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# Use of Synthetic Oligonucleotide DNA Probes for the Identification of *Bacteroides gingivalis*

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**Six different oligonucleotide probes complementary to the hypervariable regions of 16S rRNA of *Bacteroides gingivalis* were tested for specificity and sensitivity against 77 field strains of *B. gingivalis* and 105 strains of 12 other *Bacteroides* species. The data demonstrated that these probes were very specific (range, 0.85 to 1.00) and sensitive (1.00). Some limited cross-reactions with other *Bacteroides* species were observed. Four of these probes should be useful for rapid detection and identification of *B. gingivalis*.**

Periodontal diseases are a significant health problem. It has been difficult to study the causes of these diseases because of the complexity of the flora (more than 350 different species have been found in periodontal pockets [5, 13]), variations in the microbiota from site to site and time to time, and the necessary use of culture techniques and biochemical (phenotypic) criteria for identification. As a result of these difficulties, only organisms which represent more than 1% of the flora may be studied. Among the organisms which have been associated with various forms of periodontal disease, black-pigmented *Bacteroides* spp. are most often implicated (11, 13). These bacteria have been associated with other infections, particularly mixed infections of mucous membranes (11).

The taxonomy of black-pigmented *Bacteroides* spp. has undergone substantial change since 1980 (11, 19, 20), and eight species from human beings have been recognized. Recently, Shah and Collins proposed the reclassification of asaccharolytic black-pigmented *Bacteroides* spp. (*Bacteroides gingivalis*, *B. endodontalis*, and *B. asaccharolyticus*) to the new genus *Porphyromonas* (16). All three organisms have been implicated in human diseases: *B. gingivalis* in periodontal diseases; *B. endodontalis* in dental root canal infections; and *B. asaccharolyticus* in pelvic inflammatory disease, endometritis, and bite wound infections (11). Although the data are limited, *B. endodontalis* is seldom found in the gingival sulcus, and *B. asaccharolyticus* is almost never found in the oral cavity; thus, it appears that these species occupy a unique ecological niche in the human body.

Several years ago, it was demonstrated that whole-cell DNA probes could be valuable reagents for identification of pure cultures of *Bacteroides* spp. (12, 15). However, the DNA homology among the different species of oral *Bacteroides* spp. resulted in false-positive signals when subgingival plaque samples were analyzed directly (without primary culture) with whole-cell DNA probes, since two or more closely related species are usually present in such samples (B. J. Moncla, unpublished data).

Short oligonucleotide DNA probes complementary to the hypervariable regions of 16S rRNA provide an alternative to whole-cell DNA probes as a highly specific and sensitive means for detection of microorganisms (17). Probes for detection of six of the pathogens associated with periodontal disease have been developed. For a complete description of these probes, including those used in this work, see reference 3. We report here on a panel of probes for *B. gingivalis*, and as a prerequisite for their use on highly mixed samples from subgingival sulci, we have determined that they are highly specific and extremely sensitive.

## MATERIALS AND METHODS

**Organisms.** The following reference type strains were obtained from the American Type Culture Collection (ATCC) and verified biochemically: *B. gingivalis* ATCC 33277$^T$, *B. asaccharolyticus* ATCC 25260$^T$, *B. intermedius* ATCC 25611$^T$, *B. buccae* ATCC 33574$^T$, *B. bivius* ATCC 29303$^T$, and *B. disiens* ATCC 29426$^T$. Oral field strains of *Bacteroides* species were obtained from patients with various forms of periodontal disease at the University of Washington Periodontal Clinic. Subgingival plaque samples were taken from 5- to 10-mm-deep periodontal pockets with a Morse scaler, placed in 2 ml of prereduced Hanks balanced salt solution containing 10% (vol/vol) heat-inactivated horse serum, and transported to an anaerobic glove box within 10 min. The plaque samples were dispersed by a 5-s ultrasonic pulse from a microultrasonic cell disrupter (Kontes). Samples were serially diluted in the same medium and plated on prereduced blood agar plates for bacterial identification. The plates were incubated for 7 to 10 days in an anaerobic environment at 35°C, and organisms were identified to the species level as described previously (4–6). Nonoral *Bacteroides* species were provided by S. L. Hillier, Department of Obstetrics and Gynecology, University of Washington, Seattle.

