

## Multiplex PCR Using Conserved and Species-Specific 16S rDNA Primers for Simultaneous Detection of *Fusobacterium nucleatum* and *Actinobacillus actinomycetemcomitans*

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Received: May 28, 2003

Accepted: September 20, 2003

**Abstract** This study was undertaken to develop PCR primers for the simultaneous detection of *Fusobacterium nucleatum* and *Actinobacillus actinomycetemcomitans*, using two species-specific reverse primers in combination with a single conserved forward primer. These primers target the variable and conserved regions of the 16S rDNA. The primer specificity was tested against (i) four *F. nucleatum* and three *A. actinomycetemcomitans* strains and (ii) seven representatives of the different species of oral bacteria. The primer sensitivity was determined by testing serial dilutions of the purified genomic DNA of *F. nucleatum* and *A. actinomycetemcomitans*. The data indicate that species-specific amplicons could be obtained for all the *F. nucleatum* and *A. actinomycetemcomitans* strains tested, which were not found in the seven other species. The multiplex PCR could detect as little as 4 fg of chromosomal DNA of *F. nucleatum* and *A. actinomycetemcomitans* simultaneously. These findings suggest that these PCR primers are highly sensitive and are suitable for applications in epidemiological studies, diagnosis, and monitoring *F. nucleatum* and *A. actinomycetemcomitans* after the treatment of periodontitis.

**Key words:** *Actinobacillus actinomycetemcomitans*, *Fusobacterium nucleatum*, multiplex PCR, 16S rDNA

Periodontitis refers to a set of diseases that cause inflammation and the loss of the supporting structures of

the teeth [25]. The major causative factor of periodontitis is dental plaque. The major putative pathogens known to be involved in destructive periodontal diseases include *Actinobacillus actinomycetemcomitans*, *Bacteroides forsythus*, *Campylobacter rectus*, *Eikellena corrodens*, *Fusobacterium nucleatum*, *Porphyromonas gingivalis*, *Prevotella intermedia*, and spirochetes [6]. *F. nucleatum* has been frequently associated with periodontitis [3, 27, 31], and may play a role in disease by providing an anaerobic environment necessary for the growth of pathogens [7]. *F. nucleatum* strains have been shown to be able to coaggregate all species of oral bacteria tested, thereby acting as a bridge between the early and late colonizers in the development of dental plaque on tooth surfaces [16]. *F. nucleatum* was also shown to be able to modulate the secondary immune response of T-cells to *A. actinomycetemcomitans* [23]. *A. actinomycetemcomitans* is the major pathogen in localized juvenile periodontitis [15, 32] and is known to be a cause of infective endocarditis [28]. The virulence factors of this organism have been reported to be leukotoxin, cytolethal distending toxin, collagenase, chemotaxis inhibitor, and lipopolysaccharide (LPS) [9, 12, 17, 22, 26].

The major putative pathogens have to be identified in order to assess the epidemiology of the periodontal pathogens in addition to the diagnosis, treatment, and prognosis of periodontal diseases, and many studies showed several putative periodontal pathogens which could be detected by individual PCRs [1–3, 21] or by multiplex PCRs [5, 20, 26]. The use of multiplex PCR is highly economical in saving time, cost, and experimental effort, compared to the

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individual PCR as well as other bacterial identification methods, including the cell culture, DNA probe method [10], 16S rRNA sequencing, and ribotyping. Therefore, multiplex PCR has been widely applied for identifying the various bacterial species. 16S rDNA can effectively be used for multiplex PCR assays, because 16S rDNA is found universally in all prokaryotic organisms and a comparative analysis of 16S rDNA has shown that the variable sequence regions are interspersed with highly conserved regions [30].

In order to examine the combined effect of the two species on the progression of periodontitis, an accurate and simple method to identify these two bacterial species need to be developed. Therefore, the aim of this study was to develop, optimize, and evaluate multiplex PCR primers using a single 16S rDNA-directed conserved forward primer combined with two species-specific reverse primers for simultaneous detection of *F. nucleatum* and *A. actinomycetemcomitans*. After evaluating the method with four *F. nucleatum* and three *A. actinomycetemcomitans* strains as well as seven representatives of the different species of oral bacteria, multiplex PCR was compared with single PCRs by applying multiplex PCR to 20 clinical samples from subgingival plaque.

