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Use of checkerboard DNA–DNA hybridization to study complex microbial ecosystems

S. S. Socransky, A. D. Haffajee, C. Smith, L. Martin, J. A. Haffajee, N. G. Uzel, J. M. Goodson
Department of Periodontology, The Forsyth Institute, Boston, MA, USA

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It has been difficult to conduct large scale studies of microbiologically complex ecosystems using conventional microbiological techniques. Molecular identification techniques in new probe-target formats, such as checkerboard DNA–DNA hybridization, permit enumeration of large numbers of species in very large numbers of samples. Digoxigenin-labeled whole genomic probes to 40 common subgingival species were tested in a checkerboard hydridization format. Chemifluorescent signals resulting from the hybridization reactions were quantified using a Fluorimager and used to evaluate sensitivity and specificity of the probes. Sensitivity of the DNA probes was adjusted to detect 10^4 cells. In all, 93.5% of potential cross-reactions to 80 cultivable species exhibited signals <5% of that detected for the homologous probe signal. Competitive hybridization and probes prepared by subtraction hybridization and polymerase chain reaction were effective in minimizing cross-reactions for closely related taxa. To demonstrate utility, the technique was used to evaluate 8887 subgingival plaque samples from 79 periodontally healthy and 272 chronic periodontitis subjects and 8126 samples from 166 subjects taken prior to and after periodontal therapy. Significant differences were detected for many taxa for mean counts, proportion of total sample, and percentage of sites colonized between samples from periodontally healthy and periodontitis subjects. Further, significant reductions were observed post therapy for many subgingival species including periodontal pathogens. DNA probes used in the checkerboard DNA–DNA format provide a useful tool for the enumeration of bacterial species in microbiologically complex systems.

Key words: bacteria; DNA probes; microbial ecology; microbial ecosystems; microbiology; sensitivity; specificity; subgingival plaque

Sigmund S. Socransky, DDS, Department of Periodontology, The Forsyth Institute, 140 The Fenway, Boston MA 02115, USA
Tel.: +1 617 8928282;
fax: +1 617 2624021;
e-mail: ssocransky@forsyth.org
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The study of infectious diseases has traditionally focused on one or a small number of pathogens in a given infectious disease. Even when samples are taken from areas where complex mixtures of species coexist, emphasis has been placed on seeking a limited number of likely pathogens from that site. The remaining organisms are often considered to be 'normal flora'. In most instances such species might well be host-compatible, common residents of the sampled site; however, in some instances, these species might contribute to the pathogenesis of the observed condition. In addition, the absence of some host-compatible species may be as important in disease initiation or progression as the presence of one or more pathogenic species. Examination of complex mixtures of microorganisms has been delayed by at least two factors. The first is the tradition of focusing on a small number of species thought to be pathogenic and the second is the lack of useful, rapid identification/enumeration techniques to evaluate large numbers of bacterial species in large numbers of samples taken from areas where complex microbiotas exist. In the past decade, the introduction of rapid techniques, such as DNA probes, to identify bacterial species in samples of subgingival plaque has extended our knowledge of periodontal microbial ecology and the effects of periodontal treatment on the composition of the subgingival microbiota. These techniques have been employed to comprehensively examine the composition of dental plaque in health and disease (5, 13, 28, 29) and to perform studies seeking associations between plaque bacteria and local and systemic factors (14, 24) as well as studies evaluating the changes that occur in plaque composition as a result of periodontal therapy (6, 9, 10, 12, 15, 30).

Whole genomic DNA probes have been used extensively in studies evaluating the composition of subgingival plaque (1, 5, 13, 16–19, 22, 28, 29) and the microbiota associated with endodontic lesions (11, 20, 26). Whole genomic probes are constructed using the entire genome of a bacterial species as the target. One of the criticisms of these probes is that the use of the entire genome may increase the probability of cross-reactions between species because of common regions of DNA among closely related species. Other concerns have been that the whole genomic DNA probes might not detect all strains of a given species and that the probes would have a low sensitivity in terms of the numbers of cells that they detect. Investigations at The Forsyth Institute, however, using whole genomic DNA probes have indicated that many of the concerns regarding their use are unjustified or can be overcome.

DNA probes can be very effective for the detection of bacterial species, but when employed in the typical format, only limited numbers of probes can be employed to enumerate relatively large numbers of samples. Checkerboard format procedures, whether employing direct or reverse hybridization procedures, can extend markedly the number of samples evaluated for a wide range of bacterial species. The checkerboard DNA–DNA hybridization technique was first described in 1994 (25). The purpose of the present manuscript is to provide an update on the utility of this technique and to describe its sensitivity and specificity and demonstrate techniques that can be used to optimize the use of DNA probes in the identification of bacterial species in mixed populations. The method can be used, with appropriate modification, for samples from different sites in nature. This manuscript will describe methods optimized for the examination of subgingival plaque samples.

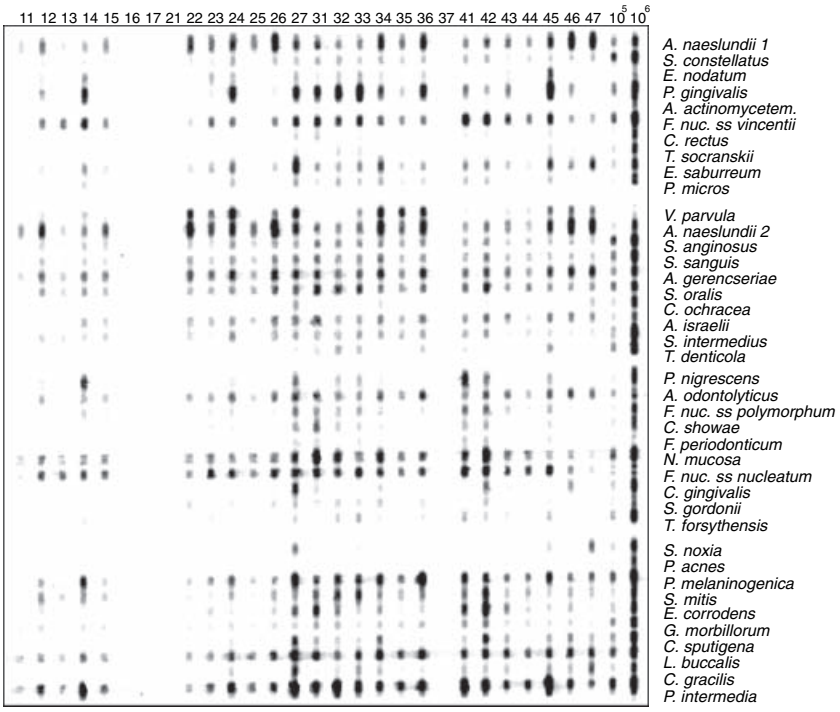


Fig. 1. Example of checkerboard DNA–DNA hybridization being used to detect 40 bacterial species in 28 subgingival plaque samples from a single patient. The vertical lanes are the plaque samples numbered from 11 (right maxillary central incisor) to 47 (right mandibular second molar). In this subject, teeth 16, 17, 21, and 37 were missing. The two vertical lanes on the right are standards containing either 10^5 or 10^6 cells of each test species. The horizontal lanes contained the indicated DNA probes in hybridization buffer. A signal at the intersection of the vertical and horizontal lanes indicates the presence of a species. The intensity of the signal is related to the number of organisms of that species in the sample. In brief, samples of plaque were placed into individual Eppendorf tubes and the DNA released from the microorganisms by boiling in NaOH. After neutralization, the released DNA was transferred to the surface of a nylon membrane using the 30 channels of a Minislot device (Immunetics). The DNA was fixed to the membrane by ultraviolet light and baking and placed in a Miniblotter 45 (Immunetics) with the lanes of DNA at right angles to the 45 channels of the Miniblotter device. Whole genomic DNA probes labeled with digoxigenin were placed in hybridization buffer into 40 of the lanes and hybridized overnight. After stringency washing, the signals were detected using phosphatase-conjugated antibody to digoxigenin and chemifluorescence substrates. Signals were compared to the standards using a Storm Fluorimager and converted to counts.