**Preparation of samples and DNA probes.** Oligonucleotide DNA probes complementary to the hypervariable regions of the 16S rRNA of *B. gingivalis* ATCC 33277 were supplied by MicroProbe Corp. (Bothell, Wash.). Oligonucleotide probes 24 bases long were end labeled with [$^{32}$P]ATP by T4 polynucleotide kinase as described previously (10; for details, see reference 3). Probes were selected for use in these studies after a preliminary screening in which potential probes were screened against a large number of organisms common to the oral cavity. From these probes, the following six were selected for further study: Bg1, Bg3, Bg5, Bg6, Bg7, and Bg8.

Total nucleic acids from pure cultures of our clinical isolates were extracted for testing against the oligonucleotide probes as follows. Organisms were removed from the surfaces of blood agar plates, suspended to a density of 0.5

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McFarland units in sterile 10 mM EDTA–10 mM ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA), and stored at −70°C. Samples were thawed, and a 250-μl volume was removed and placed in a 1.5-ml plastic centrifuge tube; 125 μl of a 75% sucrose solution containing 4 mM EDTA and 4 mM EGTA was added, followed by 37.5 μl of a freshly prepared lysozyme solution (25% [wt/vol] sucrose, 50 mM Tris buffer [pH 7.5], 10 mM EDTA, 10 mg of hen egg white lysozyme per ml). Samples were briefly mixed with a Vortex mixer and incubated in a 37°C water bath for 15 min. A 37.5-μl volume of a 10% sodium dodecyl sulfate solution was added and mixed briefly. A 37.5-μl volume of a protease suspension (type XXX; Sigma Chemical Co., St. Louis, Mo.; 10 mg/ml in 10 mM Tris buffer [pH 8.0]–10 mM EDTA–0.5% sodium dodecyl sulfate) was added and mixed, and then samples were incubated at 37°C for 30 min. A phenol-chloroform-isoamyl alcohol (24:24:1) solution (450 μl) was added to the samples, vortexed briefly, and centrifuged at 12,000 × *g* in a microcentrifuge for 15 s. The aqueous top phase was removed and reextracted with 450 μl of phenol-chloroform-isoamyl alcohol, followed by an additional extraction with 450 μl of chloroform. The aqueous phase was removed and brought to a final concentration of 1.88 M ammonium acetate, and then 2 volumes of 95% ethanol was added. The samples were chilled at −20°C for 16 h and centrifuged for 10 min in a microcentrifuge; the resulting pellet was washed with an additional 1.5 ml of 95% ethanol and dried in a lyophilizer. The resulting nucleic acid pellet was suspended in 200 μl of 10 mM Tris (pH 7.6)–1.0 M EDTA. A 3-μl volume of the solution was analyzed in an agarose minigel system as described by Maniatis et al. (10) to verify the integrity of the nucleic acids. If high-molecular-weight nucleic acids could not be verified at this step, the samples were discarded and the process was repeated with a fresh sample.

**Immobilization and hybridization of nucleic acids.** Nucleic acid concentrations were estimated from inspection of minigels stained with ethidium bromide. Nucleic acid samples were brought to a final concentration of 10 mM PIPES buffer [piperazine-*N*,*N*′-bis(2-ethanesulfonic acid), pH 7.4; Calbiochem-Behring, La Jolla, Calif.] by addition of a 40× stock solution. Samples containing approximately 1 μg of total nucleic acids were heated in a boiling water bath for 90 s and then quickly chilled on ice. The samples were applied to Nytran filters by using a slot blot manifold filter system (Schleicher & Schuell, Inc., Keene, N.H.). Samples were washed once with 200 μl of 10× SSC (1.5 M NaCl, 0.15 M sodium citrate, pH 7.0) and baked at 80°C for 1 h (10). The filters were then soaked briefly at room temperature in hybridization solution (0.6 M NaCl, 90 mM Tris, 10 mM EDTA, 30% deionized formamide, 0.5% sodium dodecyl sulfate, 5× Denhardt solution [Sigma], pH 8.0). Then, 100 μg of hydrolyzed carrier *Torulopsis utilis* RNA was added per ml of hybridization buffer and the filters were gently shaken at 42°C for 2 h. A 5-ng sample of a $^{32}$P-labeled probe containing 1,000 to 1,200 cpm was added per ml of hybridization buffer and hybridized at 42°C for at least 4 h or overnight on a platform shaker. Filters were washed twice at 55°C (or 50°C for probe Bg8) with wash solution (0.09 M NaCl, 9 mM Tris hydrochloride, 1 mM EDTA, 0.1% sodium dodecyl sulfate, pH 8.0), and the hybridized probes were detected by autoradiography at different times.

