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Identification of *Actinobacillus actinomycetemcomitans* in Subgingival Plaque by PCR

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The purpose of this study was to assess the sensitivity and specificity of the PCR in detecting *Actinobacillus actinomycetemcomitans*. The PCR's detection capability was compared with those of three other methods: culture-enhanced PCR (CE-PCR), colony hybridization (CH), and conventional culture with presumptive biochemical identification. A 285-bp stretch of the leukotoxin gene *lktA* of *A. actinomycetemcomitans* was amplified by PCR with primers TT-15 and TT-16. For CH, the PCR product was labeled with digoxigenin and used as a hybridization probe. Nucleotide sequence analysis of the PCR product of *A. actinomycetemcomitans* 1D4 and 1664 and three clinical isolates revealed complete homology among the tested strains, with only one base substitution (at position 1344) in comparison with the published sequence. With artificially infected subgingival plaque, the detection limit of PCR for *A. actinomycetemcomitans* was 10^3 CFU/ml of plaque suspension. Culturing subgingival plaque on tryptic soy-serum-bacitracin-vancomycin agar prior to PCR (CE-PCR) improved the limit of detection to 10^2 CFU/ml. Analysis of subgingival plaque samples from 35 patients with periodontal disease and 10 periodontally healthy subjects revealed that CE-PCR and CH had the highest overall rate of *A. actinomycetemcomitans* detection (both 58%), followed by PCR and culture (both 42%). With CH as the "gold standard," the sensitivities of CE-PCR, PCR, and culture were 88, 65, and 58%, respectively; the specificities were 84, 89, and 79%, respectively. The CE-PCR provided acceptable positive and negative predictive values (≥70%) when the prevalence of *A. actinomycetemcomitans* varied between 30 and 70%. PCR alone provided comparable predictive values over a narrower range of prevalence rates (30 to 50%), while culture did not afford acceptable predictive values at any prevalence rate. PCR and CE-PCR were found to be superior to culture with presumptive biochemical identification and should be the preferred methods for the detection of *A. actinomycetemcomitans* in subgingival plaque.

Although *Actinobacillus actinomycetemcomitans* has been isolated from a variety of oral and extraoral infections, its prime habitat appears to be the oral cavities of periodontally diseased patients (29). By using culture techniques, *A. actinomycetemcomitans* has been found in more than 85% of patients with juvenile periodontitis and approximately 31% of patients with adult periodontitis (23); it rarely occurs in periodontally healthy children (16).

Recently, sufficient evidence has evolved to support the hypothesis of an exogenous infection with *A. actinomycetemcomitans* in some forms of periodontitis (6). The goals of periodontal therapy have consequently been directed towards eliminating *A. actinomycetemcomitans* from the oral cavity (27). Therefore, microbiological analyses to identify *A. actinomycetemcomitans* in subgingival plaque samples are becoming increasingly important for screening, treatment planning, treatment evaluation, and monitoring of periodontal diseases.

Conventional methods used to identify *A. actinomycetemcomitans* in subgingival plaque samples include culture techniques with biochemical testing (22), immunological assays (3), and DNA probes (19). These techniques, however, are of limited specificity and sensitivity and/or are time-consuming. Recently, the PCR (26) has been described as a technique to identify *A. actinomycetemcomitans*. Although PCR appears to have great potential in the microbiological diagnosis of periodontal diseases, there is insufficient information on the value of PCR in detecting *A. actinomycetemcomitans* in subgingival plaque. Therefore, the purpose of this study was to assess the sensitivity and specificity of PCR for the detection of *A. actinomycetemcomitans* in subgingival plaque.

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MATERIALS AND METHODS

Bacterial strains. *A. actinomycetemcomitans* ATCC 29523, ATCC 33384, 1D4, and 1644 were used as reference strains (courtesy of U. Göbel, Humboldt University Berlin, and G. Conrads, University Aachen). For validation of the PCR, the following additional strains were included: *Porphyromonas gingivalis* ATCC 33277, *Prevotella intermedia* ATCC 25261, *Fusobacterium nucleatum* ATCC 23726, *Campylobacter rectus*, *Eikenella corrodens*, *Capnocytophaga sputigena* ATCC 33123, *Staphylococcus aureus*, *Clostridium histolyticum*, *Pseudomonas aeruginosa*, *Escherichia coli*, *Legionella pneumophila*, *Streptococcus salivarius*, *Streptococcus mutans*, and *Lactobacillus lactis.*

PCR. The leukotoxin gene *lktA* was selected for the identification of *A. actinomycetemcomitans* because it has been shown that non-leukotoxin-producing and leukotoxin-producing strains previously tested harbor this gene (7, 10, 26). A 285-bp segment from the middle portion of *lktA* was amplified with primers TT-15 (5′-TCG CGA ATC AGC TCG CCG-3′) and TT-16 (5′-GCT TTG CAA GCT CCT CAC C-3′) (26).

