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DEVELOPMENT AND CLINICAL APPLICATION OF DNA PROBE SPECIFIC FOR *PEPTOSTREPTOCOCCUS MICROS*

BY

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ABSTRACT

P. micros is thought to be an important pathogen in the etiology of certain inflammatory lesions, however, the role of this microorganism is uncertain due to the lack of rapid and reliable method to identify this species. The purpose of this study was to develop a DNA probe specific for the detection of *P. micros* in order to evaluate its prevalence in oral infectious lesions. The whole genomic DNA from *P. micros* was partially digested and inserted into the vector pUC 13. Four recombinant clones were selected, purified and screened against reference strains of *Peptostreptococcus* species to check the species specificity and then applied to clinical isolates. The sensitivity and specificity of the DNA probe for *P. micros* was 99.2% and 100%, respectively. *P. micros* could be detected in 7.2% of subgingival dental plaques from the patients with adult periodontitis and in 16.1% of the endodontic lesions with periapical pathosis. In the endodontic lesions, there was a good correlation between the clinical symptoms and the presence of *P. micros*. These data strongly suggested that the DNA probe can be useful in the detection of *P. micros* and that this microorganism is important in certain periodontal and endodontic lesions.

Key words: *Peptostreptococcus*, Hybridization, DNA probes, periodontal diseases, Bacterial infections

INTRODUCTION

Peptostreptococcus species are anaerobic gram-positive cocci which are frequently encountered in many infectious lesions. Little data are available, however, regarding these species in contrast to the relatively large amount of data which have been developed for the role of anaerobic gram-negative species in oral infections. Previous studies suggested that some species of *Peptostreptococcus* are concerned with the disease process. Recently, *Peptostreptococci* are thought to be etiologic in certain oral infections. *Peptostreptococcus* species are known to frequently inhabit the human oral cavity and these bacteria are present in increased numbers in the patients with periodontal disease as compared to the periodontally normal subjects (Moore et al. [1, 2]). Also, these microorganisms are found in large numbers in the endodontic lesion (Brook et al. [3]; Yoshida et al. [4]; Oguntebi et al. [5]; Konow et al. [6]; Williams et al. [7]) and in other infections throughout the body (Pien et al. [8]; Smith et al. [9]; Bourgault et al. [10]; Cofsky an Seligman [11]; Phelps and Jacobs [12]). One factor in pathogenicity of these microorganisms is its ability to degrade hyaluronic acid which is a major component of periodontal connective tissue controlling the permeability of epithelium and intercellular spaces (Last et al. [13]; Tam

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and Chan [14, 15]). Together, the data suggest that *Peptostreptococcus* species, especially *P. micros* play a role in certain oral diseases.

One reason for the paucity of information related to *Peptostreptococcus* species is the lack of rapid and reliable method to identify these organisms. Conventional biochemical characterization of these organisms is time-consuming and often difficult because of their fastidious nature (Wong et al. [16]; Lambert and Armfield [17]; Wells and Fields [18]; Socransky [19]). Recently, miniaturized substrate systems (Stargel et al. [20]; Starr et al. [21]) for the identification of anaerobic bacteria including *Peptostreptococcus* species have been used, however, results are often ambiguous and difficult to be interpreted. Antigenic relationship within *Peptococcus* and *Peptostreptococcs* species and the identification by serological methods have been explored to a limit extent (Graham and Falkler [22]; Porschen and Spaulding [23]) and coagglutination using protein A-bearing *Staphylococcus aureus* may be a future aid for a rapid presumptive identification of clinical isolates. Analysis of cellular fatty acids by gas-liquid chromatography has been of some value in differentiating between species (Lambert and Armfield [17]). Recently, high-performance liquid chromatography (HPLC) has improved the identification of *Peptostreptococcus* species based on analyzing changes in the composition of a define chemical medium, but further evaluation of the usefulness of this system is needed (Harpold and Wasilauskas [24]). These technical difficulties point to the need for a specific and rapid method to identify *Peptostreptococcus* species.

Recent advances in DNA technology enable the use of homologous DNA-DNA hybridization in detecting specific segments of DNA in microorganisms (Totten et al. [25]; Terpstra et al. [26]; Savitt et al. [30]; Schmidhuber et al. [31]). The purpose of the present study was to develop a DNA probe specific for *P. micros* and to compare its efficiency against the rapid assay systems in identifying clinical isolates from oral infections.

