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NOTE

Identification of *Actinobacillus actinomycetemcomitans* Using Species-Specific 16S rDNA Primers

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The purpose of this study was to develop species-specific PCR primers for use in the identification and detection of *Actinobacillus actinomycetemcomitans*. These primers target variable regions of the 16S ribosomal RNA coding gene (rDNA). We assessed the specificity of the primers against 9 *A. actinomycetemcomitans* strains and 11 strains (3 species) of the *Haemophilus* genus. Primer sensitivity was determined by testing serial dilutions of the purified genomic DNAs of *A. actinomycetemcomitans* ATCC 33384^T. Our obtained data revealed that we had obtained species-specific amplicons for all of the tested *A. actinomycetemcomitans* strains, and that none of these amplicons occurred in any of the other species. Our PCR protocol proved able to detect as little as 4 fg of *A. actinomycetemcomitans* chromosomal DNA. Our findings suggest that these PCR primers are incredibly sensitive, and should prove suitable for application in epidemiological studies, as well as the diagnosis and monitoring of periodontal pathogens after treatment for periodontitis.

Key words: *Actinobacillus actinomycetemcomitans*, 16S rDNA, species-specific PCR primer

Periodontitis refers to a set of diseases that cause inflammation and destruction of the supporting structures of the teeth (Tran and Rudney, 1996). Dental plaque is the principal causative factor in periodontitis. The putative pathogens primarily involved in the development of destructive periodontal diseases include: *Actinobacillus actinomycetemcomitans, Bacteroides forsythus, Campylobacter rectus, Eikellena corrodens, Fusobacterium nucleatum, Porphyromonas gingivalis, Prevotella intermedius*, and several spirochetes (Darveau *et al.*, 1997). *Actinobacillus actinomycetemcomitans* has been identified as a major pathogen in localized juvenile periodontitis (Mandell, 1984; Genco *et al.*, 1985; Zambon, 1985; Asikainen, 1986), and is also known to induce infective endocarditis (van Winkelhoff and Slots, 1999). Several virulence factors have been identified in conjunction with this organism, including: leukotoxin, cytolethal distending toxin, collagenase, chemotaxis inhibitor, and lipopolysaccharide (LPS) (Kiley and Hot, 1980; Robertson *et al.*, 1982; Lally *et al.*, 1989; Ashkenazi *et al.*, 1992; Sugai *et al.*, 1998)

In order to assess the epidemiology of periodontal pathogens, as well as the diagnosis, treatment, and prognosis of periodontal diseases, it is important to first identify the principal putative pathogens involved. Several putative periodontal pathogens have already been detected in previous studies employing DNA probe methods (Kook *et al.*, 2003) or PCR (Slots *et al.*, 1995; Ashimoto, *et al.*, 1996; Conrads *et al.*, 1999; Trans and Rudney 1999; Seong *et al.*, 2003). The advent of PCR has resulted in substantial savings with regard to time, cost, and experimental effort, as compared to other bacterial identification methods, including cell culture, the DNA probe method, 16S rDNA sequencing, ribotyping, etc. The many advantages of PCR have guaranteed its wide usage in the diagnosis and identification of bacterial species. 16S rDNA can be used effectively in multiplex PCR assays, as 16S rDNA is ubiquitous in all prokaryotic organisms, and the comparative analysis of 16S rDNA has demonstrated that variable sequence regions are interspersed with highly conserved regions (Woese 1987).

Recently, a set of PCR primers based on 16S rDNA were developed, and were then utilized in the identification of *A. actinomycetemcomitans* (Ashimoto *et al.*, 1996). However these PCR primers can also be used to detect and identify strains of the *Haemophilus* genus, when used in conjunction with $MgCl_2$ at a concentration

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of 1.5 mM. The original pioneers of this method, however, have officially recommended using an $MgCl_2$ concentration of 1.0 mM. This concentration insures, however, that the sensitivity of the primers will be less than when using 1.5 mM $MgCl_2$. In the present study, we have attempted to develop a new set of PCR primers, which could be used to detect and identify *A. actinomycetemcomitans* at an even higher level of sensitivity.