Material and methods

Bacterial strains and growth conditions

The 40 reference strains used for the preparation of DNA probes are listed in Fig. 1. The majority of strains were grown on Trypticase soy agar supplemented with 5% defibrinated sheep blood (Baltimore Biological Laboratories (BBL), Cockeysville, MD) with the following exceptions. *Tannerella forsythensis* was grown on Trypticase soy agar supplemented with 5% sheep blood and 10 μg/ml N-acetylmuramic acid (Sigma Chemical Co., St. Louis, MO). *Porphyromonas gingivalis* was grown on Trypticase soy agar supplemented with 5% sheep blood, 0.3 μg/ml menadione (Sigma) and 5 μg/ml hemin (Sigma). *Eubacterium* and *Neisseria* species were grown on Fastidious Anaerobic Agar (BBL) with 5% defibrinated sheep blood. *Treponema denticola* and *Treponema socranskii* were grown in Mycoplasma broth (Difco Laboratories, Detroit, MI) supplemented with 1 mg/ml glucose, 400 μg/ml niacinamide, 150 μg/ml spermine tetrahydrochloride, 20 μg/ml Na isobutyrate, 1 mg/ml L-cysteine, 5 μg/ml thiamine pyrophosphate and 0.5% bovine serum. All strains were grown at 35°C under anaerobic conditions (80% N_2, 10% CO_2, 10% H_2).

DNA isolation and preparation of DNA probes

Bacterial strains were grown anaerobically on the surface of blood agar plates (except the two spirochetes, which were grown in broth) for 3–7 days. The cells were harvested and placed in 1.5 ml microcentrifuge tubes containing 1 ml of TE buffer (10 mM Tris-HCl, 0.1 mM EDTA, pH 7.6). Cells were washed twice by centrifugation in TE buffer at $1300 \times \boldsymbol{g}$ for 10 min. The cells were resuspended and lysed with either 10% SDS and Proteinase K (20 mg/ml) for gram-negative strains or in 150 μl of an enzyme mixture containing 15 mg/ml lysozyme (Sigma) and 5 mg/ml achromopeptidase (Sigma) in TE buffer (pH 8.0) for gram-positive strains. The pelleted cells were resuspended by 15 s of sonication and incubated at 37°C for 1 h. DNA was isolated and purified using the method of Smith et al. (21). The concentration of the purified DNA was determined by spectrophotometric measurement of the absorbance at 260 nm. The purity of the

preparations was assessed by the ratio of the absorbances at 260 and 280 nm. Whole genomic DNA probes were prepared from each of the 40 test strains by labeling 1–3 μg DNA with digoxigenin (Boehringer Mannheim, Indianapolis, IN) using a random primer technique (8).

Sample preparation

To examine the composition of the subgingival microbiota in subjects with different states of periodontal disease or health, the following sample taking protocol was employed. Subgingival plaque samples were taken from the mesio-buccal aspect of each tooth in each subject at each monitoring visit. Counts of 40 subgingival species were determined in each plaque sample using the checkerboard DNA–DNA hybridization technique (25). After the removal of supragingival plaque, subgingival plaque samples were taken with individual sterile Gracey curettes from the mesial aspect of each tooth. The samples were placed in separate Eppendorf tubes containing 0.15 ml TE. Then 0.15 ml of 0.5 M NaOH was added to each sample and the sample boiled in a water bath for 5 min. The samples were neutralized using 0.8 ml 5 M ammonium acetate. The released DNA was placed into the extended slots of a Minislot 30 (Immunetics, Cambridge, MA) and then concentrated onto a nylon membrane (Boehringer Mannheim) by vacuum and fixed to the membrane by cross-linking using ultraviolet light (Stratalinker 1800, Stratagene, La Jolla, CA) followed by baking at 120°C for 20 min. The Minislot device permitted the deposition of 28 different plaque samples in individual 'lanes' on a single 15 × 15 cm nylon membrane as well as two control lanes containing 10^5 or 10^6 cells of each test species. The membrane with fixed DNA was placed in a Miniblotter 45 (Immunetics), with the 'lanes' of DNA at 90° to the channels of the device. A 30 × 45 'checkerboard' pattern was produced with five of the probe lanes kept empty to permit accurate localization of each species. Each channel was used as a hybridization chamber for separate DNA probes. The 40 DNA probes employed are listed in Fig. 1.

Prehybridization and hybridization

The membranes were prehybridized at 42°C for 1 h in 50% formamide, 5 × SSC (1 × SSC = 150 mM NaCl, 15 mM Na citrate, pH 7.0), 1% casein (Sigma), 5 × Denhardt's reagent, 25 mM sodium phosphate (pH 6.5) and 0.5 mg/ml yeast RNA (Boehringer Mannheim). Digoxigenin-labeled, whole genomic DNA probes were prepared using a random primer technique (8). The probes and hybridization buffer were placed in individual lanes of the Miniblotter and the whole apparatus placed in a sealed plastic bag. Membranes were hybridized overnight at 42°C in a hybridizing solution containing 45% formamide, 5 × SSC, 1 × Denhardt's reagent, 20 mM Na phosphate (pH 6.5), 0.2 mg/ml yeast RNA, 20 ng/ml of labeled probe, 10% dextran sulfate, and 1% casein. The probes were denatured by heating each probe-containing hybridization buffer at 100°C for 5 min prior to cooling on ice. Membranes were washed at low stringency to remove loosely bound probe and then at high stringency (68°C, 0.1 × SSC, 0.1% SDS, 20 min, twice) in a Disk Wisk apparatus (Schleicher and Schuell, Keene, NH).

Detection and enumeration

To detect hybrids, membranes were blocked and then incubated with a 1 : 25,000 dilution of antidigoxigenin antibody conjugated with alkaline phosphatase using the modification described by Engler-Blum et al. (7). The washed membranes were incubated in AttoPhos (Amersham, Chicago, IL) overnight at room temperature and signals detected using a Storm Fluorimager (Molecular Dynamics, Sunnyvale, CA). Two lanes in each run had standards at 10^5 and 10^6 cells of each species. Signals were converted to absolute counts by comparison with standards on the membrane. The sensitivity of this assay was adjusted to permit detection of 10^4 cells of a given species by adjusting the concentration of each DNA probe. This procedure was carried out in order to provide the same sensitivity of detection for each species. Failure to detect a signal was recorded as zero, although counts in the 1–1000 range could conceivably have been present.

If a Fluorimager is not available, after washing, the membranes may be incubated in Lumiphos 530 (Lumigen, Southfield, MI) for 45 min at 37°C, placed in a film cassette with Reflection NEF film (Dupont, Boston, MA) for 1 h at 37°C and then developed. Signals can be evaluated visually by comparison with the standards for the test species. Typically, they would be recorded as: 0, not detected; 1, $<10^5$ cells; 2, $\sim10^5$; 3, 10^5–10^6; 4, $\sim10^6$; 5 $>10^6$ cells.

Determination of sensitivity of DNA probes

To determine the sensitivity of the method, pure cultures of each probe strain were adjusted to cell concentrations of 10^6, 10^5, 10^4, 10^3, 10^2, 10^1 and 0. The suspensions were treated as described in Sample preparation and placed in individual lanes of the Minislot device. DNA probes to the species were hybridized against the test strains using the Miniblotter 45 apparatus. Membranes were stringency washed and signals detected as described above.

Determination of specificity of DNA probes

The specificity of the DNA probes was tested under 'field conditions'. Four individuals, trained in the use of the checkerboard technique, prepared their own DNA probes and reagents and separately performed the described experiment. DNA from 10^6 cells of each of 80 taxa commonly found in supra- or subgingival plaque (Fig. 1) were deposited on a series of membranes (28 species per membrane) using a Minislot device as described above. The isolates included species not commonly recovered on agar media, such as the two species of *Treponema*. Whole genomic DNA probes were prepared to 40 taxa. The probes were adjusted in concentration to detect 10^4 cells of the homologous species. The probes were employed in the checkerboard DNA–DNA hybridization format using the exact protocol described above. AttoPhos was used in the final detection step and the signals were quantified using the Storm Fluorimager. The mean and standard deviation of computed cell counts from the four experiments was determined for each probe for each of the 80 target species.

