be higher than the predicted temperatures.

The sensitivity of the Ful2-F2 and Ful2-R2 primers was found to be higher than that observed for the other two primer pairs (Fig. 3). The Ful2-F2 and Ful2-R2 primers were able to detect 4 pg of the genomic DNA of *F. nucleatum* ATCC 25586^T. In a previous study, we developed a set of multiplex PCR primers (All-F6, Fn-R6, and ChDC-AaR) for the simultaneous detection of *F. nucleatum* and *A. actinomycetemcomitans* (Kim et al., 2004). In that study, the All-Fn6 and Fn-R6 primers could detect 4 fg of the genomic DNA of *F. nucleatum* ATCC 25586^T at an annealing temperature of 68°C. The differences between the annealing temperatures and the sensitivity of the primer sets might be attributable to the G+C contents of the target genes. The G+C content of the Ful2 for the three primers designed in this study was 26.76%, but the G+C content of the 16S rDNA for All-F6 and Fn-R6 was 49.19%.

In summary, we have assessed the strain specificity of the DNA probe Ful2 for *F. nucleatum* ATCC 25586^T, and have developed three PCR primers that are specific for the strain. To our knowledge, this is the first report to address the development of strain-specific DNA probes and PCR primers for oral bacteria. Our results clearly show that the DNA probe Ful2 and the three pairs of PCR primers developed in this study may prove useful in the identification of *F. nucleatum* ATCC 25586^T, and may also be particularly helpful in the confirmation of the strain.

Acknowledgment

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