Clonal Diversity of Oral *Eikenella corrodens* within Individual Subjects by Arbitrarily Primed PCR

CASEY CHEN^{1*} AND ATSUSHI ASHIMOTO^{1,2}

University of Southern California School of Dentistry, Department of Periodontology, Los Angeles, California 90089,¹
and Department of Oral and Maxillofacial Surgery, Faculty of Medicine, Tottori University, Tottori, Japan²

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The genetic diversity of 205 *Eikenella corrodens* isolates recovered from dental plaque, mucosal surfaces, and saliva of 24 subjects was examined by arbitrarily primed PCR. Twenty-two subjects were colonized by multiple clones (range, two to eight; mean, 3.7). This study demonstrates the utility of arbitrarily primed PCR for clonal analysis of *E. corrodens* and the multiclonal colonization of *E. corrodens* in the oral cavity.

Eikenella corrodens is a gram-negative facultative anaerobe found primarily in the oral cavity and upper respiratory tract (6). This organism is implicated as a human periodontopathogen and may also cause extraoral infections (6). *E. corrodens* displays a wide spectrum of genetic, phenotypic, and antigenic diversity. Variations in biochemical reactivity, colonial morphology, electrophoretic patterns of outer membrane proteins and lipopolysaccharides, and serology have been observed (1, 3, 5). Restriction endonuclease analysis of *E. corrodens* suggests the existence of a high number of clones in humans and that isolates from different subjects are usually genetically distinct (4). Colonization of genetically distinct *E. corrodens* in the same subject has also been documented in a study involving four subjects (4).

The presence of genetically distinct clones in the same subject may be significant for the understanding of *E. corrodens* pathogenicity. Previous studies, for lack of better materials and methods, generally assumed that individual subjects were colonized by a single *E. corrodens* clone, ignoring the possibility that strains with different pathogenicities may simultaneously colonize the oral cavity. It is possible that certain *E. corrodens* strains may be virulent and capable of causing infections while others are relatively harmless and constitute a component of the normal oral flora. Therefore, the aim of the present study was to extend the observations of *E. corrodens* multiclonal colonization in the human oral cavity by using arbitrarily primed PCR (AP-PCR).

E. corrodens isolates from a previous clinical study (3) were used in the present study. The detailed information about these subjects and bacterial isolates has been published previously (3). Briefly, 10 adult periodontitis and 7 juvenile periodontitis patients and 7 periodontally healthy subjects were included. Bacterial samples were obtained from supragingival plaque (six to eight samples per subject) and subgingival plaque (six to eight samples per subject), and one sample per subject was obtained from the dorsal surface of the tongue, the lateral surface of the tongue, the buccal mucosa, and the saliva. Pure cultures of each isolate were preserved in 10% skim milk (Difco Laboratories, Detroit, Mich.) at -80°C until used.

Genomic DNA from five *E. corrodens* strains was prepared by standard methods (8) and was used as the PCR template in preliminary studies to determine the appropriate amplification

conditions and primers. Subsequently, amplification templates were prepared directly from bacterial cells. Briefly, 2 to 20 bacterial colonies of *E. corrodens* were collected and suspended in 100 μl of distilled water, boiled for 10 min, and immediately chilled on ice. Alternatively, the bacterial cells were suspended in 100 μl of lysing buffer (0.45% Nonidet P-40, 0.45% Tween 20, 10 mM Tris-HCl [pH 9.0], 50 mM KCl, 0.1% Triton X-100, 1 mM MgCl_2 , and 0.06 mg of proteinase K per ml), incubated at 50°C for 1 h, boiled for 10 min, and then chilled on ice. All samples were centrifuged at $10,000 \times g$ for 5 min in a microcentrifuge to remove large debris and unbroken cells. The supernatant was used in AP-PCR immediately.

Twenty 10-base oligonucleotide primers (OPA-1 to OPA-20; Operon Technologies, Alameda, Calif.) were used in single-primer AP-PCR experiments. The base sequences (5' to 3') of the 20 primers (from OPA-1 to OPA-20, respectively) were CAGGCCCTTC, TGCCGAGCTG, AGTCAGCCAC, AATC GGGCTG, AGGGGTCTTG, GGTCCTGAC, GAAACGG GTG, GTGACGTAGG, GGGTAACGCC, GTGATCGCAG, CAATCGCCGT, TCGGCGATAG, CAGCACCCAC, TCT GTGCTGG, TTCCGAACCC, AGCCAGCGAA, GACCGC TTGT, AGGTGACCGT, CAAACGTCGG, and GTTGCGA TCC. Preliminary studies determined that eight primers (OPA-2, -4, -9, -10, -11, -13, -14, and -19) were useful for the purpose of AP-PCR clonal analysis of *E. corrodens*.