A series of standards consisting of total nucleic acids from either *B. gingivalis* ATCC 33277$^T$ or *Escherichia coli* prepared as described above was used to normalize the test samples (see below). The concentration of nucleic acids in the standards was determined spectrophotometrically (10) and then diluted and slotted to give nucleic acid concentrations ranging from 10 μg to 5 ng per slot. The *B. gingivalis* nucleic acid dilution standard was used with each specific *B. gingivalis* probe to control for variations in specific activity, and the *E. coli* nucleic acid dilution standard was used with the clinical samples and universal probe to estimate the quantity of target material on the filters (see below).

**Normalization of filter-immobilized nucleic acids.** In slot blot experiments, the mass of the nucleic acids immobilized can vary for several reasons. (i) The concentration of total nucleic acids from each sample was not precisely known, since it was measured by detecting the sample nucleic acids by minigel electrophoresis, followed by ethidium bromide staining. This procedure was necessary, since the samples did not contain sufficient nucleic acid for spectrophotometric determination. (ii) The amount of nucleic acid actually immobilized on the Nytran filter can vary. For example, contaminating nucleases can fragment the RNA and prevent its binding to the filter. (iii) Any proteins remaining after the phenol extraction cocompete with the nucleic acids for the Nytran filter and hence may prevent their binding.

To control for variation in the amount of a sample actually immobilized on a Nytran filter, we normalized our data by using a universal probe which binds to a conserved 16S rRNA region present within all bacteria. This probe allows measurement of the total nucleic acids immobilized on each slot of the Nytran filter. By comparing the signal derived from each sample slot with the signals derived from the various diluted and immobilized standards of *B. gingivalis* or *E. coli* (see the immobilization protocol in the previous section), a direct estimate of the total amount of sample nucleic acid immobilized on the filter was obtained.

A further complication in the experiments was that the *B. gingivalis* probes were not labeled to the same specific activity after treatment with [γ-$^{32}$P]ATP and polynucleotide kinase. This variation in specific activity, however, could be compensated for by use of an internal control in the experiments. As described in the previous section, each filter contained filter-immobilized dilution standards of *B. gingivalis* nucleic acids which allowed the autoradiographic signal achieved with a *B. gingivalis* probe to be correlated with a specific amount of *B. gingivalis* nucleic acid. Thus, the relative specific activity of each probe could be roughly determined by comparing the signals achieved with various oligonucleotide probes.

In summary, detection of the total amount of nucleic acid immobilized on each slot of the Nytran filter and determination of the specific activity of each labeled oligonucleotide probe allowed normalization of our data. In turn, this normalization allowed comparison of results obtained with different lots of probes.

## RESULTS

A total of 77 strains of *B. gingivalis* and 105 strains of other *Bacteroides* species representing 12 species in all were tested to determine reactivity toward each of six different probes prepared from *B. gingivalis*. All of the DNA probes correctly identified all of the strains of *B. gingivalis* tested (sensitivity, 1.00) (Table 1). Two probes cross-reacted with other *Bacteroides* species. The Bg6 probe hybridized with 57% of the *B. asaccharolyticus* strains but none of the other *Bacteroides* species tested. Probe Bg7 demonstrated cross-reactions with *B. endodontalis*, *B. bivius*, and *B. disiens*.

Overall, the *B. gingivalis* probes were very specific and

TABLE 1. Analysis of MicroProbe oligonucleotide probes for *B. gingivalis*

| Organism tested (no. of isolates) | No. of isolates reacting with indicated probe[a] | | | | | |
|---|---|---|---|---|---|---|
| | Bg1 | Bg3 | Bg5 | Bg6 | Bg7 | Bg8 |
| *B. gingivalis* (77) | 77 | 67 (10)[b] | 68 (9)[b] | 65 (12)[b] | 77 | 71 (6)[b] |
| *B. asaccharolyticus* (14) | 0 | 0 | 0 | 8 | 0 | 0 |
| *B. endodontalis* (1) | 0 | 0 | 0 | 0 | 1 | 0 |
| *B. buccae* (4) | 0 | 0 | 0 | 0 | 0 | 0 |
| *B. capillosus* (5) | 0 | 0 | 0 | 0 | 0 | 0 |
| *B. denticola* (12) | 0 | 0 | 0 | 0 | 0 | 0 |
| *B. intermedius* I (4) | 0 | 0 | 0 | 0 | 0 | 0 |
| *B. intermedius* II (3) | 0 | 0 | 0 | 0 | 0 | 0 |
| *B. loescheii* (17) | 0 | 0 | 0 | 0 | 0 | 0 |
| *B. melaninogenicus* (8) | 0 | 0 | 0 | 0 | 0 | 0 |
| *B. bivius* (16) | 0 | 0 | 0 | 0 | 13 | 0 |
| *B. disiens* (16) | 0 | 0 | 0 | 0 | 4 | 0 |
| *B. ureolyticus* (5) | 0 | 0 | 0 | 0 | 0 | 0 |