Subtraction hybridization–polymerase chain reaction (PCR) probes

Low-level cross-reactions could be observed between closely related species or taxa. For example, cross-reactions were seen for species such as the four *Fusobacterium nucleatum/periodonticum* subspecies or between closely related species such as *Prevotella intermedia* and *Prevotella nigrescens*. Such cross-reactions could be eliminated by preparing probes using subtraction hybridization and PCR (3, 4). In brief, DNA from the probe strains was cut with *Sau*3a restriction endonuclease. A pair of primers was ligated to the cut ends to permit later amplification with PCR. Subtracter DNA was prepared by the addition of biotin using the photobiotin

reaction. The probe DNA and the subtracter DNA were hybridized for 48 h under permissive conditions at a ratio of 1 : 20,000 probe to subtracter DNA. The subtracter DNA and cross-reacting probe DNA were removed by adding streptavidin and removing the streptavidin-biotinylated DNA complex by phenol chloroform extraction. The remaining probe DNA was subjected to two more rounds of subtraction and the DNA remaining after the third round was amplified using PCR. After amplification, PCR was used to label the unlabeled DNA with digoxigenin. The resulting subtracted probes were employed in the checkerboard assay using the same conditions as the whole genomic probes.

A second method was evaluated for preparing probes by subtraction hybridization and PCR. Low-level cross-reactions were detected between the phylogenetically related (27) *Eikenella corrodens* and *Neisseria mucosa.* Probes were prepared to these species using the whole genomic subtraction hybridization kit provided by CLONTECH (PCR-Select™ Bacterial Genome Subtraction Kit, Palo Alto, CA), following the manufacturer's instructions. These probes were amplified by PCR and the resulting amplified product labeled with digoxigenin as described in the previous paragraph. The resulting probes were hybridized in the checkerboard format using the standard conditions described earlier.

Competitive hybridization

Cross-reactions were also minimized using competitive hybridization (2). In this method, high ratios of unlabeled DNA of the cross-reacting species were included in the hybridization buffer. Digoxigenin-labeled whole genomic DNA probes were prepared to the type strains of each of *Streptococcus anginosus, Streptococcus intermedius* and *Streptococcus constellatus.* Hybridization reactions were performed in the checkerboard system using these probes with and without 100 μg/ml of unlabeled DNA of both cross-reacting species.

Determination of prevalence and levels of species in clinical samples

In order to demonstrate the utility of the checkerboard DNA–DNA hybridization technique, data from two studies will be presented. All subjects in these studies had signed a Forsyth Institutional Review Board approved consent form. In the first study, a comparison was made of the microbial composition of subgingival plaque samples taken from 79 periodontally healthy subjects and 272 subjects with chronic periodontitis. Subgingival plaque samples were taken from the mesial aspect of all teeth present (excluding third molars) in the 351 adult subjects at one visit and evaluated for the levels, proportions and prevalence (% of sites colonized) of 40 bacterial taxa. The total number of samples was 8887 and the average for each subject visit was 25.3 (range 20–28). The total number of bacterial counts was 355,480. In a second study, the effect of periodontal therapy on the composition of the subgingival microbiota was examined. Periodontal therapy included scaling and root planing, instruction in proper home care procedures and, in some subjects, periodontal surgery and/or orally administered antibiotics. In this study, 166 subjects with chronic periodontitis were sampled microbiologically, as described above, prior to and 3 months post therapy. The total number of samples was 8126 and the average for each subject per visit was 24.5 (range 20–28). The total number of bacterial counts was 325,040. In both studies, the plaque samples were analyzed individually for 40 taxa using checkerboard DNA–DNA hybridization and chemifluorescence in the final detection step.

Results

Figure 1 is an example of a checkerboard from one subject. The vertical lanes represent subgingival plaque samples from 28 different teeth as well as standards of 10^5 and 10^6 cells of each species. The horizontal lanes represent the 40 DNA probes. The intensity of the signals was

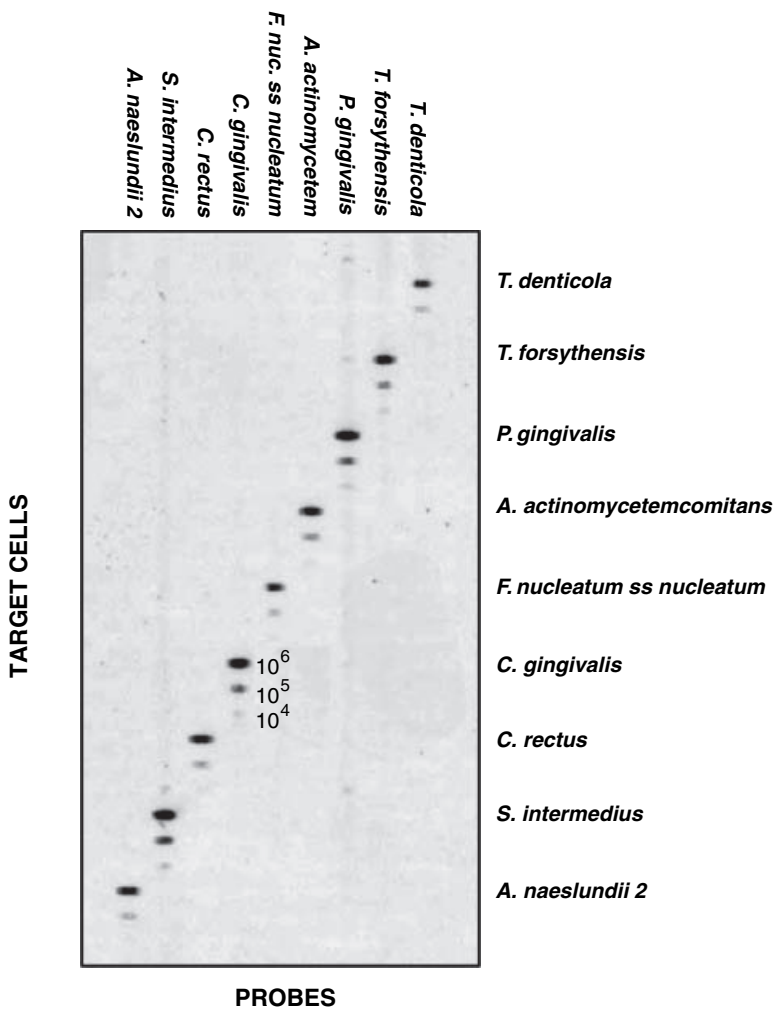


Fig. 2. Checkerboard DNA–DNA hybridization membrane demonstrating the sensitivity of nine DNA probes. The rows are the target cells of each of the test species at total numbers of 10^6, 10^5 and 10^4. The columns indicate the DNA probes employed. Samples were prepared and analyzed as described in 'Material and methods' with chemifluorescence as the final detection step. 10^4 signals not seen on the membrane image were detected using the Fluorimager.