## MATERIALS AND METHODS

### Bacterial Strains and Growth Condition

The bacterial strains used were as follows: *Fusobacterium nucleatum* subsp. *nucleatum* ATCC 25586, *F. nucleatum* subsp. *fusiforme* ATCC 51190, *F. nucleatum* subsp. *polymorphum* ATCC 10953, *F. nucleatum* subsp. *vincentii* ATCC 49256, *Actinobacillus actinomycetemcomitans* ATCC 43717, *A. actinomycetemcomitans* ATCC 43718, *A. actinomycetemcomitans* ATCC 33384, *Haemophilus aphrophilus* ATCC 33389, *H. paraphrophilus* ATCC 29241, *H. paraphrophilus* ATCC 29242, *H. parainfluenzae* ATCC 33392, *Prevotella intermedia* ATCC 25611, *P. nigrescens* ATCC 33563, *Porphyromonas gingivalis* ATCC 53978, and *Porphyromonas endodontalis* ATCC 35406. All the strains were obtained from the American Type Culture Collection (ATCC, Rockville, MD, U.S.A.). *A. actinomycetemcomitans* was grown in a medium composed of tryptic soy broth (TSB) (Difco Laboratories, Detroit, MI, U.S.A.) supplemented with 0.6% yeast extract, 5% horse serum, 75 mg/ml of bacitracin, and 5 mg/ml of vancomycin (Sigma Chemical Co., St. Louis, MO, U.S.A.). *H. aphrophilus*, *H. paraphrophilus*, and *H. parainfluenzae* were grown in TSB supplemented with 0.5% yeast extract, 0.5 mg/ml of hemin, and 2 µg/ml of vitamin K<sub>1</sub>. All the above species were grown in an incubator containing air and 5% CO<sub>2</sub> at 37°C. *F. nucleatum*, *P. intermedia*, *P. nigrescens*, *P. gingivalis*, and *P. endodontalis* were cultured at 37°C in TBS supplemented with 0.5% yeast extract, 0.05% cysteine

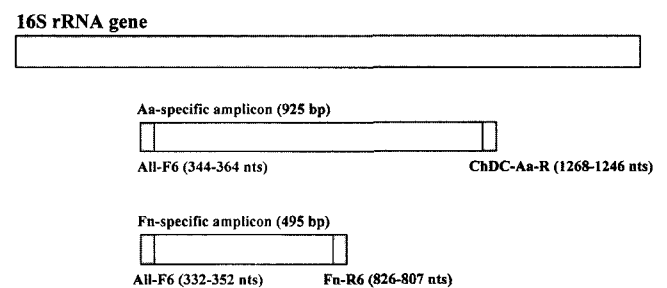
HCl-H<sub>2</sub>O, 0.5 mg/ml of hemin, and 2 µg/ml of vitamin K<sub>1</sub> in an anaerobic chamber under 10% H<sub>2</sub>, 5% CO<sub>2</sub>, and 80% N<sub>2</sub>.

### Bacterial Genomic DNA preparation

The bacteria were grown to the mid-log phase in 100 ml of medium and were harvested by centrifugation at 7,000 ×g for 10 min. The whole genomic DNA was isolated using a modification of the method of Lippke *et al.* [14]. Briefly, the cell pellets were resuspended by vortexing in 5 volumes of a cell lysis buffer [5 M guanidine isothiocyanate, 50 mM Tris-HCl (pH 7.6), 10 mM EDTA, 2% *S*-lauryl sarcosinate, 140 mM 2-mercaptoethanol]. The bacterial genomic DNAs were purified by the phenol/chloroform extraction method instead of the cesium chloride [19]. The DNA concentrations were determined spectrophotometrically at 260 and 280 nm (Ultrospec 2000, Pharmacia Biotech., Cambridge, U.K.).

### Primer Design

A forward primer, which is conserved in the 16S rDNA sequence of both *F. nucleatum* and *A. actinomycetemcomitans*, and the two reverse primers, which are each specific to *F. nucleatum* and *A. actinomycetemcomitans*, were designed using the program PRIMERSECT (DNASTAR Inc., Madison, WI, U.S.A.). The 16S rDNA sequences of *F. nucleatum* and *A. actinomycetemcomitans* strains stored in the GenBank EMBL database were used as DNA templates in PRIMERSECT. The strains and GenBank accession numbers are as follows: *F. nucleatum* subsp. *nucleatum* ATCC 25586 (AJ133496), *F. nucleatum* subsp. *vincentii* ATCC 49256 (AJ006964), *A. actinomycetemcomitans* ATCC 43718 (M75035), *A. actinomycetemcomitans* ATCC 33384 (M75039), *A. actinomycetemcomitans* ATCC 29522 (M75036), *A. actinomycetemcomitans* ATCC 29524 (M75037), and *A. actinomycetemcomitans* ATCC 29523 (M75038). The specificity of the prospective primers was tested using the program, PROBE Match, from the Ribosomal Database Project II (<http://rdp.cme.msu.edu/html/analyses.html>). No sequence completely homologous to the *F. nucleatum* and