Fifteen-microliter volumes of the bacterial suspensions were mixed with 25 μl of sterile distilled water, heated at 95°C for 10 min, and subsequently added to the PCR sample containing 30 pmol each of primers TT-15 and TT-16, 200 μM each of the four deoxynucleoside triphosphates, 5 μl of polymerase synthesis buffer (GeneAmp PCR Buffer II; Perkin-Elmer, Foster City, Calif.), and 2.0 U of *Taq* DNA polymerase for a final volume of 50 μl. Each sample was amplified by 30 cycles of 30 s at 95°C, 60 s at 65°C, and 60 s at 72°C. Eighteen microliters of each PCR product was subjected to agarose gel electrophoresis on an 1% agarose gel. The gels were stained with ethidium bromide (1 μg/ml) and assessed under UV light. Precautions as described by Kwok and Higuchi were used to prevent contamination (13).

To determine the detection limit of the PCR, a suspension of *A. actinomyce-*

temcomitans 1D4 was 10-fold serially diluted and subjected to PCR as described above. The inhibitory effect of bacteria other than *A. actinomycetemcomitans* in the PCR sample was tested by adding pure cultures of 10^7 organisms of different bacterial species (listed above) to the serial dilutions. In addition, subgingival plaque suspensions negative for *A. actinomycetemcomitans* as determined by PCR, culture-enhanced PCR (CE-PCR), colony hybridization (CH), and culture (C) were artificially infected with serial dilutions of *A. actinomycetemcomitans* 1D4. CE-PCR and CH are described below.

Nucleotide sequence analysis. The 285-bp *lktA* PCR products from *A. actinomycetemcomitans* 1D4 and 1664 and three clinical isolates from this study were excised after electrophoresis on a 1.0% agarose gel and purified with the Prep-A-Gene kit (Bio-Rad Laboratories, Richmond, Calif.) as described by the manufacturer. Two micrograms of the double-stranded PCR product was subjected to *Taq* cycle sequencing reactions by using the Prism Ready Reaction DyeDeoxy Terminator Cycle Sequencing kit (Applied Biosystems Inc., Darmstadt, Germany) according to the manufacturer's instructions, with minor modifications. Briefly, 9.5 μl of terminator premix and 4 U of *Taq* DNA polymerase (Amersham Laboratories, Little Chalfont, Great Britain), template DNA (2 μg), and 10 pM primer TT-15 were mixed in a 0.6-ml reaction tube, which was then filled with water to a final volume of 20 μl. The samples were overlaid with 1 drop of mineral oil, placed in a thermal cycler preheated to 96°C, and subjected to 25 cycles with the following parameters: 98°C for 1 s, 56°C for 15 s, and 60°C for 4 min. The cycle sequencing products were extracted once with phenol-chloroform and then ethanol precipitated. The resulting DNA pellets were dissolved in 4 μl of a mixture of formamide and 50 mM EDTA (pH 8.0) in a ratio of 5:1, denatured for 2 min at 90°C, and immediately cooled on ice. Separation of sequencing products was performed on 7% denaturing polyacrylamide gels in an automatic DNA sequencer (model 373A; Applied Biosystems, Weiterstadt, Germany). Sequence analysis was carried out with the Hitachi DNAsis Program, version 2.0 (Hitachi Software, San Bruno, Calif.).

Colony hybridization. The 285-bp fragment resulting from the amplification of the *lktA* gene of *A. actinomycetemcomitans* 1D4 with the primer pair TT-15 and TT-16 was labeled with digoxigenin and used as a hybridization probe. The probe was labeled by the PCR method described above but with substitution of a mixture of 130 mM dTTP and 70 mM digoxigenin-11-dUTP for dTTP.