Table 1. Log counts for species and sites shown in Fig. 1

11	12	13	14	15	16	17	21	22	23	24	25	26	27	31	32	33	34	35	36	37	41	42	43	44	45	46	47	Species*
4.62	5.56	4.49	4.86	5.20	–	–	–	5.93	5.51	5.97	5.39	6.12	5.87	5.81	5.21	5.18	6.00	5.46	6.06	–	5.24	5.72	5.53	4.94	5.96	6.04	6.06	*A. naeslundii 1*
0	4.22	0	4.12	4.09	–	–	–	4.23	4.13	4.62	0	4.51	4.49	4.52	4.59	4.35	4.53	4.33	4.49	–	4.51	4.53	4.19	4.29	4.37	4.39	4.48	*S. constellatus*
0	5.41	0	5.94	0	–	–	–	0	5.72	5.74	0	0	5.77	5.42	5.68	5.78	5.54	0	5.60	–	0	0	5.35	0	6.23	5.07	0	*E. nodatum*
0	4.85	0	5.83	0	–	–	–	0	4.44	5.96	0	4.43	5.90	5.85	5.91	5.98	5.58	4.58	5.94	–	5.23	4.81	4.99	0	6.04	4.91	0	*P. gingivalis*
5.00	4.94	0	5.39	0	–	–	–	4.59	4.64	5.39	0	4.95	5.35	5.42	5.41	5.50	5.12	4.59	5.25	–	5.05	5.20	4.83	0	5.51	4.77	0	*A.a..*
0	4.89	4.90	5.72	4.81	–	–	–	4.46	4.97	4.98	0	4.92	5.72	5.69	5.50	5.66	5.13	4.81	4.91	–	5.95	5.89	5.41	4.90	5.69	4.68	4.63	*F. nuc. ss vinc.*
0	0	0	0	0	–	–	–	0	0	0	0	0	5.34	5.39	0	5.01	0	0	0	–	5.17	5.10	0	0	5.15	0	0	*C. rectus*
0	0	0	0	0	–	–	–	0	0	0	0	0	5.11	0	5.08	5.02	0	0	0	–	0	0	0	0	5.10	0	0	*T. socranskii*
0	4.83	0	5.46	4.88	–	–	–	4.98	5.32	5.75	0	5.12	6.07	5.43	5.36	5.55	5.88	5.00	5.38	–	5.53	5.58	5.16	4.82	5.88	5.48	5.90	*E. saburreum*
0	0	0	5.21	0	–	–	–	0	0	5.47	0	0	5.60	5.13	5.49	5.32	5.51	4.98	5.22	–	5.37	5.36	0	0	5.39	0	0	*P. micros*
0	4.81	0	5.34	4.41	–	–	–	5.78	5.59	5.92	0	5.55	5.98	0	0	5.13	6.02	5.83	5.85	–	4.46	0	4.62	4.71	5.35	5.58	5.54	*V. parvula*
5.53	5.99	5.08	5.60	5.88	–	–	–	6.14	5.91	6.04	5.77	6.17	6.03	5.74	5.61	5.77	6.09	5.75	6.10	–	5.48	5.70	5.65	5.36	5.97	6.08	6.07	*A. naeslundii 2*
0	4.43	4.12	4.44	4.47	–	–	–	4.60	4.37	4.75	4.43	4.77	4.66	4.78	4.75	4.70	4.77	4.56	4.82	–	4.61	4.76	4.41	4.51	4.48	4.57	4.51	*S. anginosus*
0	4.60	0	4.52	4.46	–	–	–	4.83	4.53	4.88	4.57	4.94	5.16	5.24	4.87	4.87	5.17	4.65	4.91	–	4.75	5.17	4.66	4.66	4.67	4.67	4.63	*S. sanguis*
4.52	5.58	4.67	5.43	5.37	–	–	–	5.74	5.54	5.91	4.86	5.92	5.95	5.74	5.64	5.59	5.91	5.14	5.97	–	5.54	5.74	5.52	4.96	5.80	5.89	5.92	*A. gerencseriae*
0	4.82	4.46	4.81	4.64	–	–	–	4.73	4.70	4.91	4.69	5.46	5.57	5.86	5.92	5.68	4.96	4.89	5.38	–	5.26	5.96	4.81	4.90	4.81	4.77	4.82	*S. oralis*
0	0	0	0	0	–	–	–	0	0	0	0	0	5.49	0	0	0	4.96	0	0	–	0	4.86	0	0	0	0	5.18	*C. ochracea*
0	0	0	5.49	5.18	–	–	–	5.49	5.36	5.66	5.18	5.87	5.92	6.04	5.66	5.57	5.77	5.44	5.95	–	5.62	5.91	5.75	4.96	5.78	5.76	5.65	*A. israelii*
0	4.79	4.66	4.77	4.87	–	–	–	4.88	4.57	5.44	4.00	5.21	5.28	5.05	5.19	5.22	5.17	4.93	5.29	–	5.23	5.35	4.59	4.77	4.78	4.66	4.69	*S. intermedius*
0	0	0	0	0	–	–	–	0	0	0	0	0	4.52	4.54	5.00	4.79	0	0	4.75	–	4.52	0	0	0	4.98	0	0	*T. denticola*
0	5.06	0	5.94	0	–	–	–	0	0	5.10	0	0	5.77	0	5.38	5.42	4.95	0	4.90	–	6.05	5.76	0	0	5.44	0	0	*P. nigrescens*
0	4.94	0	5.11	4.89	–	–	–	5.14	5.06	5.91	4.66	5.83	6.18	6.04	5.87	5.43	5.90	5.45	6.15	–	5.79	5.99	5.86	4.93	5.94	6.05	5.80	*A. odontolyticus*
0	0	5.00	5.26	5.00	–	–	–	0	5.14	5.23	0	5.09	5.77	5.63	5.41	5.52	5.20	0	5.14	–	5.78	5.83	5.31	5.06	5.45	4.95	0	*F. nuc. ss polym.*
0	0	0	0	0	–	–	–	0	4.86	4.83	0	0	5.59	5.87	5.27	5.06	4.88	0	0	–	5.45	5.50	0	0	5.00	0	4.82	*C. showae*
0	0	0	5.38	0	–	–	–	0	0	0	0	0	5.78	5.84	0	5.58	0	0	0	–	5.92	5.92	0	0	5.68	0	0	*F. periodontic.*
4.08	4.85	4.29	4.66	4.68	–	–	–	4.65	4.83	4.76	4.72	4.84	5.71	5.98	5.84	4.88	5.74	4.85	5.74	–	5.80	6.02	4.97	4.96	4.71	4.66	4.32	*N. mucosa*
4.43	5.88	5.79	5.89	5.89	–	–	–	5.55	6.15	6.08	5.56	6.15	6.26	6.15	6.08	6.15	6.03	5.75	6.06	–	6.25	6.23	5.97	6.00	6.21	5.69	4.97	*F. nuc. ss nucl.*
0	0	0	0	0	–	–	–	0	0	0	0	0	5.90	4.93	0	0	4.91	0	0	–	0	5.68	4.74	0	0	5.44	0	*C. gingivalis*
0	0	0	0	0	–	–	–	5.26	0	5.23	0	5.22	5.56	5.30	5.41	5.22	5.47	0	5.26	–	0	5.56	0	0	4.94	0	0	*S. gordonii*
0	0	0	4.65	0	–	–	–	0	4.76	0	0	0	4.80	0	4.82	4.99	4.58	0	4.65	–	4.97	4.82	4.66	0	4.92	0	0	*T. forsythensis*
0	0	0	0	0	–	–	–	0	0	0	0	0	5.66	0	0	4.83	0	0	0	–	0	0	0	0	5.08	0	5.67	*S. noxia*
0	0	0	0	0	–	–	–	0	0	0	0	0	0	0	0	0	0	0	0	–	0	0	0	0	0	0	0	*P. acnes*
0	4.59	0	5.55	4.42	–	–	–	4.37	4.74	5.21	4.36	4.88	5.94	4.95	5.65	5.39	5.63	4.86	6.12	–	5.70	5.56	4.87	4.85	5.66	4.87	4.95	*P. melaninogen.*
0	5.51	5.03	5.58	5.17	–	–	–	4.84	4.96	5.45	4.80	5.43	5.67	5.77	5.88	5.89	5.54	5.32	5.73	–	5.70	6.00	5.20	5.38	5.21	5.36	5.25	*S. mitis*
0	4.89	0	0	0	–	–	–	0	4.99	4.82	0	4.96	5.76	5.93	5.67	5.16	5.36	4.70	4.99	–	5.88	6.09	5.55	4.84	4.92	5.19	4.67	*E. corrodens*
0	4.52	0	4.51	4.46	–	–	–	4.42	4.45	4.64	4.33	4.62	4.77	4.88	4.86	4.48	4.71	4.64	4.81	–	4.95	5.45	4.56	4.55	4.50	4.62	4.40	*G. morbillorum*
0	0	0	0	0	–	–	–	0	0	4.54	0	0	5.76	4.89	4.74	4.59	5.48	0	4.47	–	4.72	5.86	4.89	0	4.90	5.27	4.83	*C. sputigena*
4.45	4.69	4.40	4.89	4.67	–	–	–	4.60	4.80	5.45	4.78	5.57	6.10	5.86	4.96	5.42	5.91	5.09	6.05	–	5.53	5.76	5.47	4.90	5.61	5.67	5.51	*L. buccalis*
0	0	0	0	0	–	–	–	0	0	5.22	0	0	6.01	4.74	5.01	5.52	5.25	4.79	4.97	–	5.35	5.17	5.18	4.68	5.46	5.23	5.82	*C. gracilis*
4.35	4.92	4.47	6.06	4.80	–	–	–	4.47	4.89	6.00	4.67	4.86	6.23	5.11	6.26	5.53	5.38	5.32	6.07	–	6.25	6.04	4.90	4.96	6.16	5.03	4.81	*P. intermedia*

– represents a missing tooth. A zero value represents counts below 10^4.