**Fig. 1.** Multiplex PCR using the conserved and species-specific 16S rDNA primers for the simultaneous detection of *F. nucleatum* (Fn) and *A. actinomycetemcomitans* (Aa).

A schematic drawing of the location where the primers anneal to the bacterial 16S rDNA is shown. The approximate sizes of the species-specific amplicons generated are also depicted.

*A. actinomycetemcomitans* reverse primers was found in the 16S rDNAs for the other oral bacterial species in these databases. The nucleotide sequences of the three selected primers were as follows: conserved forward primer (All-F6), 5'-CGG GAG GCA GCA GTG GGG AAT-3'; *F. nucleatum* reverse primer (Fn-R6), 5'-TTG CTT GGG CGC TGA GGT TC; and *A. actinomycetemcomitans* reverse primer (ChDC-AaR), 5'-CAT CGC TGG TTG GTT ACC CTC TG-3'. The expected product lengths were 495-bp and 923-bp for *F. nucleatum* and *A. actinomycetemcomitans*, respectively (Fig. 1).

### Specificity and Sensitivity of the PCR

PCR was performed to validate the primer specificity and to determine the detection limit of the PCR primers. The specificity of PCR was evaluated by testing three *A. actinomycetemcomitans* and four *F. nucleatum* strains, as well as seven representatives of the different species of oral bacteria (aliquots of 4 ng of nucleic acid). For the sensitivity test of the multiplex PCR, the lower limit of detection was defined as the smallest amount of bacterial genomic DNA that could be detected by the multiplex PCR. This was determined by a serial dilution of a mixture of genomic DNA from *F. nucleatum* ATCC 25586 and *A. actinomycetemcomitans* ATCC 33277, and was found to range from 4 ng to 4 fg by 10-fold dilutions.

The designed oligonucleotide primers were obtained from BIONEER Corp., Seoul, Korea. The PCR was performed using an *AccuPower*<sup>®</sup> PCR PreMix (BIONEER Corp., Daejeon, Korea), which contained 5 nmole each of deoxynucleoside triphosphate, 0.8  $\mu$ mole KCl, 0.2  $\mu$ mole Tris-HCl (pH 9.0), 0.03  $\mu$ mole MgCl<sub>2</sub>, and 1 unit of *Taq* DNA polymerase. The bacterial genomic DNA and 20 pmoles each of primer were added to a PCR PreMix tube. PCR was carried out in a final volume of 20  $\mu$ l. The PCR reaction was run for 32 cycles on a Peltier thermal cycler (Model PTC-200 DNA engine<sup>™</sup>, MJ Research Inc., Watertown, MA, U.S.A.) under the following conditions: denaturation at 94°C for 1 min, primer annealing at 68°C for 30 sec, and extension at 72°C for 1 min. The final cycle included an additional 10 min extension at 72°C. A 2  $\mu$ l aliquot of the reaction mixture was 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 100 V for 30 min. The amplification products were stained with ethidium bromide and visualized by UV transillumination.

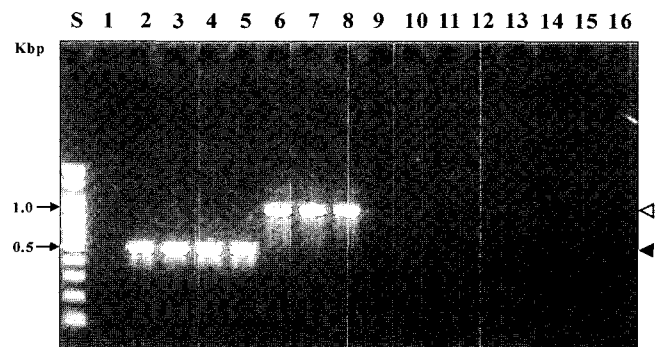
### Coincidence Test of Multiplex PCR and Single PCR Using Plaque Samples