[a] Sensitivity = number of true-positive results/(number of true-positive results + number of false-negative results); specificity = total true-negative results/(total true-negative results + total false-positive results). All of the probes had a sensitivity of 1.00. Bg1, Bg3, and Bg5 had a specificity of 1.00, and the specificities of Bg6, Bg7, and Bg8 were 0.93, 0.85, and 1.00, respectively.

[b] Number of samples not included in analyses because of loss of material; see text for details.

sensitive (Table 1). A sensitivity of 1.00 was observed for all probes, and specificity ranged from 0.85 (Bg7) to 1.00 (Bg1, Bg3, Bg5, and Bg8).

The appropriate use of the DNA probes and the controls is demonstrated in Fig. 1. The universal probe (μ′) was used to estimate the quantity of nucleic acid in each sample by comparison with the *E. coli* nucleic acid standard (lane A, slots 1 to 10). Under the conditions used, the μ′ probe detected 5 ng of nucleic acid. Next, the samples were evaluated for hybridization with the different probes by comparison of the sample with *B. gingivalis* nucleic acid dilution standards. For example, the quantity of nucleic acid found in the sample in lane B, slot 5, was estimated at 0.01 to 0.05 μg by using μ′ (compare lane B, slot 5, with the *E. coli* DNA standards, lane A, slots 8 and 9). When a duplicate filter was tested with the Bg7 probe, the reaction corresponded to that observed with 0.05 μg of *B. gingivalis* DNA (compare lane D, slot 5, with the reactions observed with the probe and the *B. gingivalis* standard, lane C, slot 8). Therefore, this sample was scored as positive. The sample in lane B, slot 4, had less than 0.01 μg of DNA (compare lane B, slot 4, with the *E. coli* standards in lane A, slot 9); since this value was less than the quantity detected by probe Bg7, the sample was not included in our tabulation of data (Table 1).

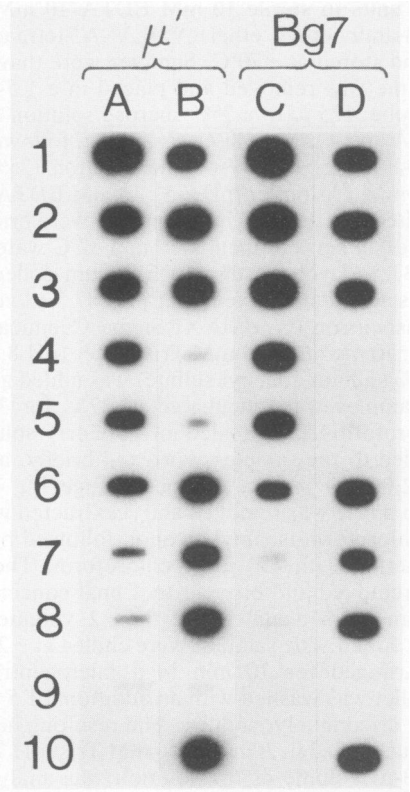


FIG. 1. Detection of *B. gingivalis* clinical strains by oligonucleotide DNA probes. Lane A contained *E. coli* nucleic acid in the following decreasing concentrations per slot: 10, 5, 2.5, 1.25, 1.0, 0.5, 0.1, 0.05, 0.01, and 0.005 μg (slots 1 to 10, respectively). Lane C contained *B. gingivalis* ATCC 33277 nucleic acid ranging in concentration from 10 μg to 5 ng. Lanes B and D are replicate filters that contained nucleic acids from samples of clinical strains of *B. gingivalis* prepared as described in the text. The probes used are indicated at the top. Lanes A and B were probed with the universal probe (μ′), and lanes C and D were probed with probe Bg7. Each pair of filter strips was exposed to X-ray film for different periods of time: lanes A and B, 3 days; lanes C and D, 2 h.