All bacterial colonies were transferred to Zeta-probe GT membranes (Bio-Rad Laboratories) by replica plating from tryptic soy-serum-bacitracin-vancomycin (TSBV) agar (21) plates with 30 to 300 colonies and incubated for 4 to 5 days. After removal of the membranes, bacteria were lysed in 0.5 M NaOH containing 1.5 M NaCl and subsequently dried on Whatman 3MM filters. The above-described procedure was repeated twice before the membranes were placed for 15 min on Whatman 3MM filters soaked in 0.5 M NaOH containing 0.2% Triton X-100. Membranes were then neutralized on Whatman 3MM filters soaked in 1 M Tris-HCl containing 1.5 M NaCl (pH 7.5), incubated in twofold SSC buffer (twofold SSC is 300 mM NaCl plus 30 mM sodium citrate [pH 7.0]), air dried for 10 min, and baked at 80°C for 2 h. The filters were then washed at 50°C in SSC buffer containing 1% sodium dodecyl sulfate (SDS) and rubbed off with a sterile cotton swab. The filters were then washed twice in SSC buffer containing 0.1% SDS with gentle agitation at 50°C for 20 min. Finally, the filters were washed in SSC buffer alone. After the cell debris was removed, the filters were placed between two 3MM papers and directly used for prehybridization or air dried for storage (15). Hybridization and detection of *lktA*-positive colonies on the TSBV agar plates were performed by using a Boehringer Mannheim detection kit according to the manufacturer's instructions (2). Highly stringent hybridization conditions (>95%) were used to prevent hybridization of genes that had some homology with *lktA* (12), e.g., the leukotoxin gene from *Pasteurella haemolytica* and the alpha-hemolysin genes from *E. coli* and *Actinobacillus pleuropneumoniae*. To assess the detection limit of CH, 10-fold serial dilutions of *A. actinomycetemcomitans* 1D4 were added to plaque samples negative for *A. actinomycetemcomitans* (as determined by C, PCR, CE-PCR, and CH).

Detection of *A. actinomycetemcomitans* in subgingival plaque. Supragingival plaque was removed, and the supragingival area was dried with sterile gauze. By using a sterile curette, subgingival plaque samples were then taken from the four deepest periodontal pockets (9) of 22 patients with early-onset periodontitis and 13 patients with adult periodontitis. Plaque samples were also taken from four sites of 10 periodontally healthy subjects. The samples were pooled and then placed in 1 ml of reduced transport fluid (24). After dispersion of the bacterial suspension, one aliquot was saved for PCR analysis. The other aliquot was serially diluted 10-fold (dilution range, 10^{-1} to 10^{-3}), and 0.1 ml of the suspension was cultured on TSBV agar in an H_2-CO_2 atmosphere at 37°C for 4 to 5 days (Gas Pack Anaerobic System; BBL, Cockeysville, Md.). Undiluted and diluted plaque samples were cultured on three TSBV agar plates. On the first agar plate, small adherent colonies characteristic for *A. actinomycetemcomitans* were identified and enumerated (21). The presumptive biochemical identification included positive catalase and negative β-galactosidase reactions (1, 20). The second agar plate was used for CH, and the third plate was used for CE-PCR, i.e., colonies were washed off with 1 ml of sterile 0.85% NaCl and the suspension was analyzed by PCR.

TABLE 1. Detection of *A. actinomycetemcomitans* in subgingival plaque samples from 45 subjects simultaneously analyzed by PCR, CE-PCR, C, and CH

Result by CH[a]	No. of samples with indicated result by[b]:						Total
	PCR		CE-PCR		C		
	+	−	+	−	+	−	
+	17	9	23	3	15	11	26
−	2	17	3	16	4	15	19
Total	19	26	26	19	19	26	45

[a] +, *A. actinomycetemcomitans* detected; −, *A. actinomycetemcomitans* not detected.

[b] Sensitivities and specificities were as follows: PCR, 65 and 89%, respectively; CE-PCR, 88 and 84%, respectively; C, 58 and 79%, respectively.

RESULTS

Specificity of primers for detection of *lktA*. To assess the specificity of the PCR, the 285-bp PCR products from *A. actinomycetemcomitans* 1D4 and 1664 and three clinical isolates from this study were subjected to nucleotide sequence analysis. The sequences of all five tested strains were identical. Comparison with the published sequence of *lktA* (accession no. X16829) revealed only one base substitution at position 1344, with cytosine being replaced by thymine (11). However, this base substitution did not result in a change of the respective amino acid residue. The primers TT-15 and TT-16 shared no significant homology with other leukotoxin genes or any other genes listed in the EMBL data bank.