In order to determine whether the results derived from multiplex PCR and single PCRs were coinciding, the PCRs were performed with subgingival plaque samples collected from 20 adult periodontitis patients by following procedures approved by the University of Chosun Institutional Review Board. A subgingival plaque sample

was collected from a molar tooth of each subject; the teeth met all of the following criteria for periodontitis: gingival index of 3 and a probing depth of  $\geq 6$  mm. The plaque samples were taken using sterile paper point, and placed in 500  $\mu$ l 1 $\times$  PBS. Bacterial DNAs from the plaque sample were extracted using the direct DNA extraction method described by Lee *et al.* [12]. A 50  $\mu$ l aliquot of a plaque sample was mixed with 50  $\mu$ l of a 2 $\times$  Lysis buffer (2 mM EDTA-1% Triton X-100), and the mixture was boiled for 10 min. Three 4  $\mu$ l aliquots of the plaque lysate were prepared and transferred to three PCR premix tubes; one tube for multiplex PCR and two tubes for single PCRs, each with primers All-F6 and Fn-R6, and with All-F6 and ChDC-AaR. The PCR conditions were the same as described above.

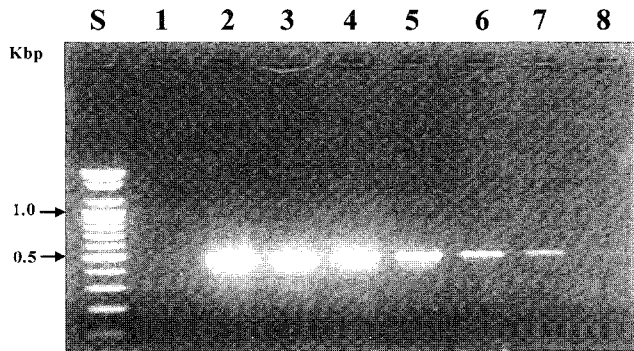
## RESULTS AND DISCUSSION

The specificity of the multiplex PCR primers was tested by performing PCR, using genomic DNAs purified from seven other representative strains of oral species chosen for this study. The PCR products showed that the DNA bands of the predicted size were 495 and 925 bp for the *F. nucleatum* and *A. actinomycetemcomitans* genomic DNAs, respectively (Fig. 2). On gel electrophoresis, the PCR products displayed DNA bands with predicted sizes of 495 and 925 bp for the *F. nucleatum* and *A.*



**Fig. 2.** Specificity test of the multiplex PCR performed with primers All-F6, Fn-R6, and ChDC-AaR.

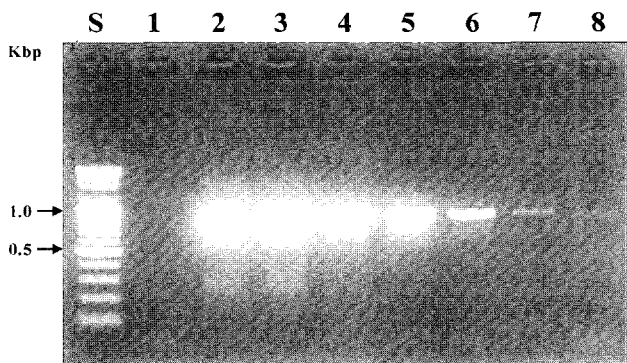
Agarose gel electrophoresis of the amplification products obtained by the three primers was performed. Each (4 ng) of the bacterial genomic DNA was used as the PCR template. Lanes: S, 100 base pair DNA ladder (Bioneer Corp.); 1, sterilized deionized water (negative control); 2, *F. nucleatum* ATCC 25586; 3, *F. nucleatum* ATCC 10953; 4, *F. nucleatum* ATCC 49256; 5, *F. nucleatum* ATCC 51190; 6, *A. actinomycetemcomitans* ATCC 43717; 7, *A. actinomycetemcomitans* ATCC 43718; 8, *A. actinomycetemcomitans* ATCC 33384; 9, *H. aphrophilus* ATCC 33389; 10, *H. paraphrophilus* ATCC 29241; 11, *H. paraphrophilus* ATCC 29242; 12, *H. parainfluenzae* ATCC 33392; 13, *P. intermedia* ATCC 25611; 14, *P. nigrescens* ATCC 33563; 15, *P. gingivalis* ATCC 53978; 16, *P. endodontalis* ATCC 35406. The open and filled arrowheads indicate the PCR products of *A. actinomycetemcomitans* (925 bp) and *F. nucleatum* (495 bp), respectively.