The type strains and reference strains of the bacteria used in this study were as follows: *Actinobacillus actinomycetemcomitans* ATCC 43717, *A. actinomycetemcomitans* ATCC 43718, *A. actinomycetemcomitans* ATCC 33384^T, *Haemophilus aphrophilus* ATCC 33389^T, *H. paraphrophilus* ATCC 29241^T, *H. paraphrophilus* ATCC 29242, *H. parainfluenzae* ATCC 33392^T, *Prevotella intermedia* ATCC 25611^T, *Prevotella nigrescens* ATCC 33563^T, *Porphyromonas gingivalis* ATCC 33277^T, *Porphyromonas endodontalis* ATCC 35406^T, *Fusobacterium nucleatum* ATCC 25586^T, and *Camphylobacter rectus* ATCC 33238^T. All strains were obtained from the American Type Culture Collection (ATCC, USA). Six *A. actinomycetemcomitans* strains (ChDC A110, ChDC A111, ChDC A113, ChDC A115, ChDC A117 and ChDC A121), four *H. aphrophilus* (ChDC PV-F30, ChDC PV-F36, ChDC PV-F39, and ChDC PV-F41), and one *H. paraphrophilus* (ChDC A108) strains were isolated from the dental plaque samples of six Korean periodontitis patients; all of these strains were identified at the species level, via 16S rDNA sequencing.

A. actinomycetemcomitans was grown in a tryptic soy broth (TSB) (Difco, USA) medium, which had been supplemented with 0.6% yeast extract, 5% horse serum, 75 μg/ml of bacitracin, and 5 μg/ml of vancomycin (Sigma, USA) at 37°C, in an anaerobic chamber, under the following atmospheric conditions of 10% H_2, 5% CO_2, and 85% N_2. *H aphrophilus*, *H. paraphrophilus*, and *H. parainfluenzae* were allowed to grow in TSB medium, which had been supplemented with 0.5% yeast extract, 0.5 mg/ml of hemin, and 2 μg/ml of vitamin K_1 at 37°C, in an incubator with an air atmosphere containing 5% CO_2.

Whole genomic DNA was isolated according to the method described by Lippke *et al.* (1987), with some modifications (Kook *et al.*, 2003); bacterial genomic DNAs were purified by the phenol/chloroform extraction method, rather than by the cesium chloride extraction method. DNA concentrations were determined at 260 and 280 nm by UV-spectrophotometry (Ultrospec 2000, GE Healthcare Bioscience, USA).

A pair of PCR primers, specific to the 16S rDNA sequence of *A. actinomycetemcomitans,* was designed using the program PRIMERSECT (DNASTAR Inc., USA). All of the 16S rDNA sequences of the *A. actinomycetemcomitans* strains stored in the GenBank EMBL database were used for DNA templates in PRIMERSECT. Strains and GenBank accession numbers were as follows: *A. actinomycetemcomitans* ATCC 43718 (M75035), *A. actinomycetemcomitans* ATCC 33384^T (M75039), *A. actinomycetemcomitans* ATCC 29522 (M75036), *A. actinomycetemcomitans* ATCC 29524 (M75037), and *A. actinomycetemcomitans* ATCC 29523 (M75038). The specificities of the prospective primers were evaluated using the PROBE Match program from the Ribosomal Database Project (http://rdp.cme.msu.edu/html/analyses.html). No sequences that were completely homologous to the *A. actinomycetemcomitans* primers was found in the 16S rDNAs of the other oral bacterial species in these databases. The nucleotide sequences of the selected primers were as follows: forward primer (ChDC-Aa-F), 5'-TAG CCC TGG TGC CCG AAG C-3'; and reverse primer (ChDC-Aa-R), 5'-CAT CGC TGG TTG GTT ACC CTC TG-3'. The expected product length for *A. actinomycetemcomitans* was 428 bp.

PCR was then performed, in order to validate primer specificity, and also to determine the detection limits of the PCR primers. Primer specificity was tested against three *A. actinomycetemcomitans* strains, three *Haemophilus* species, and five representatives of different oral bacteria species (4 ng aliquots of genomic DNA). For the PCR sensitivity test, we defined the lower limit of detection as the smallest amount of bacterial genomic DNA detectable by our PCR. This, in turn, was determined by serial dilutions of the genomic DNA from *A. actinomycetemcomitans* ATCC 33384^T. These ranged from 4 ng to 4 fg, by 10-fold dilutions. In addition, in order to compare the sensitivity of the PCR primers (ChDC-Aa-F and ChDC-Aa-R) and Ashimoto's PCR primers (Ash-Aa-F and Ash-Aa-R), we also conducted PCR with a 1.5 mM concentration of $MgCl_2$.