Materials and Methods

I. Bacterial strains and culture conditions

A total of 95 bacterial strains were used in this study (Table 1), including 83 strains isolated and identified by other investigators as species of *Peptostreptococcus*. In addition, *Escherichia coli* strain Jm 83 was used as a host and plasmid pUC 13 as a vector in cloning experiments. All strains were maintained on enriched tryptic soy agar (Difco Laboratories, Detroit, Mich.) supplemented with 5% sheep defibrinated blood, 5 μg/ml hemin and 1 μg/ml vitamin K_1. Prior to testing with the rapid assay systems, all strains were subcultured on Columbia blood agar supplemented with 5% sheep defibrinated blood according to manufacturer's recommendations. All cultures were incubated at 37°C in anaerobic environment consisting of 5% carbon dioxide, 10% hydrogen and 8% nitrogen using Forma Anaerobic Chamber (Forma Scientific, Marietta, Ohio). Batch cultures were grown in 1/2 strength Brain Heart Infusion (1/2 BHI) broth.

II. Isolation of genomic DNA and plasmid DNA

Genomic DNA was isolated and purified using a modification of the method of Silhavy et al. [32]. Briefly, 3 liters batch culture, grown in 1/2 BHI broth, was harvested at later log phase by centrifugation at 10,000 g for 10 minutes and resuspended in 50 mM Tris (pH 8.0), 50 mM EDTA, 50 mM NaCl. Cells were disrupted using Mickel-Nossal glass bead pulverizer (Quigley, Inc., Rochester, NY)

Table 1. Strains of Anaerobic and Aerobic Cocci Examined

Species	No. of strains	Source*	Location in Fig. 2
Anaerobic gram-positive cocci			
P. micros	30	1.2.3.5.	A1-C10
P. anaerobius	10	1.2.3.	D1-10
P. asaccharolyticus	12	1.3.4.6.	E1-10, I5-6, J8-9**
P. indolicus	7	1.3.	F1-7
P. prevotii	8	1.3.6.	F9-10, I1-4
P. magnus	11	1.3.	G1-9
P. tetradius	5	1.3.	H1-5
P. productus	1	1	F8
P. niger	1	1	J7
R. flavefacience	1	1	I8
C. evtactus	1	1	I9
S. ventriculi	1	1	J6
Aerobic gram-positive cocci			
M. luteus	1	1	J4
S. aureus	1	6	J3
S. mutans	1	6	J2
S. sanguis	1	1	J1
L. mesenteroides	1	1	J5
P. damnosus	1	1	I10
Anaerobic gram-negative cocci			
V. paruvla	1	6	I7

* Source: 1, ATCC; American Type Culture Collection, Rockville, Md., 2, G. Sundqvist, University of Umea, Sweden., 3, H. Jousimies-Somer, National Public Health Institute, Helsinki, Finland., 4, G. Dahlen, University of Goteborg, Goteborg, Sweden., 5, A. J. Van Winkelhoff, University of Vrije, Amsterdam, Holland., 6, J. J. Zambon, SUNY/Buffalo, N. Y..

** Same strains (15–6) were dot blotted at J8–9.

for 2-minute intervals for a total of 8 minutes (Marmur [33]). The cell suspension was mixed with a fresh lysozyme solution (1 mg/ml) and incubated for 15 minutes at 38°C. Sodium dodecyl sulfate was then added to give a final concentration of 2%. After incubation for 60 minutes at 37°C, proteinase K was added to the final concentration of 50 μg/ml and reincubated for an additional 90 minutes at 65°C. Cell lysate was extracted by adding an equal volume of buffered phenol followed by centrifugation at 10,000 g for 10 minutes. The aqueous layer was removed and phenol extraction repeated. One-tenth volume of 3 M sodium acetate and two volumes of ethanol were added and mixed by repeated inversion of tube. DNA was then removed by spooling it onto a glass rod and transferred to a clean tube. The extracted DNA was dissolved in 50 mM Tris (pH 7.5), 1 mM EDTA, 200 μg/ml RNase A solution by continuously rocking the tube overnight at 4°C. This was followed by extraction, using phenol and chloroform and centrifugation at 10,000 g for 10 minutes. The aqueous layer was saved and the precipitated DNA was completely dissolved in 15 mM NaCl (pH 7.0), 1.5 mM sodium citrate solution. Plasmid DNAs were also purified by the method described by Maniatis et al. [34] or by the mini-preparation procedure by Birnboim and Doly [35].

III. DNA labelling

The genomic and plasmid DNAs were labelled with ^{32}P by incorporating ^{32}P-d ATP (3,000 ci/mmol; ICN, Biomedicals, Inc., Irvine, CA) to a specific activity of 2 to 3×10^8 cpm per μg of DNA with nick translation kit (International Biotechnologies, Inc., New Haven, CT)(Maniatis et al. [34]; Birnboim and Doly [35]). The radiolabelled DNA probes were purified by chromatography on Nu-clean D 50 disposable spun columns (International Biotechnologies, Inc., New Haven, CT).