*Abbreviations: *A. a., Actinobacillus actinomycetemcomitans; F. nuc.* ss *vinc, Fusobacterium nucleatum* ss *vincentii; F. nuc.* ss *polym., Fusobacterium nucleatum* ss *polymorphum; F. periodontic, Fusobacterium periodonticum; F. nuc.* ss *nucl., Fusobacterium nucleatum* ss *nucleatum; P. melaninogen., Prevotella melaninogenica.*

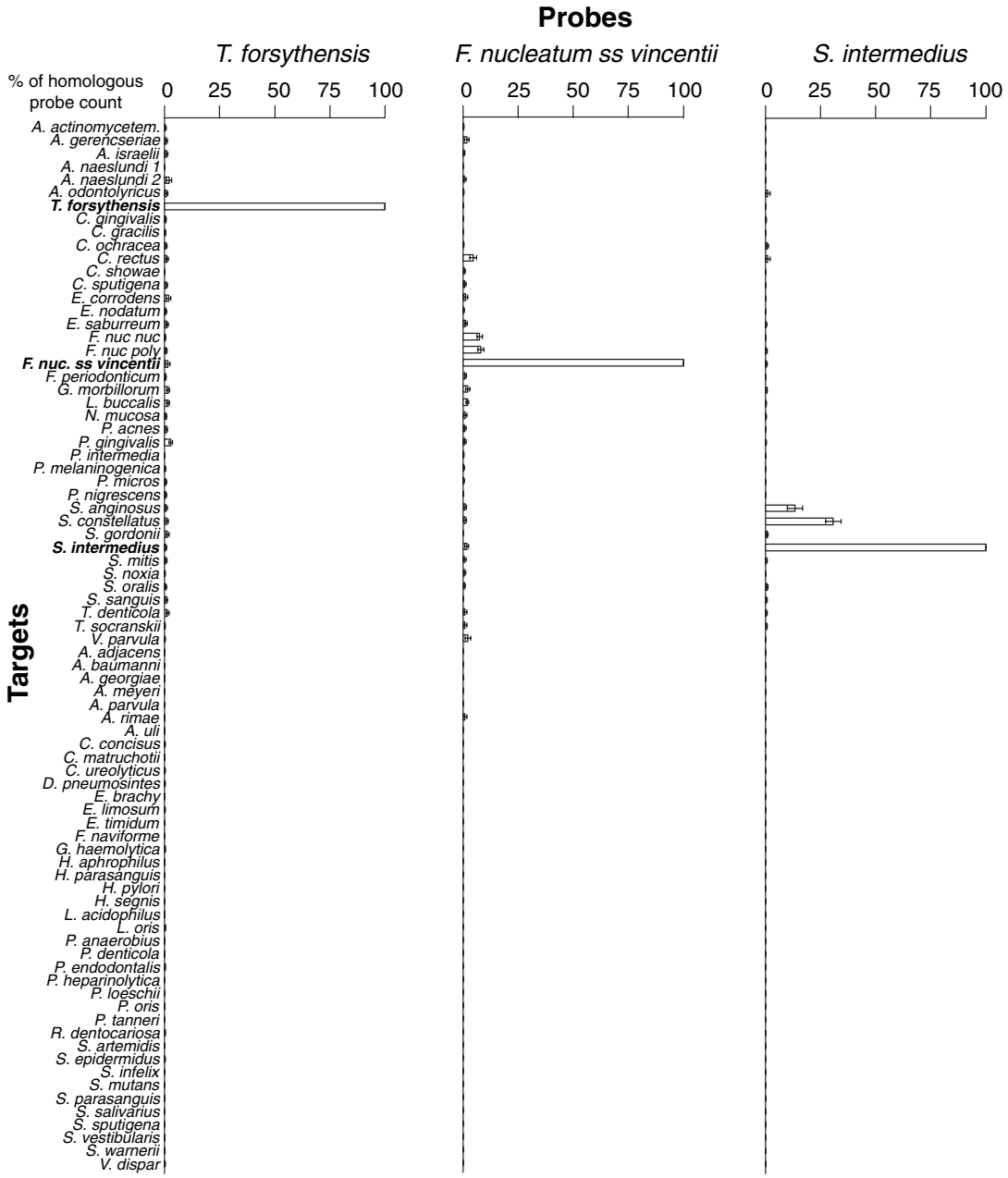


Fig. 3. Bar charts of the mean percentage of the homologous DNA probe count for probes to *T. forsythensis, F. nucleatum* ss *vincentii* and *S. intermedius.* DNA representing 10^6 cells of each of 80 test taxa was placed on nylon membranes and hybridized against probes to the 40 test taxa. The data represent the mean ± SEM of four experiments performed by four different operators as described in 'Material and methods'.

measured using a Fluorimager and converted to log counts. Table 1 provides the log counts for the signals presented in Fig. 1.

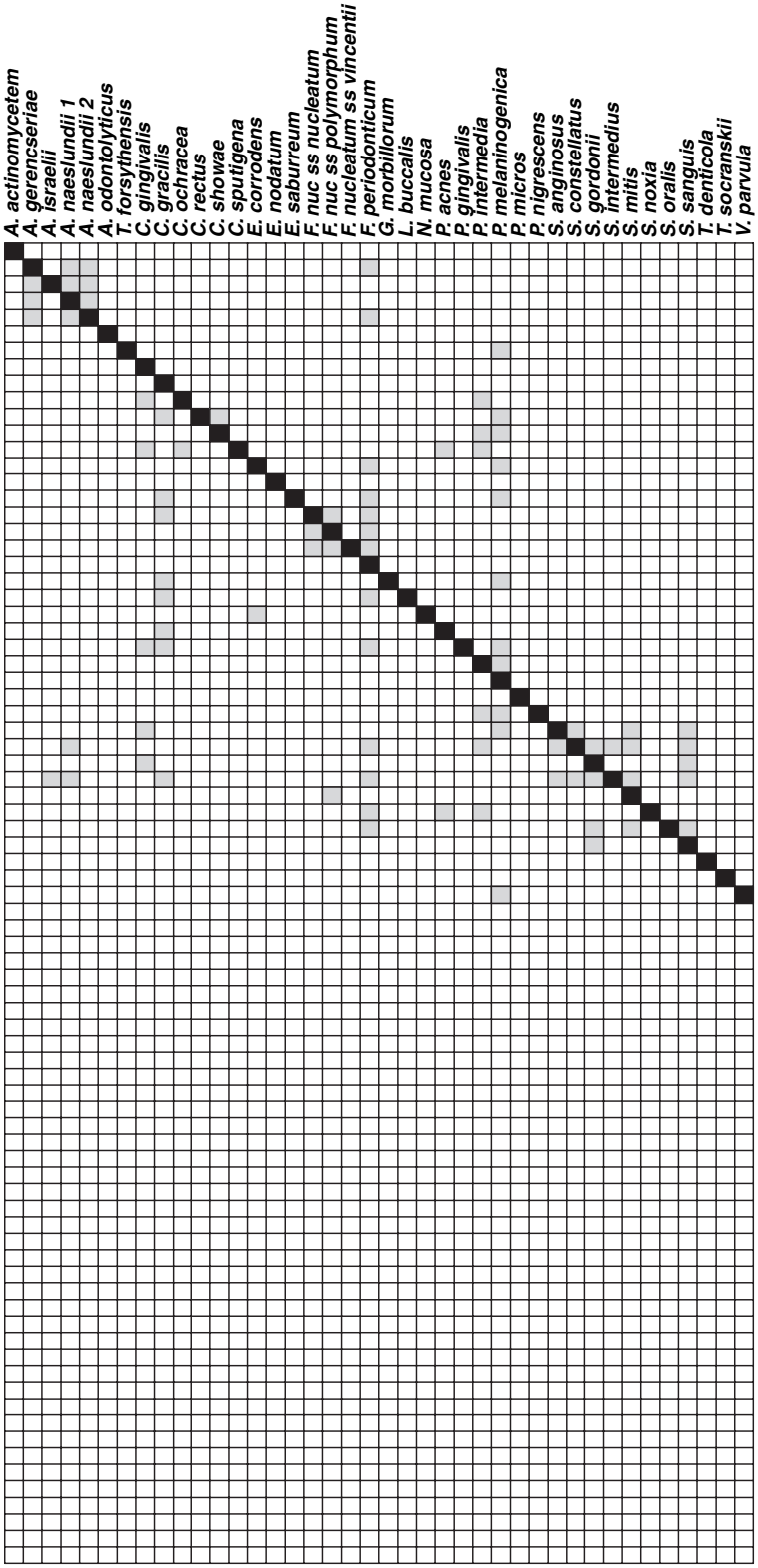
Sensitivity of DNA probes

Figure 2 provides an example of the sensitivity of nine DNA probes using chemifluorescent detection. 10^4 cells of each species could be detected (some signals were not visible on the image but could be detected by the Fluorimager). The conditions of the assay could be adjusted to detect 10^3 cells by increasing probe concentration and concentration of antibody conjugate (data not shown). However, this adjustment prevented distinction of counts at levels > 10^6.