The designed oligonucleotide primers were obtained from the Bioneer Corp., in Korea. PCR was carried out with an *AccuPower*® PCR PreMix (Bioneer, Korea), which contained 5 nmoles of each 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, as well as 20 pmoles of each primer, was then added to a PCR PreMix tube. PCR was then carried out in a final volume of 20 μl. The PCR reaction was run for 32 cycles on a Peltier thermal cycler (Model PTC-200 DNA Engine™, MJ Research Inc., USA) under the following conditions: 1 min of denaturation at 94°C, 30 sec of primer annealing at 68°C, and 1 min of extension at 72°C. 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 via 1.5% agarose gel electrophoresis in a Tris-acetate buffer (0.04 M Tris-acetate, 0.001 M EDTA, pH 8.0) for 30 min at 100 V. The amplification products were stained with ethidium bromide, and were finally visualized by UV transillumination.

On gel electrophoresis, the PCR products displayed DNA bands for the genomic DNAs of *A. actinomycetemcomitans* strains which were the expected size, 428 bp (Fig. 1). However, the PCR amplicons did not occur during our test of the genomic DNAs of the *Haemophilus* spp.. Our PCR primers (ChDC-Aa-R and ChDC-Aa-R) proved able to clearly differentiate *A. actinomycetemcomitans* from the closely-related *Haemophilus* spp. We also confirmed the specificity of the PCR primers for *A. actinomycetemcomitans* with the genomic DNAs of type strains of six different representative oral bacteria species; *Prevotella intermedia* ATCC 25611^T, *Prevotella nigrescens* ATCC 33563^T, *Porphyromonas gingivalis* ATCC 33277^T, *Porphyromonas endodontalis* ATCC 35406^T, *Fusobacterium nucleatum* ATCC 25586^T, and *Camphylobacter rectus* ATCC 33238^T (data not shown). In addition, neither of our species-specific primers evidenced complete homology with the 16S rDNA of any of the other oral species in the current sequence databases. This indicates that similar results would probably have been obtained if we had evaluated pure cultures of additional species.

Under optimal conditions, PCR with the ChDC-Aa-F and ChDC-Aa-R primers proved able to detect as little as 4 fg of the purified genomic DNA of *A. actinomycetemcomitans* ATCC 33277 (Fig. 2A). The results of the specificity test of the PCR obtained using cell lysates were identical to those obtained using the purified genomic DNAs (data not shown). However, the results of the sensitivity tests of both of the PCR protocols involving bacterial cell lysates were 10-fold lower than those observed when using the purified genomic DNAs (data not shown). This may be attributable to an inhibition of the PCR by cellular components of the lysates, or by detergents present in the lysis buffer.

According to the comparison test, the sensitivity of our PCR primers (ChDC-Aa-F and ChDC-Aa-R) was 10-fold higher than the sensitivity of Ashimoto's PCR primers (Ash-Aa-F and Ash-Aa-R) when assessed with a 1.5 mM concentration of $MgCl_2$ (Fig. 2). In addition, the results of our specificity tests of Ashimoto's PCR primers (in 1.0 mM $MgCl_2$) and our PCR primers (in 1.5 mM $MgCl_2$)