IV. Colony and DNA dot blot and hybridization with DNA probes

Bacterial cells and genomic and plasmid DNAs were absorbed onto nylon filters (Optiblot™ positively charged nylon membranes; International Biotechnologies, Inc., New Haven, CT, or Nylon membranes, charge modified; SIGMA Chemical Company, ST. Louis, MO) using a vacuum filtration apparatus (Bio-Dot; Bio-Rad Laboratories, Richmond, CA) or an appropriate amount of bacterial cells was removed from the surface of blood agar plates (ETSA plates) and simply applied to the nylon filter. The filters were air dried and treated with 10% sodium dodecyl sulfate for 3 minutes. The cells were denatured with 1.5 M NaCl-0.5 M NaOH for 5 minutes and neutralized with 1.5 M NaCl-0.5 M Tris. HCl (pH 8.0) for 5 minutes in order to lyse the cells in situ. The filters were baked at 80 °C for 2 hours to fix DNA onto the filter. Hybridization of filterbound DNA to ^{32}P-labelled DNA probes was carried out under the conditions described by Totten et al. [25] with some modifications. Briefly, nylon filters were incubated at 42°C for 4 hours in 10 ml of prehybridization solution. The stock prehybridization solution consisted of 50% formamide, 1% glycine, 50 mM sodium phosphate buffer pH 6.5, 5× SSPE and 5× Denhardt's solution containing 100 μg of denatured salmon sperm DNA per ml. The filters were removed, drained and added 2 ml of the hybridization mixture containing heat denatured ^{32}P-labelled DNA probes (5×10^6 cpm/ml) and further incubated overnight at 42 °C. The stock hybridization solution consisted of 50% formamide, 1% glycine, 20 mM sodium phosphate buffer pH 6.5, 5×SSPE, 1× Denhardt's solution, and 10% dextran sulfate containing 100 μg of denatured salmon sperm DNA per ml. The filters were washed three times with 2× SSC containing 0.1% sodium dodecyl sulfate for 15 minutes at room temperature and one time with 1× SSC containing 0.1% sodium dodecyl sulfate for 60 minutes at 68°C, air dried and exposed to an X-ray film (XAR film; Eastman Kodak Company, Rochester, NY) overnight.

V. Construction of DNA probes

In order to obtain the DNA fragments specific for only *P. micros*, 50 μg of the genomic DNA of *P. micros* were partially digested with the restriction enzyme *Sau* 3A (Boehringer Mannheim Biochemicals, Indianapolis, IN) and the DNA fragments ranging in size from 1 to 4Kb were collected using sucrose density gradient centrifugation at 26,000 rpm for 24 hours at 15°C in Beckman SW 27 rotor (Maniatis et al. [34]; Yasui et al. [37]). These DNA fragments were ligated to *Bam* H1 (Boehringer Mannheim Biochemicals, Indianapolis, IN) treated vector plasmid pUC 13 by using T4 ligase (Boehringer Mannheim Biochemicals, Indianapolis, IN). After transformation of *E. coli* strain JM 83, 48 white colonies cultured on X-gal plates containing ampicillin (50 μg/ml) were transferred to 300 μl volumes of L Broth in microcentrifuge tubes and incubated at 37°C overnight. Same amount of cell suspension were transferred onto two filters with the vacuum filtration apparatus. Screening was done with the ^{32}P-

labelled whole genomic DNA probes from *P. micros* (ATCC33270^T) as well as from *P. magnus* (ATCC15794^T) to check the cross reactivity. The plasmids containing *Sau* 3A fragments of *P. micros* DNA were further purified and screened against a number of related strains to check specificity and sensitivity.

VI. Clinical subjects

Eleven patients (36 sites) with various types of periodontitis and 24 patients (26 sites) with periapical pathosis were studied (Table 2). All subjects were generally in good health (*i. e.* lack of systemic disease), demonstrated a loss of periodontal attachment and visible gingival inflammation or presence of periapical pathosis by clinical and radiographical examination and absence of antibiotic medication at least 2 months prior to sampling. For the patients with periapical pathosis, following symptoms were also recorded. 1. Pain, which was defined as any spontaneous discomfort ranging from dull to sharp. 2. Swelling, which was defined as local redness, softtissue enlargement, or pain elicited by sight or finger touch at mucosa over tooth apex. 3. Sinus tract was determined by sight of purulent discharge. 4. Induced pain or discomfort by tooth percussion. 5. Foul odor, which was defined as the detection of an odor when pulp chamber was opened. 6. Exudate of either clear or purulent fluid through the root canal.