AP-PCR methods previously used for clonal analysis of *Actinobacillus actinomycetemcomitans* and *Porphyromonas gingivalis* (2, 7, 9) were adapted and used in this study. The amplification mixture (50- μl final volume) consisted of 5 μl of template, 0.2 mM (each) deoxynucleotide triphosphate (Pharmacia LKB Biotechnology, Piscataway, N.J.), 2.5 U of *Taq* DNA polymerase (Promega, Madison, Wis.), and 0.4 μM primer in 10 mM Tris-HCl (pH 9.0)–50 mM KCl–0.1% Triton X-100–4 mM MgCl_2 . Amplification was performed in a thermal cycler (PTC-100; MJ Research, Watertown, Mass.). The temperature profile was 94°C for 5 min for initial denaturation and 36 cycles of 94°C for 1 min, 32°C for 2 min, and 72°C for 2 min. After the last cycle, the amplified products were extended for another 10 min at 72°C . Twenty microliters of the amplified products was separated by agarose gel electrophoresis (1.5% agarose gel containing 0.5 μg of ethidium bromide per ml). After electrophoresis, the DNA banding patterns were visualized under UV illumination. One microgram of a 1-kb ladder (Life Technologies, Gaithersburg, Md.) was used as the molecular size marker.

Identical AP-PCR patterns of different intensities were generated with variable amounts of either genomic DNA (50 to

* Corresponding author. Mailing address: DEN 4335, USC School of Dentistry, Department of Periodontology, Los Angeles, CA 90089. Phone: (213) 740-1075. Fax: (213) 740-2194. Electronic mail address: ccchen@hsc.usc.edu.

TABLE 1. Clonal diversity of *E. corrodens* within subjects

Subject ^a	No. of isolates examined	No. of AP-PCR types ^b
D1	11	3
D2	13	4
D3	13	2
D4	16	7
D5	13	4
D6	4	2
D8	11	4
D9	4	2
D10	20	6
D11	8	4
J1	5	2
J2	10	2
J3	7	3
J4	6	5
J5	11	8
J6	12	7
J7	10	4
H3	6	1
H4	2	2
H5	8	1
H7	5	3
H8	4	2
H9	4	2
H10	2	2

^a D, adult periodontitis patient; J, juvenile periodontitis patient; H, periodontally healthy subject.

^b *E. corrodens* isolates from different subjects nearly always had different AP-PCR patterns. A total of 80 distinct AP-PCR patterns were found in 205 isolates from 24 subjects. Among these distinct AP-PCR patterns, only two were shared by isolates from different subjects (H3 and H8, J5 and J6).

500 ng) or cells (10 to 100 colonies) in AP-PCR. AP-PCR of lower numbers of cells (from one colony) yielded essentially identical but very faint banding patterns. AP-PCR using templates prepared in water or in lysing buffer yielded identical patterns. However, the quality of the banding patterns from the latter method was usually better. The reproducibility of the method was confirmed by performing AP-PCR analysis of the same samples on different occasions and also by AP-PCR analysis of templates prepared from different batches of cells.

AP-PCR of *E. corrodens* using primer OPA-4 yielded 3 to 10 strong DNA bands. These patterns were highly discriminative and were used throughout the experiments to distinguish *E. corrodens* isolates. Isolates with identical or nearly identical AP-PCR patterns generated with primer OPA-4 were subjected to amplification with one or more primers (OPA-2, -9, -10, -11, -13, -14, and -19) to verify the results.

Differences in the distribution of individual *E. corrodens* clones in supragingival, subgingival, and mucosa or saliva sites within each subject were analyzed by the chi-square test. One-way analysis of variance was used to compare the total numbers of clones identified within individuals and to compare the ratios of the number of clones to the number of isolates in individuals from the three subject groups. When the null hypothesis was rejected, the Tukey method for multiple comparison was used to determine the differences between individual groups.