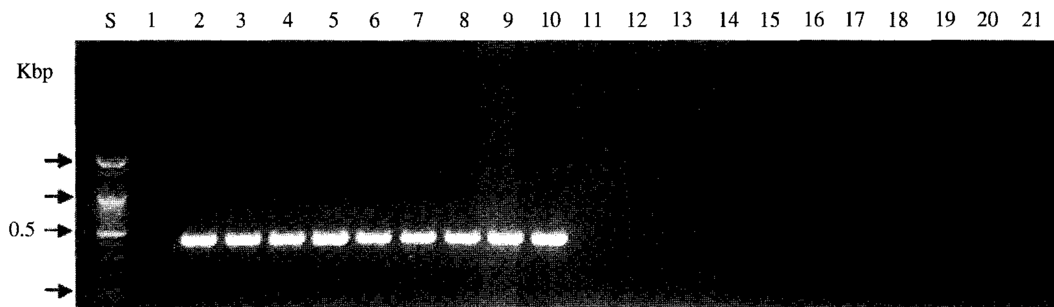

Fig. 1. Specificity test of PCR with the ChDC-Aa-F and ChDC-Aa-R primers and purified genomic DNA of the type strains. 4 ng of each bacterial genomic DNA were used as PCR templates. The PCR reaction products were electrophoresed on 1.5% agarose gel. Lanes: S, 100 base pair DNA ladders (Bioneer, Korea); 1, sterilized deionized water (negative control); 2, *A. actinomycetemcomitans* ATCC 43717; 3, *A. actinomycetemcomitans* ATCC 43718; 4, *A. actinomycetemcomitans* ATCC 33384^T; 5, *A. actinomycetemcomitans* ChDC A110 ; 6, *A. actinomycetemcomitans* ChDC A111; 7, *A. actinomycetemcomitans* ChDC A113; 8, *A. actinomycetemcomitans* ChDC A115; 9, *A. actinomycetemcomitans* ChDC A117; 10, *A. actinomycetemcomitans* ChDC A121; 11, *H. aphrophilus* ATCC 33389^T; 12, *H. paraphrophilus* ATCC 29241^T; 13, *H. paraphrophilus* ATCC 29242; 14, *H. parainfluenzae* ATCC 33392^T; 15, *H. parainfluenzae* ATCC 7901; 16, *H. aphrophilus* ChDC A1; 17, *H. aphrophilus* ChDC PV-F30; 18, *H. aphrophilus* ChDC PV-F36; 19, *H. aphrophilus* ChDC PV-F39; 20, *H. aphrophilus* ChDC PV-F41; 21, *H. paraphrophilus* ChDC A108.

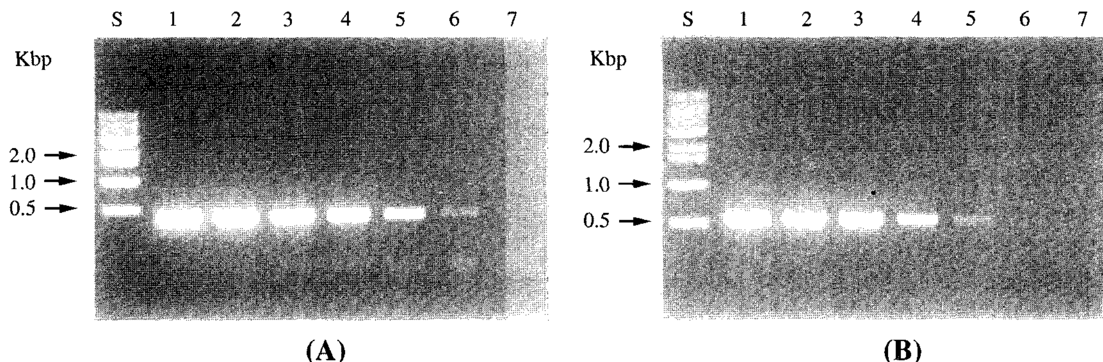

Fig. 2. Comparison of the sensitivity of (**A**) our primer set (ChDC-Aa-F and ChDC-Aa-R), with (**B**) Ashimoto's primer set, with the purified genomic DNA of *A. actinomycetemcomitans* ATCC 33384^T with a 1.5 mM concentration of $MgCl_2$. All PCR reactions were electrophoresed on 1.5% agarose gel. Lanes: S, 1 Kb DNA ladders (Bioneer, Korea); 1 through 7, purified genomic DNA serially diluted 10-fold, from 4 ng to 4 fg.

were identical (data not shown). Therefore, our PCR primers may be, on the whole, more useful in the detection and identification of *A. actinomycetemcomitans.*

In order to detect *A. actinomycetemcomitans* strains, several PCR primers were designed, based on 16S rDNA (Ashimoto *et al.*, 1996) and the leukotoxin gene (Tonjum and Haas, 1993). The PCR primers reported in this study will constitute a valuable addendum to the list of primers which are already available for the identification of *A. actinomycetemcomitans*. In our laboratory, two or three PCR primer sets are routinely used to identify bacteria grown on agar plates at the species level, and the resulting PCR data generally tend to be consistent with the 16S rDNA cloning and sequencing data.

In summary, the data gleaned from this study revealed that the PCR primer set, ChDC-Aa-F and ChDC-Aa-R, was both highly sensitive and highly specific. These primers might have potential applications in epidemiological studies. Also, they may prove useful in the diagnosis and monitoring of *A. actinomycetemcomitans* in dental plaque samples, both before and after periodontal treatments.

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