VII. Microbiological procedure

The sample site was first isolated with cotton rolls and allowed site to air dry. Supragingival plaque was then carefully removed using sterile cotton pellets. Sterile paper points (Extra Fine Absorbent Points, Johnson & Johnson, East Windsor, NJ) were then inserted into the periodontal pocket until resistance was met, kept in place for 10 seconds and removed. These paper points were placed in 3 ml of prereduced anaerobically sterilized (PRAS) Ringer's solution and vortexed on Vortex Genie Mixer (Scientific Products, McGawq Park, IL) at the maximum setting for 60 seconds. For the samples from root canals, all bacteriological procedures were performed using the system according to the method of Berg and Nord [38]. Briefly, the tooth to be sampled was thoroughly cleaned and isolated with a rubber dam, disinfected with a tincture of iodine, and dried. After opening the pulp chamber using sterile roud bur and rinsing the access canal with sterile saline, sterile paper points (Extra Fine Absorbent Points, Johnson & Johnson, East Windsor, NJ) were inserted into the pulp chamber canal, close to the apex, maintained for 10 seconds and removed. These paper points were then placed in 3 ml of prereduced anaerobically sterilized (PRAS) PY medium and vortexed for 60 seconds. The samples were immediately serially diluted in PRAS Ringer's solution or PRAS PY medium and plated on ETSA. These plates were incubated anaerobically at 37°C. After seven days of incubation, 36 to

Table 2. Number of Samples Collected

Sample site	Type of disease	No. of patients	No. of samples
Periodontal pocket			
	Adult periodontitis	9	31
	Localized juvenile periodontitis	1	3
	Rapidly progressive periodontitis	1	2
Root canal			
	Periapical pathosis	24	26

40 colonies from the periodontal pocket samples were randomly selected and subcultured. In addition, one of each colony morphotype was subcultured from the root canal samples.

VIII. Identification procedure

1. Biochemical method

All clinical isolates were grown aerobically and anaerobically to determine purity and aerotolerance. Gram-stains were performed on all obligate anaerobes. All anaerobic cocci were examined for catalase production, indole production, acid end products as determined by gas-liquid chromatography (GLC), and enhancement of growth by lactate. All anaerobic, gram-positive cocci were further identified using regular biochemical characterization (Holdeman et al. [39]; Sneath and Mair [40]), including API AN-Ident System and 20A Anaerobe System (API Laboratory Products Ltd., Quebec, Canada).

2. DNA hybridization method

Colonies from the subculture plates were streaked onto a nylon filter (Nylon Membranes, Charge Modified, SIGMA Chemical Company, St. Louis, MO), lysed with alkali, fixed and hybridized with the DNA probe specific for *P. micros*, as described above.

IX. Comparison of the results

If the results from the rapid assay systems conflicted with those of the DNA hybridization method, strains were further characterized by using the method described in VPI Anaerobic Manual (Holdeman et al. [39]) or Bergey's manual of systematic bacteriology (Sneath and Mair [40]).

X. Data analysis

Data was summarized and the patients were divided into two groups according to the presence or absence of *P. micros*. These results were then compared with the recorded symptoms. Fisher's exact test was used for statistical analysis.

Results

P. micros showed little cross reactivity with the other species of *Peptostreptococcus* by DNA-DNA homology studies (Fig. 1). However, because of the biochemical and morphological similarities of *P. micros* to *P. magnus*, it was decided to use the DNA probe from *P. magnus* for initial screening of clones. As a result, four clones were selected that demonstrated a strong reaction to homologous strain of *P. micros* while showing weak reactions to *P. magnus* (ATCC 15794^T). Fig. 1 shows the cross reactivity of the cloned plasmid DNA probes with each species of *Peptostreptococcus*. All four cloned plasmid probes hybridized only with homologous strain of *P. micros* and showed very high specificity compared with the whole genomic DNA probe of *P. micros* (ATCC33270^T). Each plasmid DNA was digested with *Eco* R1 and *Pst* 1 and electrophoresed on a slab-gel to determine the inserted fragment size which ranged from 1.0 to 2.6 Kb. The plasmid with the largest fragment size (clone 11G3; fragment size 2.6 Kb) was chosen for further testing with a number of related strains of other genera and reference strains of *Peptostreptococcus* species. Eighty-three reference strains of *Peptostreptococcus* species including 30 strains of *P. micros*, were used. Fig. 2 shows the autoradiogram of the hybridization reaction. The first three lanes on the left side (A-C) were dot blotted with the strains received as *P. micros*. The DNA probe (11G3) hybridized with 29 out of 30 strains. *P. asaccharolyticus* strains 15 and J8 (same strain as 15) showed a strong reaction on the autoradiogram and this strain was also estimated as *P. micros* by this method. These two strains (B2 and 15) that gave conflicting results were characterized by the conventional biochemical test. Strain B2 was speciated as *Streptococcus*