A total of 205 *E. corrodens* isolates were recovered from samples for AP-PCR clonal analysis. These included 113 isolates from 10 adult periodontitis patients, 61 isolates from 7 juvenile periodontitis patients, and 31 isolates from 7 periodontally healthy subjects. The mean number of isolates re-

covered per subject was 8.5 (range, 2 to 20 isolates per subject) (Table 1). Thirty-two isolates (from 10 subjects) were catalase positive and two isolates (from 2 subjects) were lysine decarboxylase negative.

AP-PCR of *E. corrodens* typically yielded banding patterns consisting of 5 to 10 DNA bands which were highly discriminatory among strains. The AP-PCR patterns of eight *E. corrodens* isolates from an adult periodontitis subject (D11) are shown as an example (Fig. 1). Four distinct patterns were distinguished, indicating the presence of at least four different clones of *E. corrodens* in subject D11. Of the 24 subjects, 22 were colonized by multiple clones of *E. corrodens* (range, two to eight; mean, 3.7) (Table 1). The remaining two periodontally healthy subjects were each colonized by a single clone. The utility of AP-PCR for clonal analysis was supported by the identical results from using this method and from performing restriction endonuclease analysis of *E. corrodens* isolates from four subjects (D1, D4, H5, and H8) in a previous study (4).

Isolates from different individuals commonly displayed different AP-PCR patterns. A total of 80 AP-PCR patterns were identified among 24 subjects, with 2 AP-PCR patterns each shared by isolates from two different individuals. The observed genetic diversity of *E. corrodens* isolated from different individuals in this study is similar to that in our previous study (4). Because of the enormous genetic diversity of *E. corrodens*, no conclusion about the association between specific clones and periodontal disease can be made. In addition, we did not observe common AP-PCR patterns among catalase-positive *E. corrodens* isolates.

The average numbers of clones within each subject group were 3.8, 4.4, and 1.9 for adult periodontitis and juvenile pe-

FIG. 1. AP-PCR clonal analysis of *E. corrodens* from adult periodontitis patient D11, with OPA-2 (upper panel) or OPA-4 (lower panel) as the random primer. Lanes 1 and 11 of each gel contained DNA size markers. Lanes 2, *E. corrodens* type strain ATCC 23834; lanes 3, strain 514; lanes 4, strain 517; lanes 5, strain 508; lanes 6, strain 522; lanes 7, strain 524; lanes 8, strain 509; lanes 9, strain 513; lanes 10, strain 519. Strains in lanes 5 to 7 and lanes 8 to 10 exhibited identical AP-PCR profiles.

riodontitis patients and periodontally healthy subjects, respectively. There is a significant difference in the numbers of clones among three subject groups (analysis of variance, $P < 0.05$; Tukey test, significant differences between juvenile periodontitis patients and periodontally healthy subjects at the 0.05 level and between adult periodontitis patients and periodontally healthy subjects at the 0.1 level). We also compared the ratios of the number of clones to the number of isolates examined and found similar ratios among three subject groups (analysis of variance, $P > 0.05$). Therefore, the higher number of clones observed in diseased subjects may be explained by the higher numbers of isolates examined. Further study is necessary to determine whether a higher degree of clonal diversity is associated with the presence of periodontal disease.

Since we did not know a priori that different clones of *E. corrodens* may be recovered from a single sample, only one *E. corrodens* isolate per site was usually recovered for clonal analysis, in spite of the possibility that genetically distinct clones with indistinguishable colonial morphologies could exist. Occasionally isolates from the same samples with apparently different colonial morphologies were selected from primary cultures. In 15 instances (from nine adult periodontitis or juvenile periodontitis patients) in which multiple isolates were recovered from supra- or subgingival sites for clonal analysis, 10 samples each harbored two distinct clones. We also did not find a specific distribution pattern for any clones in sample sites within individual subjects (chi-square test, $P > 0.05$). Although we cannot be certain how often different clones might concomitantly colonize the same periodontal pockets, the data suggested a random distribution of individual *E. corrodens* clones in different oral sites and a consequential coexistence of different clones in the same sites.

In summary, this study demonstrates the utility of AP-PCR for clonal analysis of *E. corrodens* isolates. The presence of

multiple clones of *E. corrodens* in the oral cavity appears to be very common. No specific association between clones and various oral sites was found. The possibility that distinct clones may elicit different levels of humoral immune response in individuals is currently under investigation.

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