NOTES

Differentiation of Strains of *Actinobacillus actinomycetemcomitans* by Arbitrarily Primed Polymerase Chain Reaction

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The present study describes a method for amplifying DNA in *Actinobacillus actinomycetemcomitans* by using short, synthetic oligonucleotides of random sequence as primers in the polymerase chain reaction. Genomic DNA from each of 20 human isolates of *A. actinomycetemcomitans* was successfully amplified in a thermal cycler with a single synthetic primer (GGGTAACGCC) and reproducibly produced 14 different DNA amplification profiles (amplitypes). *A. actinomycetemcomitans* isolates from the same subject revealed the same amplitype. The arbitrarily primed polymerase chain reaction appears to be useful in characterizing human isolates of *A. actinomycetemcomitans* for studies of epidemiology and bacterial transmission.

Actinobacillus actinomycetemcomitans is a gram-negative coccobacillus that has been strongly implicated in the etiology of certain forms of periodontal diseases and has also been isolated from serious extra-oral infections (3, 10). Therefore, increasing attention has been paid to the epidemiology of *A. actinomycetemcomitans* infections, particularly, the route by which this microorganism infects the human oral cavity. Epidemiological studies of this nature require the ability to differentiate strains within the species. However, *A. actinomycetemcomitans* strain variability has been shown to be limited. Thus, biochemical assays for the fermentation of four key sugars has differentiated *A. actinomycetemcomitans* into 10 different biotypes (7), while antigenic analysis can distinguish among only five serotypes of this microorganism (1, 11). Restriction endonuclease analysis (4, 12) has also been used to track *A. actinomycetemcomitans* infections but, like the other methods, has been hampered by the limited number of easily distinguishable patterns (12). In contrast, DiRienzo and Slots (2) found differences sufficient for epidemiological studies of *A. actinomycetemcomitans* based on restriction fragment length polymorphisms. These conflicting data urge the development of novel molecular techniques with which the epidemiology of this important periodontal pathogenic species may be studied.

Recently, Welsh and McClelland (8) and Williams et al. (9) proposed a method to distinguish procaryotic (8) and eucaryotic (8, 9) organisms based on the variability in the genomes detected by using oligonucleotide primers of random sequence in the polymerase chain reaction (PCR). With this method, Welsh and McClelland (8) were able to demonstrate variability in genomic DNA from strains of *Streptococcus pyogenes* and *Staphylococcus aureus*.

Both serology and restriction endonuclease analysis-restriction fragment length polymorphism have so far demon-

strated limited variability among different *A. actinomycetemcomitans* strains. Since there is a need for other techniques in order to study the epidemiology of this periodontal pathogen, the purpose of the present study was to examine whether the arbitrarily primed PCR could be adapted to studies of *A. actinomycetemcomitans* and prove helpful in distinguishing among strains of this species.

Twenty thoroughly characterized isolates of *A. actinomycetemcomitans* from various sources were examined (Table 1). Four pairs of isolates (State University of New York

TABLE 1. *A. actinomycetemcomitans* strains used in this study

Strain ^a	Serogroup	Source
SUNYaB 30	a	Human dental plaque
SUNYaB 32	a	Human dental plaque
SUNYaB 75	a	Human dental plaque
SUNYaB 76	a	Human dental plaque
SUNYaB 114	a(c) ^b	Human dental plaque
SUNYaB 122	a	Human dental plaque
SUNYaB 334	a	Human dental plaque
SUNYaB 359	a	Human dental plaque
SUNYaB 13	b	Human dental plaque
SUNYaB 23	b	Human dental plaque
SUNYaB 54	b	Human dental plaque
SUNYaB 57	b	Human dental plaque
SUNYaB 62	b	Human dental plaque
SUNYaB 82	b	Human dental plaque
SUNYaB 333	b	Human dental plaque
FDCY4 ^a	b	Human dental plaque
SUNYaB 67	c	Human dental plaque
SUNYaB 69	c	Human dental plaque
SUNYaB 113	c(a) ^b	Human dental plaque
SUNYaB 360	c	Human dental plaque
ATCC 33384	c	Lung abscess

^a SUNYaB, State University of New York at Buffalo; FDCY, Forsyth Dental Center.