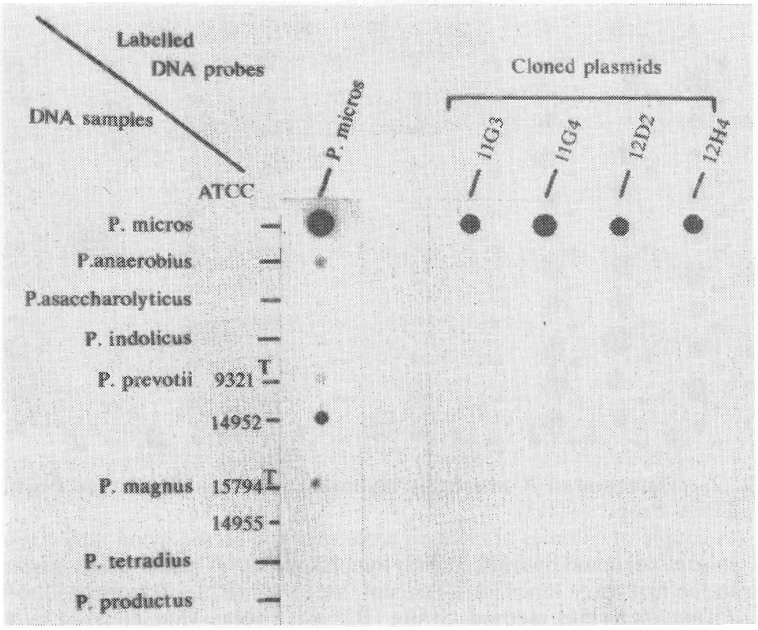

Fig. 1. Autoradiogram of Secondary Screening of Plasmid DNA Probes
One hundred ng of whole genomic DNAs from *Peptostreptococcus* species were dot blotted onto the filter and hybridized with whole genomic DNA probe from *P. micros* (ATCC 33270^T) and plasmid DNA probes from the selected four clones. The cross reactivity with the other species of *Peptostreptococcus* was eliminated with the plasmid DNA probes.

species and strain 15 was speciated as *P. micros*. As seen in this figure, positive and negative reactions are very clear. Table 3 shows the results of the identification of *P. micros* from the clinical isolates by the DNA probe method and the rapid assay systems. By Gram-stain observation, 436 colonies were identified as cocci. A total of 114 strains were identified as *P. micros* (12 strains were identified to the genus level) and a total of 270 strains were identified as other species (64 strains did not match with data base) by both methods. A total of 11 strains were identified as *P. micros* by only the DNA probe method, and 41 strains were identified as *P. micros* (27 strains to the genus level) by the rapid assay systems only. Strains which gave conflicting results were submitted to the conventional biochemical test. Eleven strains identified as *P. micros* by the DNA probe method only were all characterized as species of *P. micros*. Of the 41 strains

Table 3. Identification of *P. micros* -Comparison Between DNA Probe Method and Rapid Assay Systems

		DNA probe method	
		Positive*[1]	Negative*[2]
Rapid assay systems	+*[3](+)*[4]	102(12)	14(27)
	−*[5](N.F.)*[6]	1(10)	206(64)

*[1]: Spots could be seen on the autoradiogram.
*[2]: Spots could not be seen on the autoradiogram.
*[3]: Profile number which gave the name of *P. micros*.
*[4]: Profile number which gave the name of *Peptostreptococcus* species include *P. micros*.
*[5]: Profile number which gave another name.
*[6]: Profile number which was not found in the data base.