**Fig. 3.** Sensitivity test of the PCR performed with primers All-F6 and Fn-R6, and the purified genomic DNA of *F. nucleatum* ATCC 25586.

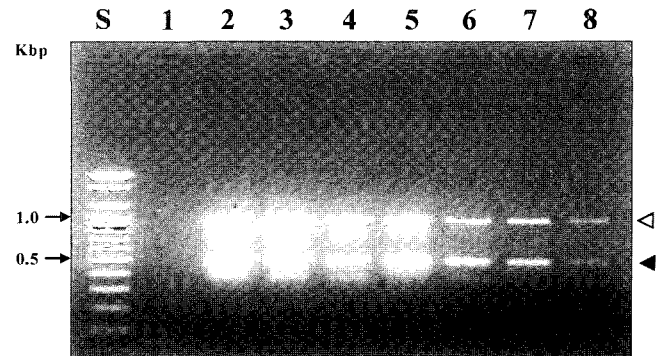
The PCR products were electrophoresed in 1.5% agarose gel. Lanes: S, 100 base pair DNA ladder (Bioneer Corp.); 1, sterilized deionized water (negative control); 2 through 8, purified genomic DNA serially diluted 10-fold from 4 ng to 4 fg.

*actinomycetemcomitans* genomic DNAs, respectively (Fig. 2). However, the PCR amplicons did not occur with the seven other bacterial species. The multiplex PCR could clearly distinguish *A. actinomycetemcomitans* from closely related *Haemophilus* spp. [8]. In addition, neither of our species-specific reverse primers showed a complete homology to the 16S rDNA of any other oral species found in the current sequence databases. This suggests that similar results would have been obtained if pure cultures of additional species had been evaluated. Under optimal conditions, PCR with primers All-F6 and Fn-R6 detected as little as 4 fg of the purified genomic DNA of *F. nucleatum* ATCC 25586 as well as the other four strains of *F. nucleatum* (Fig. 3). In addition, PCR with primers All-F6 and ChDC-AaR also detected as little as 4 fg of the purified genomic DNA of *A. actinomycetemcomitans*



**Fig. 4.** Sensitivity test of the PCR performed with the primers All-F6 and ChDC-AaR, and the purified genomic DNA of *A. actinomycetemcomitans* ATCC 33277.

The PCR products were electrophoresed in 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.



**Fig. 5.** Sensitivity test of the multiplex PCR performed with primers All-F6, Fn-R6, ChDC-AaR, and the purified genomic DNA of *F. nucleatum* ATCC 25586 and *A. actinomycetemcomitans* ATCC 33277.

The PCR products were electrophoresed in 1.5% agarose gel. Lanes: S, 100 base pair DNA ladder (Bioneer Corp.); 1, sterilized deionized water (negative control); 2 through 8, a mixture of the purified genomic DNAs from both species, serially diluted 10-fold from 4 ng to 4 fg. The open and filled arrowheads indicate the PCR products of *A. actinomycetemcomitans* (925 bp) and *F. nucleatum* (495 bp), respectively.

ATCC 33277 (Fig. 4). The sensitivity of the multiplex PCR with primers All-F6, Fn-R6, and ChDC-AaR was the same as that of the single PCRs (Fig. 5), thus manifesting that the multiplex PCR primer set could detect the strains of *F. nucleatum* (genome size; 2.17 Mb) and *A. actinomycetemcomitans* (genome size; 2.1 Mb) simultaneously.

Since the DNA extraction method for the bacterial pure culture was different from that for dental plaque, it seems likely that the conditions of the DNAs prepared from the two sources may be dissimilar. As described in Materials and Methods, a direct DNA extraction method was employed to expedite the processing of DNAs from plaque samples. Therefore, there may be different detection limits for the multiplex PCR, depending on the sources of DNAs prepared. It was recently reported that a direct DNA extraction method could be applied to clinical specimens to detect *Vibrio vulnificus* by PCR [13]. In that experiment, clinical specimens or bacterial cells were boiled in 1 mM EDTA-0.5% Triton X-100 buffer and the lysates were used in PCR. The present study tested whether the 1 mM EDTA-0.5% Triton X-100 lysis buffer was effective in preparing the cell lysate from a single colony of *F. nucleatum* or *A. actinomycetemcomitans* for PCR, and found that the specificity of multiplex PCR with the cell lysate was identical to those obtained using the purified genomic DNAs (data not shown). However, the sensitivity of both single and multiplex PCRs carried out with the bacterial cell lysate was 10-fold lower. This might have been due to an inhibition of PCR by cellular components of the lysates or by the detergents present in the lysis buffer.