Specificity of DNA probes

Figure 3 presents quantitative data for three example probes to *T. forsythensis, F. nucleatum* ss *vincentii* and *S. intermedius.* Certain species such as *T. forsythensis* showed virtually no measurable cross-reactions to any of the test taxa. The probe to *F. nucleatum* ss *vincentii* exhibited weak cross-reactions with *F. nucleatum* ss *nucleatum* and *F. nucleatum* ss *polymorphum* as well as a weak reaction to *Campylobacter rectus*. The probe to *S. intermedius* exhibited virtually no cross-reactions except for the expected reactions with the two other members of the 'streptococcus milleri group', *S. anginosus* and *S. constellatus*.

Figure 4 presents the results of the test of the specificity of the DNA probes which was carried out by four individu-



Actinobacillus actinomycetemcomitans	43718, 29523
Actinomyces gerencseriae	23860
Actinomyces israelii	12102
Actinomyces naeslundii 1	12104
Actinomyces naeslundii 2	43146
Actinomyces odontolyticus	17929
Tannerella forsythensis	43037
Capnocytophaga gingivalis	33624
Campylobacter gracilis	33236
Capnocytophaga ochracea	33596
Campylobacter rectus	33238
Campylobacter showae	51146
Capnocytophaga sputigena	33612
Eikenella corrodens	23834
Eubacterium nodatum	33099
Eubacterium saburreum	33271
Fusobacterium nucleatum ss nucleatum	25586
Fusobacterium nucleatum ss polymorphum	10953
Fusobacterium nucleatum ss vincentii	49256
Fusobacterium periodonticum	33693
Gemella morbillorum	27824
Leptotrichia buccalis	14201
Neisseria mucosa	19696
Propionibacterium acnes	11827, 11828
Porphyromonas gingivalis	33277
Prevotella intermedia	25611
Prevotella melaninogenica	25845
Peptostreptococcus micros	33270
Prevotella nigrescens	33563
Streptococcus anginosus	33397
Streptococcus constellatus	27823
Streptococcus gordonii	10558
Streptococcus intermedius	27335
Streptococcus mitis	49456
Selenomonas noxia	43541
Streptococcus oralis	35037
Streptococcus sanguis	10556
Treponema denticola	B1
Treponema socranskii	S1
Veillonella parvula	10790
Abiotrophia adjacens	49175
Acinetobacter baumannii	19606
Actinomyces georgiae	49285
Actinomyces meyeri	35568
Atopobium parvula	33793
Atopobium rimae	49626
Atopobium uli	49627
Campylobacter concisus	484
Corynebacterium matruchotii	14266
Campylobacter ureolyticus	33387
Dialister pneumosintes	GBA27
Eubacterium brachy	33089
Eubacterium limosum	8486
Eubacterium timidum	33093
Fusobacterium naviforme	25832
Gemella haemolytica	10379
Haemophilus aphrophilus	33389
Haemophilus parasanguis	29242
Helicobacter pylori	43504
Haemophilus segnis	33393
Lactobacillus acidophilus	4356
Lactobacillus oris	49062
Peptostreptococcus anaerobius	27337
Prevotella denticola	35308
Porphyromonas endodontalis	35406
Prevotella heparinolytica	35895
Prevotella loeschii	15930
Prevotella oris	33573
Prevotella tannerae	51259
Rothia dentocariosa	17931
Selenomonas artemidis	43528
Staphylococcus epidermidis	14990
Selenomonas infelix	43523
Streptococcus mutans	25175
Streptococcus parasanguis	15912
Streptococcus salivarius	27945
Selenomonas sputigena	35185
Streptococcus vestibularis	49124
Staphylococcus warneri	27836
Veillonella dispar	17748

Fig. 4. Grid plot demonstrating homologous and heterologous DNA probe hybridizations between 40 test whole genomic DNA probes and 80 target species DNA. The probes are in the vertical lanes and the target species in the horizontal lanes. The black boxes represent the homologous probe-target signal, which was considered to be 100%. The shaded boxes represent heterologous reactions between 10 and 20% of the homologous signals. 97.4% of the 3160 probes-heterologous species signals were <10% of the homologous signals. The four- and five-digit numbers to the right of the Figure represent the American Type Culture Collection (ATCC) strain numbers, the remaining designations represent isolates from The Forsyth Institute collection.

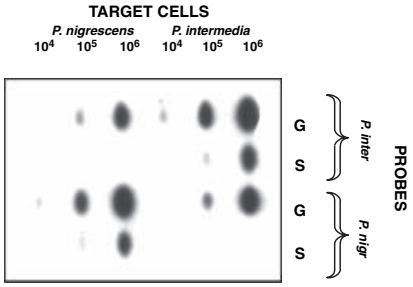


Fig. 5. Checkerboard DNA–DNA hybridization membrane demonstrating the use of subtraction hybridization to minimize cross-reactions between *P. intermedia* and *P. nigrescens.* The columns indicate test species deposited on the membrane at counts of 10^6, 10^5 and 10^4 cells. The rows indicate whole genomic DNA probes (G) and probes prepared using subtraction hybridization and PCR (S) as described by Bjourson et al. (3, 4). Hybridization was performed as described in 'Material and methods' except that the stringency wash temperature was lowered to 62°C to maximize potential cross-reactions.

als who prepared their own DNA probes and separately performed the experiment. The top half of Fig. 4. presents the 40 probes tested against the battery of species used to make the probes, while the bottom half of presents the same probes run against 40 additional species. In all, 97.4% of all probe-heterologous species reactions did not exhibit cross-reactions >10% of the homologous probe signal; 93.5% were <5% of the homologous species probe signal, and 82.1% were <1% of the homologous probe signal. Probes to certain species showed essentially no cross-reactions to other species at a level >10% of the homologous probe signal. These included probes to *Actinobacillus actinomycetemcomtans*, *Actinomyces odontolyticus*, *T. forsythensis*, *C. rectus*, *Capnocytophaga sputigena, Eubacterium nodatum, Eubacterium saburreum, F. nucleatum* ss *vincentii, Gemella morbillorum, Leptotrichia buccalis, N. mucosa, P. gingivalis, Peptostreptococcus micros, P. nigrescens, Selenomonas noxia, Streptococcus oralis, T. denticola, T. socranskii* and *Veillonella parvula.*

Subtraction hybridization–PCR probes

Figures 5 and 6 compare signals obtained using whole genomic probes and probes generated to the same species using subtraction hybridization and PCR. In the membranes presented, the stringency of hybridization and washing were lowered to purposely produce cross-reactions with the whole genomic probes in order to evaluate the efficacy of the improved probes. Cross-reactions between *P. nigrescens* and *P. intermedia,* between *F. nucleatum* subspecies (data not shown) and between

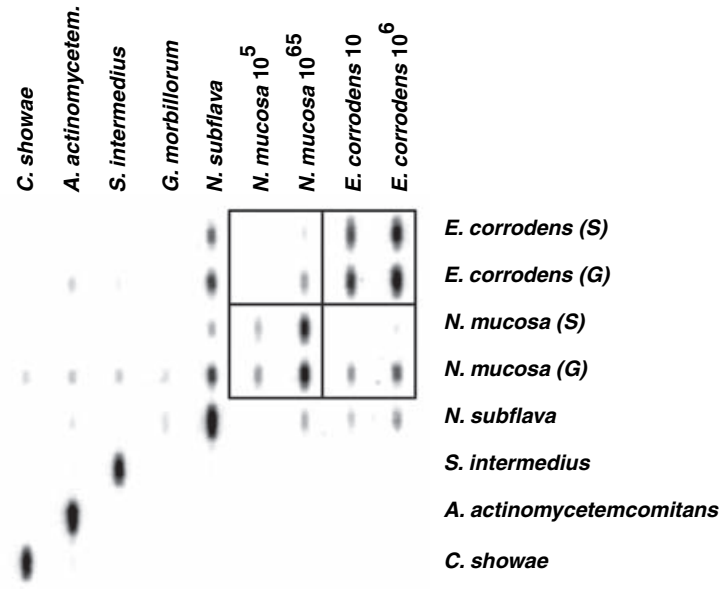


Fig. 6. Checkerboard DNA–DNA hybridization membrane demonstrating the use of subtraction hybridization to minimize cross-reactions between *N. mucosa* and *E. corrodens.* The columns indicate test species deposited on the membrane at counts of 10^6 and 10^5 cells. The rows indicate whole genomic DNA probes (G) and probes prepared using subtraction hybridization and PCR (S) using the CLONTECH kit. Hybridization was performed as described in 'Material and methods' except that the stringency wash temperature was lowered to 62°C to maximize potential cross-reactions.