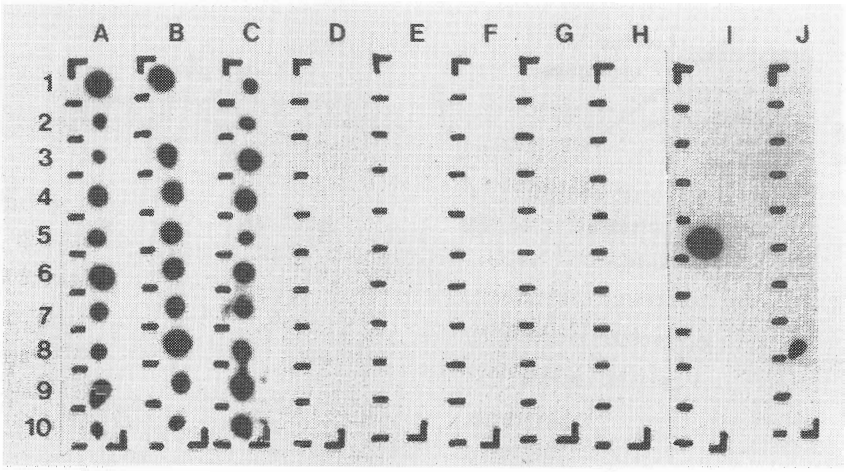

Fig. 2. Detection of *P. micros* by Hybridization with ^{32}P-labelled Plasmid DNA Probe (11G3)
A couple of colonies of bacteria were streaked on the nylon filters, lysed in situ, fixed and hybridized with the plasmid DNA probe. Each bacteria in the first three lanes (A-C) except one strain (B2) was estimated to be *P. micros* by this method. Strain (B2) was further characterized by the conventional biochemical test and it was not speciated as that species. I5 (the same strain was inoculated at J8) received as *P. asaccharolyticus* was found to be *P. micros* by this method. The samples which were dot blotted onto the filters are listed in Table 1.

which were identified as *P. micros* by the rapid assay systems alone, only one strain was characterized as *P. micros*. Of the other 40 strains, 2 strains were characterized as *P. productus*, 8 strains could not be categorized, 2 strains were non-viable, and the other 28 strains were phenotypically very close to the species of *P. micros*, *P. magnus*, or *P. prevotii* according to the procedures outlined in the VPI manual. The autoradiogram in Fig. 3 shows the DNA-DNA homology between the reference strains of *P. micros*, clinical isolates of *P. micros* that were verified by both methods and *P. micros* identified by the rapid assay systems alone. The whole genomic DNA probe from the type strain of *P. micros* (ATCC 33270^{T}) was hybridized with 4 reference strains and 5 strains identified as *P. micros* by both methods. There was no cross reaction with the other 5 strains which were identified as *P. micros* by the rapid assay systems alone. The DNA probe from *P. micros* (04D1) which was identified as that species by the rapid assay systems alone, was hybridized with all of this group of isolates and did not show cross reactivity with the other groups of *P. micros*. This group of bacteria was phenotypically very close but genetically remote from the typical *P. micros*. By using the DNA probes, 125 out of 126 clinical strains of *P. micros* were identified. By the rapid assay systems, 103 out of 126 strains were identified to the species level, 115 out of 126 strains were identified to the genus level and 40 strains were misidentified as *P. micros*. One strain which did not react was confirmed as *P. micros* by reexamination of DNA probe method.

The recovery proportion of *P. micros* from the various lesions is summarized in Table 4. From the pockets in the adult periodontitis patients, *P. micros* were reco-