Sensitivity of primers for detection of *lktA*. For *A. actinomycetemcomitans* isolates, the detection limit of PCR was approximately 10^3 CFU/ml. Adding large numbers of bacteria other than the test organism (listed in Materials and Methods) did not reveal any inhibitory effect on the PCR result. The detection limit of PCR was also approximately 10^3 CFU/ml in subgingival plaque samples artificially infected with *A. actinomycetemcomitans*. Culturing subgingival plaque samples on TSBV agar prior to PCR (i.e., CE-PCR) improved the detection limit to approximately 10^2 CFU/ml of plaque suspension. CH demonstrated a detection limit of approximately 10^2 CFU/ml for plaque samples artificially infected with *A. actinomycetemcomitans.*

Detection of *A. actinomycetemcomitans* in subgingival plaque. A total of 45 pooled plaque samples from periodontally healthy or diseased subjects were simultaneously assessed for the presence of *A. actinomycetemcomitans* by PCR, CE-PCR, CH, and C. In the healthy group, only 1 of 10 subjects tested positive for *A. actinomycetemcomitans* by both CE-PCR and CH. For the remaining nine periodontally healthy subjects, *A. actinomycetemcomitans* was not detected by any of the methods used. In the group with periodontitis, *A. actinomycetemcomitans* was detected by PCR in samples from 19 patients (54%), by C in samples from 19 patients (54%), by CE-PCR in samples from 25 patients (71%), and by CH in samples from 25 of 35 patients (71%). The overall detection rates for all subjects were 42% for PCR and C and 58% for CE-PCR and C.

The sensitivities of CE-PCR, PCR, and C in detecting *A. actinomycetemcomitans* in subgingival plaque were 88, 65, and 58%, respectively, with CH as the "gold standard." In most cases, false-negative results for PCR and C occurred with plaque samples harboring less than 10^3 *A. actinomycetemcomitans* CFU/ml. The specificities for CE-PCR, PCR, and C were 84, 89, and 79%, respectively (Table 1).

Because the predictive values of a test depend on the prev-

TABLE 2. Positive and negative predictive values for the detection of *A. actinomycetemcomitans* by PCR, CE-PCR, and C in populations with different prevalences of *A. actinomycetemcomitans* infection[a]

Prevalence of A.a. (%)	PCR		CE-PCR		C	
	PV +	PV -	PV +	PV -	PV +	PV -
1	6	100	5	100	3	99
10	40	96	38	99	23	94
20	60	91	58	97	41	88
30	72	86	70	95	54	81
40	80	79	79	92	65	74
50	86	72	85	88	73	65
60	90	63	89	83	81	56
70	93	52	93	76	87	45
80	96	39	96	65	92	32
90	98	22	98	45	96	17
99	100	3	100	7	100	2

[a] Brackets indicate the range over which tests show a positive predictive value (PV+) of ≥70% or a negative predictive value (PV−) of ≥70%; shaded areas indicate the range over which tests show a PV+ of ≥70% and a PV− of ≥70%. The predictive values were calculated by using the following formulas, with sensitivity and specificity values obtained by comparing each test result with that for CH:

$$PV+ = \frac{\text{sensitivity} \times \text{prevalence}}{[\text{sensitivity} \times \text{prevalence}] + [(1 - \text{specificity}) \times (1 - \text{prevalence})]}$$

$$PV- = \frac{\text{specificity} \times (1\text{-prevalence})}{[\text{specificity} \times (1 - \text{prevalence})] + [(1 - \text{sensitivity}) \times \text{prevalence})]}$$

Values are expressed as percentages. A.a., *A. actinomycetemcomitans*.

alence of infection in a population, positive and negative predictive values were calculated for populations with different prevalences of *A. actinomycetemcomitans* infection (5). As shown in Table 2, CE-PCR provided positive and negative predictive values of 70% or greater at a prevalence of *A. actinomycetemcomitans* infection between 30 and 70%. The prevalence at which PCR showed predictive values of 70% or greater ranged from 30 to 50%, whereas C did not show comparable predictive values at any *A. actinomycetemcomitans* prevalence rate.

DISCUSSION

The study described here confirmed the high specificity of PCR analysis with primers TT-15 and TT-16 for detection of *lktA* in *A. actinomycetemcomitans* (7, 26). With the exception of one base substitution, the nucleotide sequence analysis of the PCR product revealed complete homology with the published sequence. Since the substitution of cytosine by thymine at position 1344 did not result in a different amino acid residue, it appears that the amplified *lktA* stretch in *A. actinomycetemcomitans* is highly conserved. Thus, the identification of *lktA* appears to be a good indicator for detection of *A. actinomycetemcomitans* in subgingival plaque.