## DISCUSSION

Our understanding of periodontal diseases is limited by our ability to detect and identify the putative pathogens present. Culture techniques are the only available methods for such studies, but they are time-consuming and expensive and require highly trained technicians and specialized equipment. One potential solution to the problem of identification is the use of polyclonal and monoclonal antibodies for identification of these organisms. Such an approach has been used to study the roles of *Actinobacillus actinomycetemcomitans* and *Bacteroides* species (1, 21). However, such reagents may have one or more drawbacks; for example, they may not detect all members of a given species (2, 18). More recently, the concept of using bacterial enzyme activities as an indicator of disease has gained recognition (9). Periodontal disease activity is tested by assaying for specific enzymes believed to be important bacterial virulence factors, such as the trypsinlike enzyme of *B. gingivalis* (9) or the sialidase activity associated with many putative periodontal pathogens (B. J. Moncla and P. Braham, J. Dent. Res. **67**:179, 1988). Because it is virtually impossible to find an enzyme unique to one particular organism, such an approach would not be useful for detection of specific etiologic agents.

DNA hybridization can detect pathogens either from primary cultures or directly from clinical specimens, such as feces, pus, or vaginal washes (14). Whole-cell DNA probes are specific and reliable reagents for identification of *B. gingivalis* and other *Bacteroides* species (12, 15). These organisms generally have less than 35% interspecies DNA-DNA homology (7, 15); thus, whole-cell DNA probes work well for members of the genus *Bacteroides* in pure culture under controlled conditions of cell concentration and hybridization. Subgingival plaque samples usually contain many closely related species capable of hybridizing with whole-cell probes; therefore, when subgingival plaque samples are

analyzed directly, these interspecies homologies result in numerous false-positive signals for both *A. actinomycetemcomitans* and *Bacteroides* species (B. J. Moncla, unpublished data).

The data presented here suggest that the oligonucleotide probes complementary to the hypervariable regions of 16S rRNA provide an alternative to whole-cell DNA probes and conventional methods for study of pure cultures or plaque samples.

Use of the universal probe to detect target material in each sample is a significant advance in DNA probe technology, since it allows for reasonable interpretations of some weak reactions. Without these controls, we would have reported some false-negative results. It should also be noted that by washing filters at higher temperatures (wash temperature plus 10°C), it is possible to remove the test probes from the filters while retaining the target nucleic acid (unpublished data). The sample may then be tested with the universal primer probe to confirm the presence of target material or with other probes.

The large percentage of samples rejected for analysis because of loss of nucleic acid during storage (0 to 15%) may be surprising on first inspection. However, it should be noted that many of these samples had been stored for 2 or more years at −70°C before processing. In other studies, we have determined that there can be substantial loss of nucleic acid with time in the sample solution used (EDTA-EGTA) (unpublished data). This problem can be eliminated by limiting the storage time to 3 months.

The specificities of all of the probes examined were greater than 0.8, and four probes were 100% specific (Bg1, Bg3, Bg5, and Bg8). The reactions with probes Bg6 and Bg7 with non-*B. gingivalis* strains is interesting from a taxonomic standpoint, as it may suggest phylogenetic relatedness (8). The reactions of these probes with the nonoral isolates does not conflict with their applicability for detection of periodontal pathogens in that they are virtually never found in the gingival sulcus. Shah and Collins recently proposed the reclassification of *B. gingivalis*, *B. endodontalis*, and *B. asaccharolyticus* in a new genus, *Porphyromonas* (16). It is not surprising that 57% of the *B. asaccharolyticus* isolates reacted with probe Bg6, since these organisms form a phylogenetic group. It is interesting that the cross-reactions observed were restricted to this one probe. The cross-reaction observed between probe Bg7 and *B. bivius* (81% of isolates) and *B. disiens* (25% of isolates) is also interesting and may shed light on the changes in rRNA structures that occur with the evolution of the species, as well as the diversity of structure within a species.

Oligonucleotide probes were shown to be sensitive and specific for identification of *B. gingivalis* clinical strains. Use of the universal primer sequences as a probe not only makes it possible to control for loss of nucleic acids but will facilitate the study of highly mixed flora by allowing measurement of the total bacterial nucleic acids in a sample directly, as well as the total quantity of a given species.

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