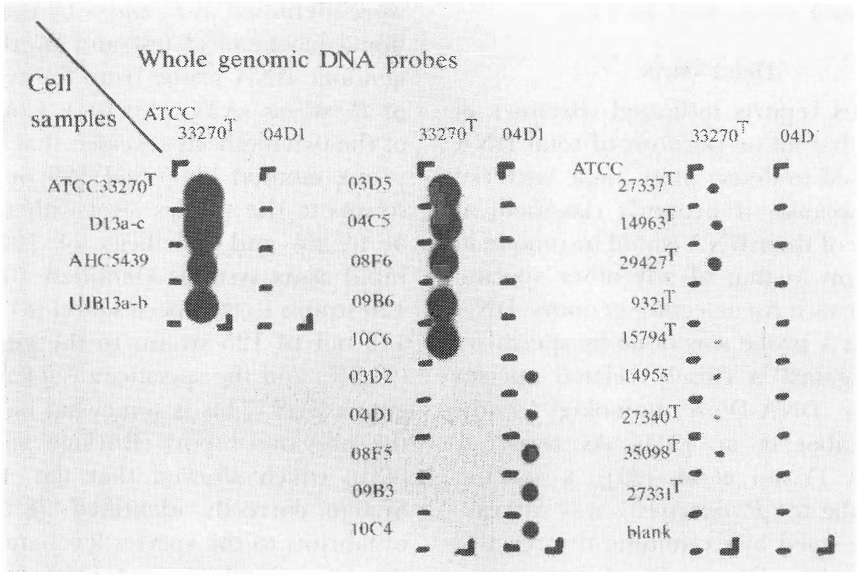

Fig. 3. DNA-DNA Homology Study Between Reference Strains and Clinical Isolates of *P. micros*
Each strain was dot blotted onto the filter and hybridized with the whole genomic DNA probe from a type strain of *P. micros* (ATCC33270^T) or a clinical isolate (04D1). Four strains in the first lane were received as *P. micros* from different institutes, the upper 5 strains in the second lane are clinical isolates which were verified as *P. micros* by both methods and the lower 5 strains were identified as that species by the rapid assay systems alone. Nine strains in the third lane are different species of *Peptostreptococcus* and *Peptococcus* (ATCC 27337^T; *P. anaerobius*, ATCC 14963^T; *P. asaccharolyticus*, ATCC 29427^T; *P. indolicus*, ATCC 9321^T; *P. prevotii*, ATCC 15794^T and ATCC 14955; *P. magnus*, ATCC 27340^T; *P. productus*, ATCC 35098^T; *P. tetradius*, ATCC 27331^T; *P. niger*).

Table 4. Percent of *P. micros* in Total Microflora from Various Lesions

Sample from	*P. micros*	
	Percent*	Site/total site
Adult periodontitis	7.2	23/31
Localized juvenile periodontitis	25.0	3/3
Rapidly progressive periodontitis	1.4	1/2
Periapical pathosis	16.1	14/26

*Percent of total viable cells.

vered from 23 out of 31 sites and the proportion was 7.2%. From the pockets in the localized juvenile periodontitis patients, it was recovered from 3 out of 3 sites and comprised 25.0%. From the pockets in the patients with rapidly progressive periodontitis, it was recovered from 1 out of 2 sites and constituted 1.4%. From the root canals with periapical pathosis, *P. micros* could be recovered from 14 out of

26 sites and comprised 16.1%.

Discussion

Previous reports indicated (Brenner et al. [41]) that all or portions of total DNA can be used to detect most single bacterial species because, if properly classified, at least 30% of their DNA would be unique in comparison to that of any other species. One approach for selecting genomic DNA for use as a probe was done by specificity testing against a closely related species based on DNA-DNA homology study (Schmidhuber et al. [31]). As reported previously (Yasui et al. [37]), a specific DNA probe for *P. anaerobius* was successfully developed by examining the reactivity against the other species of *Peptostreptococcus* using the information of DNA-DNA homology study. *P. micros* showed low level of cross reactivity with the various species of *Peptostreptococcus*, but the differentiation of *P. micros* from *P. magnus* has been difficult because these known phenotypic properties are similar except phosphatase production and electrophoretic pattern of soluble proteins, and both of these species are thought to be important pahogens (Sneath and Mair [40]). Because of the reasons described above, *P. magnus* was selected for the initial screening. Recombinant clones from *P. micros* did not show any cross reactivity with the other species of *Peptostreptococcus*. This "shotgun method" was successful, in part, because levels of DNA-DNA homology of *P. micros* with the other species of *Peptostreptococcus* were rather low. If the level of DNA-DNA homology between *P. micros* and the other species is high, modification of this method by the selective enrichment of specific restriction fragments with biotinylated probe and avidin-agarose gel method could be used (Schmidhuber et al. [31]; Welcher et al. [42]).

Of the 436 clinical isolates, 126 strains were identified as *P. micros* by the conventional biochemical test and by the whole genomic DNA probe from the type strain of *P. micros* (ATCC33270^T). Comparison of the two methods revealed that the DNA probe method identified 125 out of 126 strains to the species level with sensitivity of 99.2% and specificity of 100%. The rapid assay systems identified 103 out of 126 strains to the species level (81.7%) and 115 out of 126 strains to the genus level (91.3%) and the specificity is 74.2% at the genus level. This is somewhat better than the previous report (Burlage and Ellner [43]), which showed that the AN-Ident System correctly identified 78% of the organisms to the species level and 88% to the genus level. One possible explanation for the differences is that the types of the bacteria examined were quite different. In the present study, all the test bacteria were gram-positive cocci while in the previous study most were Bacteroides species with very few gram-positive cocci (9 strains). Once a pure culture inoculum becomes available, these two rapid assay systems were quite easy to get results. The AN-Ident System can identify an anaerobic isolate within 4–5 hours while the API 20 A system requires more than 48 hours. The fact that the color of the substrate often gave borderline reactions (Burlage and Ellner [43]) which can lead to misidentification. Compared with the rapid assay systems, relatively small amount of bacteria is enough to identify this species by the DNA probe method. The rapid assay systems require cell numbers greater than those of a No. 3 McFarland for API 20A Anaerobe System and a No. 5 McFarland for AN-Ident Systm. This translates into an area of almost half a standard size agar plate. The DNA probe method, in contrast, requires only a single bacterial colony directly from the primary culture plate. However, a significant drawback is the

necessity for ^{32}P in the DNA hybridization method. This radioisotope gives the assay a high level of specificity and sensitivity, but it has problems, for instance, a short functional half-life, the expense and radioisotope disposal problem and limit of the use of this technique for routine application in clinical settings (Bialkowska-Hobrzanska [27]). Recently several nonisotopic labelling methods were developed (Chollet and Kawashima [44]; Forster et al. [45]; Langer et al. [46]), but these methods often show severe background and further improvement is required to apply this method.