In addition, two sets of single PCR performed with primers All-F6 and Fn-R6, and All-F6 and ChDC-AaR,

could be used in detecting *F. nucleatum* and *A. actinomycetemcomitans*, respectively. For the detection of *A. actinomycetemcomitans*, several PCR primers were designed based on the 16S rDNA [1] and leukotoxin gene [24]. As for *F. nucleatum*, PCR primers were also designed based on the 16S rDNA [11, 18]. The PCR primers reported in the present study would be a valuable addendum to the list of already available primers for the two bacterial species. In the laboratory, two or three sets of PCR primers are routinely used to identify the bacteria grown on agar plates at the species level, and the resulting PCR data generally coincided with the 16S rDNA cloning and sequencing data.

In the coincidence test, the intensity and size of the amplicons of the multiplex PCR were similar to those of

the two sets of single PCR (Fig. 6), indicating that the multiplex PCR can be used to simultaneously detect and identify *F. nucleatum* and *A. actinomycetemcomitans* from specimens such as dental plaques.

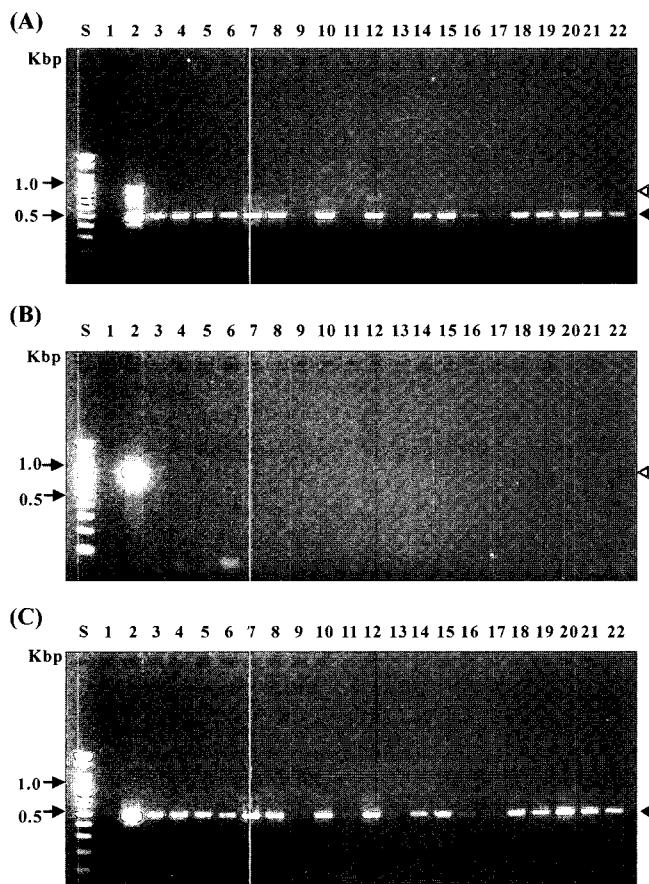
In summary, the present data reveal that the multiplex PCR primer set (All-F6, Fn-R6, and ChDC-AaR) is highly sensitive and specific. These primers could potentially be used in epidemiological studies, diagnosis, and monitoring *F. nucleatum* and *A. actinomycetemcomitans* from dental plaque samples before and after periodontal treatments.

## Acknowledgment

This work was supported by Korea Research Foundation Grant (KRF-2001-002-F00153) (J.-K. Kook and B.-O. Kim).

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**Fig. 6.** Coincidence test of the multiplex PCR and single PCRs performed with the plaque samples.

Multiplex PCR for simultaneous detection of *F. nucleatum* and *A. actinomycetemcomitans* (A), single PCRs for the detection of *F. nucleatum* (B), and *A. actinomycetemcomitans* (C) were performed with the plaque samples from 20 periodontitis patients. Lane: S, 100 base pair DNA ladder; 1, sterilized deionized water (negative control); 2, a mixture (4 ng) of equal amounts of genomic DNA from *F. nucleatum* ATCC 25586 and *A. actinomycetemcomitans* ATCC 33384 (positive control); 3 through 22, plaque samples from 20 subjects (patients 1 to 20). The open and filled arrowheads indicate the PCR products of *A. actinomycetemcomitans* (925 bp) and *F. nucleatum* (495 bp), respectively.

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