^b Cross-reactive strain.

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FIG. 1. Amplicypes generated with primer 1 in eight human serotype a (a), eight serotype b (b), and four serotype c (c) strains of *A. actinomycetemcomitans*. Lanes (in consecutive order from left to right): SUNYaB 30, 32, 75, 359, 76, 114, 122, and 334 (a); SUNYaB 13, 23, 54, 57, 62, 82, and 333 and FDC Y4 (Y4) (b); and molecular weight (M.W.) marker II (23.130, 9.416, 6.557, 4.361, 2.322, 2.027, 564, and 125 bp (from top to bottom) (Boehringer Mannheim, Indianapolis, Ind.), SUNYaB 67, 69, 113, and 360, molecular weight marker VI (2.176, 1.766, 1.230, 1.033, 653, 517, 453, 394, and 298 bp (from top to bottom) (Boehringer Mannheim) (c). SUNYaB 75–76, 13–54, 67–69 and 113–114 are pairs of strains isolated from each of four different subjects. Strains SUNYaB 13–54 and SUNYaB 23 were isolated from two siblings. (d) Amplicypes generated with primer 2 (GTTGCGATCC) in seven human serotype a strains of *A. actinomycetemcomitans*. Lanes (in consecutive order from left to right): SUNYaB 30, 32, 75, 359, 114, 122, and 334 and molecular weight (M.W.) marker VI. Strains 30 and 32 are from different patients, although they showed the same amplicype. This primer verified the identity of amplicypes in SUNYaB 30 and 32 and the distinction between SUNYaB 334 and SUNYaB 75–76 that was suggested by, but not clear from, the amplicypes generated by primer 1 as shown in panel a.

at Buffalo [SUNYaB] 75 and 76, 13 and 54, 67 and 69, and 113 and 114) originated in pairs from each of four human subjects, and another three strains, SUNYaB 13, SUNYaB 54, and SUNYaB 23, had been obtained from two siblings that suffered from localized juvenile periodontitis. Following culture on TSBV agar (6), isolates were confirmed as *A. actinomycetemcomitans* by colony morphology, Gram stain, catalase reaction, and rRNA analysis (5). The isolates were characterized by using serotype-specific antisera as previously described (11).

Each of the 20 *A. actinomycetemcomitans* isolates was cultured for 48 h in 100 ml of 1/2-strength brain heart infusion broth (Difco Laboratories, Detroit, Mich.) containing 5 mg of hemin per liter, 1 g of sodium bicarbonate per liter, and 10 µg of vitamin K₁ per ml in an atmosphere of 5% CO₂ in air at 37°C. The bacterial cells were harvested by centrifugation (7,000 × g for 15 min). The cells were lysed, and the DNA was isolated as previously described (12).

Each of five different 10-mer synthetic oligonucleotide primers (GGGTAACGCC, GTTGCATCC, CAGGCCCTTC, CAAACGTCGG, and TCTGTGCTGG) selected from a random primer kit (Operon Technologies, Alameda, Calif.) was used in the arbitrarily single-primed PCR, as well as the M13-40 forward sequencing primer (5'-d[GTTTCCCAGTCACGAC]-3') (Pharmacia-LKB, Piscataway, N.J.). Bacterial DNA (25 µl) was amplified in GeneAmp reaction tubes (Perkin-Elmer Cetus, Norwalk, Conn.) containing 10 mM Tris-Cl (pH 8.3), 50 mM KCl, 5 mM MgCl₂, 100 µM each dATP, dCTP, dGTP, and dTTP (Pharmacia-LKB), 0.2 µM primer, 0.5 U of *Taq* polymerase (Pharmacia-LKB), and 0.05 µg of genomic DNA. Amplifications were performed in a thermal cycler (Coy Manufacturing, Ann Arbor, Mich.) programmed as follows: one cycle of 5 min at 94°C, 2 min at 42°C, and 2 min at 72°C; 30 cycles of 1 min at 94°C, 2 min at 42°C, and 2 min at 72°C; and one cycle of 1 min at 94°C, 2 min at 42°C, and 10 min at 72°C, with the fastest available transition between temperatures. The *Taq* polymerase was added to each reaction after the initial 5-min denaturation. Amplification products were analyzed by electrophoresis in 1% submarine agarose gels, run at 45 V for 5 h, and detected by staining with ethidium bromide.