Interestingly, 41 strains -almost one-fourth of the *P. micros* found in the clinical samples- were identified as *P. micros* by the rapid assay systems alone. Most of these strains showed low level of DNA-DNA homology with the type strain of *P. micros* and also did not show cross reactivity with the whole genomic DNA from type strain of *P. prevotii* (ATCC 9321^T) and *P. magnus* (ATCC 15794^T) (data not shown). Phenotypically, this group of non-reacting bacteria was similar to *P. micros, P. magnus,* or *P. prevotii*. They were asaccharolytic and produced a small amount of acetic acid and butyric acid as determined by gas-liquid chromatography. By the AN-Ident System, the only difference between this group of bacteria and the typical *P. micros* was lack of alkaline phosphatase production. Previous studies described that *P. prevotii* (ATCC 9321^T) (Harpold and Wasilauskas [24]; Ezaki et al. [47, 48]) showed variability in sugar utilization, urease, esterase, β-glucosidase positive in contrast to clinical isolates of *P. prevotii*. Furthermore, DNA-DNA homology between the type strain and these clinical isolates ranged from 35 to 73%. Taken together, the group of strains non-reactive with the DNA probe may represent either a distinct DNA homology group or a new species.

As previously reported, *P. micros* is frequently encountered not only in periodontal pockets but also in infected root canals with periapical pathosis where it is thought to be a predominant species. As shown in this study, *P. micros* can be recovered in high proportions from both types of lesions. It was found in a proportion as high as 7.2% in the periodontal pockets of adult periodontitis patients and 16.1% in the root canals with periapical pathosis. *P. micros* can also be recovered from the subgingival dental plaques with rapidly progressive periodontitis and with localized juvenile periodontitis. In the latter, almost one-fourth of the anaerobic bacteria were identified as *P. micros*. Our findings in the adult periodontitis patients are consistent with the previous reports that *P. micros* was founded in proportion of 4.45% and 4.3% in the subgingival plaque from persons with severe and moderate periodontitis, respectively, in the studies by Moore et al. [1, 2]. *P. micros* was included in a group of 22 bacterial species associated with the periodontal disease in these studies (Moore et al. [49]). Dzink et al. [50] also reported in their study, if any species or combination of species including *A. actinomycetemcomitance, B. forsythus, B. gingivalis, B. intermedius, W. recta,* or *P. micros* was present in a lesion in increased numbers, the likelihood of that site showing activity increased dramatically. In the endodontic lesion, *P. micros* is frequently encountered and can comprise a high proportion of the microflora. In an experimental animal model, Sundqvist et al. [51] found that the periapical inflammation could be induced by the specific combination of bacteria including *B. melaninogenicus, B. asaccharolyticus,* and *P. micros*.

Table 5 shows the correlation between the presence of *P. micros* and clinical symptoms. Previously, Sundqvist reported

Table 5. Correlation Between the Presence of *P. micros* and Various Symptoms

Symptom	No. of patients with *P. micros*(14 patients)	No. of patients without *P. micros*(12 patients)	Total No. of patients (26 patients)	P
Pain	5	3	8	N.S.
Swelling	8	2	10	<0.05
Sinus tract	7	0	7	<0.005
Pain/or sinus tract	10	3	13	<0.05
Percussion	10	3	13	<0.05
Odor	2	3	5	N.S.
Exudate	8	4	12	N.S.

N.S.=Not significant.

a 100% correlation of pain with the presence of *B. melaninogenicus* and Michael et al. also reported the relationships between *B. melaninogenicus* and the presence of clinical symptoms. The present study shows significant correlations between the presence of *P. micros* and swelling, sinus tract, pain plus sinus tract and sensitivity on percussion. These results strongly suggest that *P. micros* play an important role in the endodontic lesions. But, there is also a possibility that number of bacteria simply arose in the response to changed environment cannot be neglected (Moore et al. [2]).

In conclusion, the DNA probe can be used to detect *P. micros* with levels of sensitivity and specificity higher than that seen using the rapid assay systems. The high prevalence and proportion of *P. micros* in both the periodontal pockets and root canals with periapical pathosis point to the importance of this microorganism in these diseases.

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