The PCR's detection limit for *A. actinomycetemcomitans* was approximately 10^3 CFU/ml of suspension in pure culture, in a mixture with various laboratory strains, and in artificially infected subgingival plaque. The detection limit could be further improved to 10^2 CFU/ml of plaque suspension by applying CE-PCR, i.e., culturing subgingival plaque samples on TSBV prior to PCR. The detection limit for CE-PCR was similar to that for CH and was lower than detection limits previously reported for DNA probe analysis (10^3 cells per ml) (19), indirect immunofluorescence microscopy (10^3 cells per ml) (3), and bacterial concentration fluorescence immunoassay (10^6 cells per ml) (28).

A. actinomycetemcomitans could be detected more frequently in subgingival plaque by CE-PCR and CH than by C. This is consistent with other study results which have demonstrated that the culture technique may often fail to detect *A. actinomycetemcomitans* in subjects harboring this microorganism (3, 19). Consequently, in studies using culture techniques, the actual prevalence of *A. actinomycetemcomitans* in healthy subjects as well as in patients with various periodontal diseases may be higher than reported.

Compared with CH, CE-PCR revealed the highest degree of sensitivity (88%) for the detection of *A. actinomycetemcomitans* in subgingival plaque, followed by PCR (65%) and C (58%). The lower sensitivities of PCR and C were related to frequent false-negative results due to their poorer limit of detection. Compared with CH, the specificities of PCR (89%) and CE-PCR (84%) were slightly higher than the specificity of C (79%); PCR and CE-PCR demonstrated only a few false-positive results (4 and 7%, respectively). The high frequency of false-positive results (up to 77%) by PCR in detecting *Mycobacterium tuberculosis* in sputum, saliva, or water recently reported was related to cross-contamination of samples during processing (17). To prevent cross-contamination of samples for PCR, the sequence of operations from sample pretreatment to purification of DNA should be closely monitored.

The value of a microbiological test depends not only on its sensitivity and specificity, but also on the prevalence of infection with the target microorganism in the population. The lower the prevalence of infection, the more specific a test must be in order to be clinically useful (5). Compared with PCR and CE-PCR, the positive predictive value for C was considerably lower in subjects with a low (i.e., 40% or less) prevalence of *A. actinomycetemcomitans* infection. Consequently, PCR and CE-PCR appear to be superior to the culture technique in detecting *A. actinomycetemcomitans* in a population with a low prevalence of *A. actinomycetemcomitans* infection, such as periodontally healthy subjects (16), patients with adult periodontitis (23), and patients tested after periodontal therapy (18). Conversely, if there is a high prevalence of infection with a target microorganism, a microbiological test must be very sensitive to be useful. Therefore, for subjects with a high (i.e., 60% or greater) prevalence of *A. actinomycetemcomitans* infection, such as patients with early-onset periodontitis (23), CE-PCR appears to be superior to PCR and C in identifying *A. actinomycetemcomitans* in subgingival plaque.

Although the quantity of *A. actinomycetemcomitans* that has a metabolic or toxic impact on periodontal tissues is by and large unknown (14), previous study results suggest that there is low risk of periodontal breakdown at sites where *A. actinomycetemcomitans* represents less than 0.01% of the cultivable subgingival microflora (4). For PCR or CE-PCR, to detect *A. actinomycetemcomitans* at a level above 0.01% of the cultivable microflora would require plaque samples to contain at least 10^6 or 10^5 CFU/ml, respectively. Plaque samples usually contain between 10^2 and 10^7 microorganisms, depending on the quantity of subgingival plaque and the sampling method used. Thus, for maximum detection of *A. actinomycetemcomitans* a sampling method that removes a high proportion of the subgingival microflora, i.e., curettes, should be preferred (25). For quantification of *A. actinomycetemcomitans* in subgingival plaque by PCR to be possible, the technique used in this study needs to be further developed. Currently, CH appears to allow

adequate quantification of viable *A. actinomycetemcomitans* in subgingival plaque (8).

The results of this study suggest that PCR is a moderately sensitive and highly specific technique to detect *A. actinomycetemcomitans* in subgingival plaque. The sensitivity of PCR can be further enhanced by prior culturing of plaque samples, i.e., CE-PCR. The CE-PCR provided acceptable (≥70%) positive and negative predictive values when the prevalence of *A. actinomycetemcomitans* infection varied between 30 and 70%. PCR alone provided comparable predictive values over a narrower range of prevalence rates (30 to 50%), while C did not afford acceptable predictive values at any prevalence rate. PCR and CE-PCR were found to be superior to C with presumptive biochemical identification and should be the preferred methods for detection of *A. actinomycetemcomitans* in subgingival plaque.

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