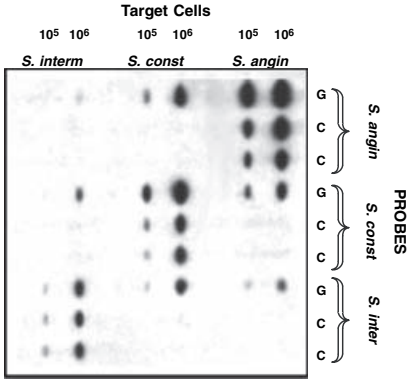


Fig. 7. Checkerboard DNA–DNA hybridization membrane demonstrating the use of competitive hybridization to minimize cross-reactions between *Streptococcus* species. The columns indicate test species deposited on the membrane at counts of 10^6 and 10^5 cells. The rows indicate whole genomic DNA probes (G) and whole genomic probes used in the presence of 100 μg/ml of un-labeled competing DNA (C). Hybridization was performed as described in 'Material and methods' except that the stringency wash temperature was lowered to 62°C to maximize potential cross-reactions.

N. mucosa and *E. corrodens* were virtually eliminated when the subtracted-PCR probes were employed.

Competitive hybridization

Figure 7 is an example of the use of competitive hybridization to minimize cross-reactions between closely related *Streptococcus* species. As may be observed in Fig. 7, whole genomic probes (G) cross-reacted to some extent under the permissive conditions of the assay employed. These cross-reactions were minimized in the lanes where the competitive unlabeled DNAs were included (C).

Composition of subgingival microbiota in periodontal health and disease

Figure 8 presents the mean (± SEM) counts, proportions and prevalence of 40 taxa in subgingival plaque samples from periodontally diseased and periodontally healthy individuals. The dominant species subgingivally were the *Actinomyces*. The majority of taxa had significantly higher mean counts and a greater prevalence in the periodontally diseased subjects compared with healthy subjects. Particularly noteworthy were the high levels and prevalence of species associated with periodontal diseases, members of the 'red' and 'orange' complexes (22), in the peri-

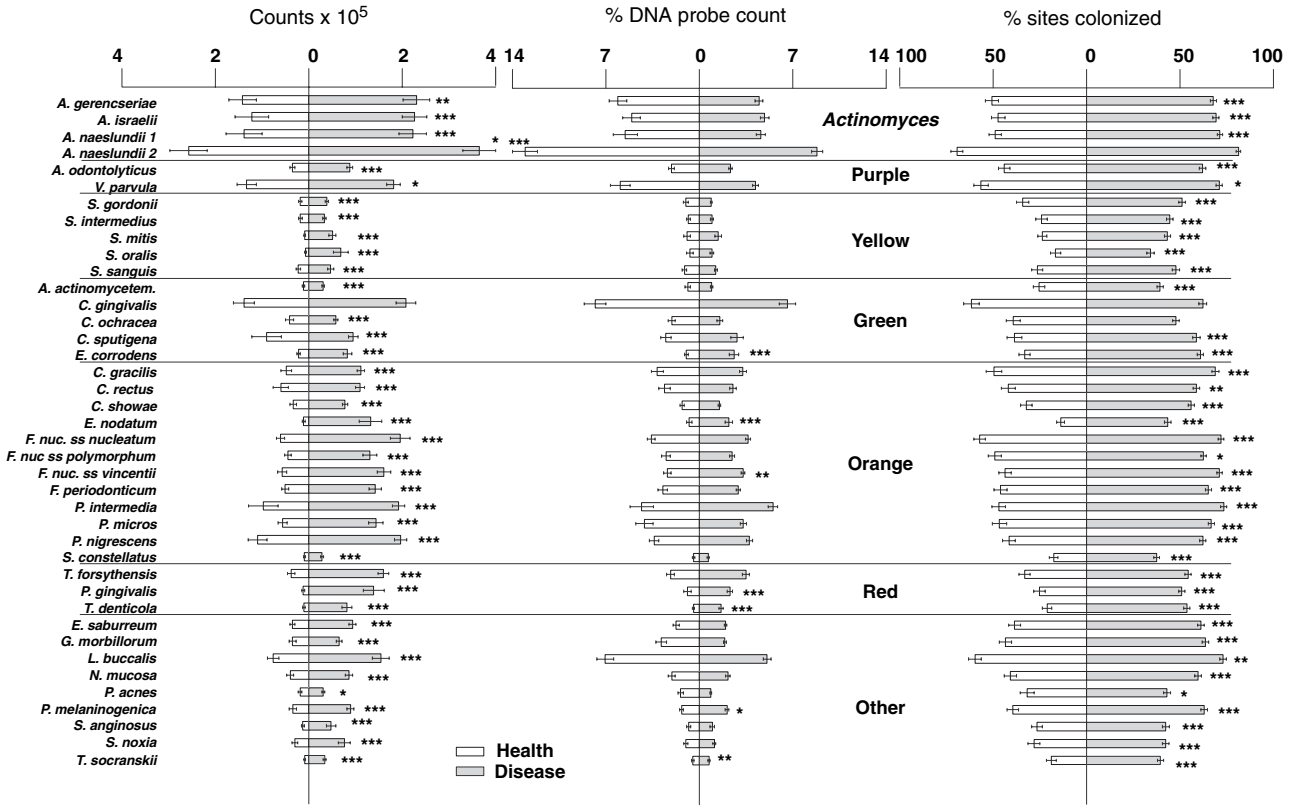


Fig. 8. Bar charts of the mean (± SEM) counts ($\times 10^5$), proportions and percentage of sites colonized in subgingival plaque samples taken from 79 periodontally healthy and 272 subjects with chronic periodontitis. The bars represent the mean values and the whiskers, the SEM. Subgingival plaque samples were taken from the mesial surface of each tooth, excluding third molars and individually processed for their content of 40 bacterial species using checkerboard DNA–DNA hybridization. The lefthand bars represent health and the righthand shaded bars, disease. The species are arranged within microbial complexes described by Socransky et al. (22). The significance of difference between health and disease was determined using the Mann–Whitney test and adjusted for multiple comparisons (Socransky et al. (23)) (*****P <0.05, ******P <0.01, *******P <0.001). The asterisks are placed beside the bars showing the higher mean value.

odontitis subjects. The differences in proportions of species between health and disease were less striking, although marked differences were observed for the red complex species such as *P. gingivalis* and *T. denticola.* There was also a significantly higher mean proportion of *A. naeslundii* genospecies 2 in the subgingival plaque samples from periodontally healthy subjects.

Effect of periodontal therapy on the composition of the subgingival microbiota

The effect of periodontal therapy on the composition of the subgingival microbiota is demonstrated in Fig. 9. The major effect on the composition of the microbiota appeared to be a significant reduction in mean counts of 28 of the species evaluated. Striking reductions were observed in the mean counts of the pathogenic 'red' and 'orange' complexes. Species in these complexes also showed a significant decrease in the mean percentage of sites colonized. Changes in mean proportions were less dramatic, with significant decreases observed only for *T. forsythensis, P. gingivalis*, and *P. nigrescens* and a significant increase in *Capnocytophaga gingivalis.*

Discussion

The checkerboard DNA–DNA hybridization technique outlined in this manuscript offers a number of advantages for the study of multiple species of bacteria in large numbers of samples containing complex mixtures of microorganisms. The technique is rapid, sensitive, and relatively inexpensive. It overcomes many of the limitations of cultural microbiology including loss of viability of organisms during transport, the problem of enumerating difficult to cultivate (or even uncultivable) species, and the difficulty encountered in speciating certain taxa that are difficult to grow or which exhibit few positive phenotypic traits. Another advantage is that the entire sample may be employed without dilution or amplification (with appropriate regard to total sample size), overcoming problems in quantification imposed by either serial dilution or PCR amplification procedures. Finally, the technique provides quantitative data which may be important in treatment studies of biofilm infections where species levels and proportions may be markedly decreased but the species not eliminated. It has been found that one laboratory technician can routinely prepare and evaluate about 12 checkerboards, or 312 samples, per week for their content of 40 bacterial species. The major cost in materials is the membranes and the constituents used in making or detecting the DNA probes. The cost of membranes has been minimized by evaluating 28 samples for their content of 40 taxa (i.e. 1120 bacterial counts) on a single 15×15 cm nylon membrane. Costs of DNA probes are minimal since the total volume occupied by each DNA probe–hybridization buffer lane is 150 µl. Another advantage of the technique is that membranes may be stripped and re-probed