The reproducibility of the method was examined by comparing ampotypes obtained with three different preparations of target DNA from *A. actinomycetemcomitans* SUNYaB 57 (serotype b), SUNYaB 67 (serotype c), and ATCC 33384 (serotype c). The stability of the DNA preparation over time was tested by assaying the same DNA preparation obtained from these three strains in seven different arbitrarily primed PCRs performed monthly for 6 months. The stability of the ampotype over time was tested by comparing the ampotypes of *A. actinomycetemcomitans* strains originally isolated in 1979 (SUNYaB 67) and 1989 and stored in liquid nitrogen with fresh isolates obtained in 1991 from the same subject. All reactions were performed in duplicate.

Only one of the six primers (GGGTAACGCC) generated amplification products (17 to 23 bands) which were useful in discriminating among the *A. actinomycetemcomitans* strains (Fig. 1). The other primers either failed to generate any PCR products or produced only a small number of bands which were insufficient for distinguishing among all the test strains. In one case, another set of ampotypes generated with a second primer (GTTGCATCC) was used to verify a weak differentiation between *A. actinomycetemcomitans* SUNYaB 334 and SUNYaB75-76 made with primer 1 (Fig. 1a and d). However, in general, this second primer did not discriminate sufficiently among the other strains.

FIG. 2. Reproducibility testing of amplification of DNA polymorphisms in strain *A. actinomycetemcomitans* SUNYaB 57 (lanes 1 to 4) and ATCC 33384 (lanes 5 to 8). Lanes 1, 2, 5, and 6, reactions from the same day with the same DNA batch for each strain extracted in the spring of 1991; 3 and 7, amplification reactions from different days but with the same DNA batches as in lanes 1, and 2, 5, and 6, respectively; 4 and 8, reactions from DNA batches extracted from the same strains, SUNYaB 57 and ATCC 33384, respectively, in 1989 and kept frozen until this experiment. The amplifications shown in lanes 4 and 8 were run on days different from those for the other lanes.

Six amplification patterns were found among the eight serotype a (Fig. 1a and d) and serotype b (Fig. 1b) strains. Three different ampotypes were identified among the four serotype c strains. Each pair of *A. actinomycetemcomitans* strains that had been isolated from each of four patients (SUNYaB 13 and 54, 67 and 69, 75 and 76, and 113 and 114) exhibited in pairs identical ampotypes. *A. actinomycetemcomitans* SUNYaB 23 showed the same ampotype as SUNYaB 13 and 54. These strains, SUNYaB 23 and SUNYaB 13-SUNYaB 54, had been isolated from two siblings. Two of the 20 strains, SUNYaB 30 and 32, although isolated from two (to our knowledge) unrelated individuals, were shown to exhibit identical ampotypes. The identity of these two strains was also verified by comparing ampotypes generated by other primers (Fig. 1d).

With this single primer, the arbitrarily primed PCR was reproducible in different DNA preparations from the same strain and in repeated assays of the same DNA preparation (Fig. 2). DNA preparations from *A. actinomycetemcomitans* strains isolated more than 13 years ago exhibited the same ampotype as fresh isolates from the same patients. Arbitrarily primed PCR with the same DNA preparation assayed every month for 6 months generated identical ampotypes.

This study demonstrated that certain randomly selected nucleotide sequences may be used as primers in the PCR to amplify regions of genomic DNA from human isolates of *A. actinomycetemcomitans*. The DNA template concentration was critical in this technique. Too much DNA resulted in smears, while too little DNA resulted in nonreproducible ampotypes (data not shown). An initial denaturation cycle of 5 min prevented the development of amplification products during the initial ramp up to the denaturing temperature and enhanced reproducibility.

The present data are consistent with a single previous report indicating the presence of several genetically distinct groups within *A. actinomycetemcomitans* (2). The overall

amplotype diversity within *A. actinomycetemcomitans* and the finding of the same amplotype in related subjects point to the utility of this method in studying the epidemiology of *A. actinomycetemcomitans* infections.

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