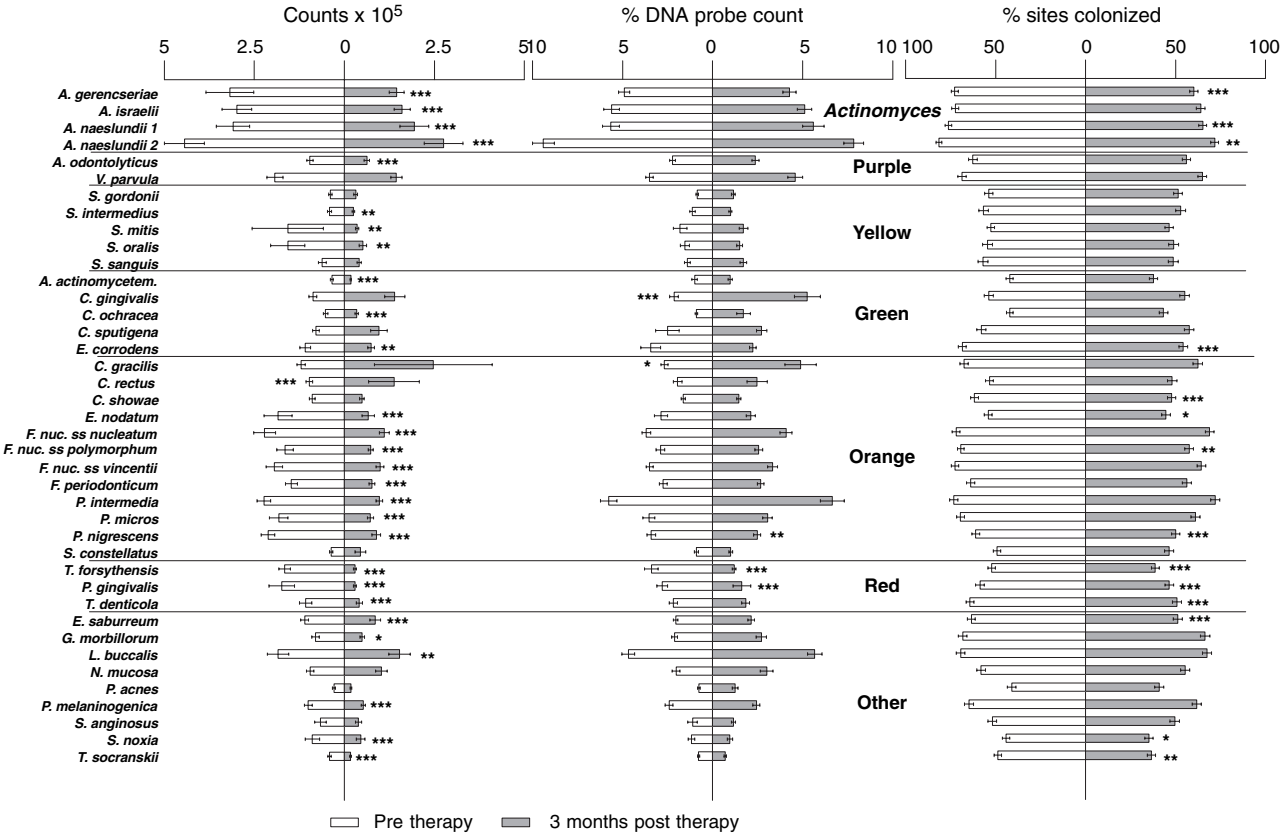


Fig. 9. Bar charts of the mean (± SEM) counts ($\times 10^5$), proportions and percentage of sites colonized in subgingival plaque samples taken from 166 subjects with chronic periodontitis prior to and 3 months after periodontal therapy. The bars represent the mean values and the whiskers, the SEM. Subgingival plaque samples were taken at each visit as described in Fig. 8. The lefthand bars represent pretreatment values and the righthand shaded bars, 3 months post-therapy data. The species are arranged within microbial complexes (22). The significance of difference between pre- and post-therapy values was determined using the Wilcoxon signed ranks test and adjusted for multiple comparisons (23) (*****P <0.05, ******P <0.01, *******P <0.001). The asterisks are placed beside the bars showing the lower mean value.

with a new set of 40 different DNA probes. Re-stripping and re-probing have been successfully employed for four sets of probes, i.e. 160 bacterial taxa (data not shown).

The technique is sensitive since it can routinely detect 10^4 cells of a given species in a sample. The conditions of hybridization can be altered to detect as few as 10^3 cells as well as higher numbers of cells by changing the probe concentration and the concentration of the phosphatase-conjugated antibody to digoxigenin. Such alterations change the 'window' of enumeration. Adjusting the assay to be more sensitive impairs the ability to accurately enumerate large numbers of cells of a given species.

As far as is known, the probes identify 100% of isolates of species to which the probe is directed. This conclusion is based on studies of 3200 pure cultures isolated from subgingival samples taken from 64 subjects. To identify the fresh isolates, 109 probes to subgingival taxa were employed. When isolates were detected that could not be identified by any of the probes, they were phenotypically characterized and their 16S rRNA sequenced. In no instance was an isolate detected that could be identified by phenotypic tests or 16S rRNA sequence as a species in the probe battery (data not shown). These data suggest that the probes did not miss any fresh isolate of a species in the test battery. The results were not surprising since whole genomic probes were used.

For the most part, the whole genomic DNA probes were remarkably specific: 93.5% of probe:heterologous species cross-reactions were less than 5% of the homologous probe signal. Cross-reactions could be minimized by competitive hybridization or by using probes prepared using subtraction hybridization and PCR techniques. The latter method seems preferable for large scale studies since it obviates the need to add specific DNAs singly or in combination to a variety of DNA probe lanes. Subtraction hybridization by the method of Bjourson et al. (3, 4) and the CLONTECH kit both worked well. However, probes developed using the CLONTECH kit were easier to prepare.

The checkerboard DNA–DNA hybridization technique does have limitations. The technique can detect only species for which DNA probes have been prepared. Thus, novel pathogens or environmentally important species which might be detected in culture or by other molecular techniques would not be detected by this method. The technique must be optimized for a given biological or environmental site. The use of probes developed for subgingival plaque samples is unlikely to be optimum for samples for other body sites or other sites in nature. The probes must be used to detect organisms in samples of the appropriate size. Probes optimized to detect species in the 10^4–10^7 range often will provide cross-reactions if much larger samples are employed. A note of caution to potential users of the technique. On occasion, a clinical collaborator may feel that the samples that he/she provides are too small unless large clumps of visible plaque can be observed in the microcentrifuge tube. This would be devastating to the assay as described. An overly large

sample would not be lysed by the amount of NaOH employed due the buffering provided by the sample's proteins. Such samples will provide artificially low signals in the assay unless reagent volumes are altered. The possibility of nonspecific binding of either digoxigenin or the phosphatase antibody conjugate to non-DNA cellular or abiotic debris is a real concern when the proportion of bacterial DNA to other macromolecules is low. Such constituents might absorb the digoxigenin labeled probes or antibody-phosphatase conjugate, providing false-positive signals. For this reason, sterile samples of an untested target site should be tested for this possibility prior to utilizing the technique.

When properly employed, checkerboard DNA–DNA hybridization and other rapid microbiological techniques permit investigation of etiologic, therapeutic, and environmental problems which could not be approached by other means. The data from clinical samples presented in this paper demonstrate the feasibility of examining the microbiota in different disease states and health. In addition, species abundance, species diversity, and community structure can be computed from data derived using this technique (22). Further, effects of therapeutic modalities on major segments of the microbiota can be examined, as shown in Fig. 9. These data indicate not only whether a target species was diminished, but whether other members of the microbiota were affected. It seems likely that this approach could be extended to studies of environmental samples, although such applications were not tested in this investigation. As new and improved DNA probes are developed, and rapid microbiological techniques are employed, an understanding of the ecologic relationships of complex microbial communities can be developed at a level hitherto beyond our reach.

Acknowledgments

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