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"Reverse" DNA hybridization method for the rapid identification of subgingival microorganisms

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A "reverse" hybridization method is described, in which whole chromosomal DNA was extracted from 10–20 colonies of "unknown" strains in pure culture and labelled with digoxigenin by a random primer technique. DNA probes were prepared from a total of 23 strains and hybridized with targets containing 100 ng purified, denatured DNA from 38 reference strains fixed to nitrocellulose. 21/23 digoxigenin-labelled DNA probes successfully detected all members of the homologous species present on filters. Probes to *Fusobacterium nucleatum* strains 364 and MG detected 3/4 and 1/4 members of this species, respectively; 13/23 probes were 100% specific, but cross reactions between 10 probes and DNA targets from closely related, heterologous species occurred in 15/834 possible instances. False-positive reactions that occurred between closely related species were, however, easily distinguished and did not prevent the accurate identification of probe strains. Digoxigenin-labelled probes were capable of detecting 100 pg of homologous DNA. The reverse hybridization procedure allows identification or grouping of a large number of isolates within 3 days and provides a more economical means of characterizing subgingival isolates than predominant cultivable techniques and conventional phenotypic testing. This method could be adapted for the direct identification of microorganisms in subgingival plaque samples

Key words: subgingival microorganisms; reverse hybridization; digoxigenin-labelled DNA probes.

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The identification of pure cultures from subgingival plaque samples is often a time-consuming, labor-intensive process requiring specialized laboratory techniques and considerable expertise. Even when these ideal conditions prevail, problems such as the failure of fastidious organisms to grow in available media, a paucity of well-defined phenotypic markers for many species, or variability in test results may contribute to equivocal identifications. Attempts have been made to overcome these difficulties using DNA probes as an adjunct to, or a replacement for, conventional characterization (5, 7, 8). The classical approach to DNA probe identifications is to place cells of the test species on a solid support, such as nitrocellulose, and use a series of treatments to lyse the cells, denature and bind the released DNA to the solid support (3, 10). Filters carrying the test organisms' DNAs are from purified reference strain DNA. Although it has a number of advantages, this procedure also has several drawback. Unless the investigator has a reasonable idea of the identity of the test species, the number of filters prepared and probes employed could be quite large before the organism is successfully identified. Furthermore, in its classical form, the technique is not well suited to the identification of small numbers of isolates, since the work involved in preparing and hybridizing 96 unidentified strains on a filter is comparable to that involved in identifying a single strain by the same method. In such situations, the investigator may choose to wait until sufficient strains have accumulated to make filter preparation and hybridizations worthwhile.

A more practical approach to strain identification would be to prepare a single strain for hybridization in such a tested against virtually any number of reference species DNAs. To achieve this goal, we investigated the concept of "reverse" hybridization, in which a small number of cells of "unknown" test species (in pure culture) were treated to release, denature and label their DNAs with digoxigenin. Thus, the unknown isolate became the probe, which was then hybridized with a range of purified, denatured reference strain DNAs fixed to nitrocellulose. In this way, unknown isolates could be identified or grouped by positive reactions appearing at appropriate sites on the filters. Previous experiments (unpublished observations) indicated that inclusion of hexadecyltrimethyl ammonium bromide (CTAB; 11) in the extraction of DNA from 10^8 cells produced DNA which could be biotin-labelled by a random primer technique (2). The present study examined the possibility of using this technique to

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with DNA extracted from small numbers of cells in the same manner.

Material and methods

Sources and cultivation of strains

Thirty-three gram negative and 5 gram positive strains were used in this study (Table 1). Strains were grown on Trypticase soy agar plates supplemented with 5% sheep blood (Baltimore Biological Laboratories, Cockeysville, MD), in an atmosphere of 80% N_2, 10% H_2 and 10% CO_2 at 35°C. Gram negative strains were incubated for 3–5 days and gram positive organisms for 1–2 days; plates inoculated with *Bacteroides forsythus* were incubated for 5–7 days in parallel with a supporting strain of *Fusobacterium nucleatum* (FDC 364). Strains belonging to the species *Wolinella, Campylobacter* and *Bacteroides gracilis* were grown on brain heart infusion agar (Baltimore Biological Laboratories), supplemented with 0.2% sodium formate, 0.3% disodium fumarate and 1% hemin.

Preparation of filters

Confluent growth was removed from the surface of 1–2 agar plates and test strain DNAs were purified as previously described (9). Aliquots of the 38 DNA preparations were placed in the wells of microtiter plates (see Fig. 1 legend) and transferred to nitrocellulose (BA 85, 0.45 μm, Schleicher & Schuell, Inc., Keene, NH) using an MIC inoculator (Dynatech Inc., Alexandria, VA.). Each "spot" contained 100 ng of purified target DNA. Filters were soaked for 10 min in 0.5 M NaOH, 1.5 M NaCl (lysing/denaturing solution), blotted dry, and then soaked in 1.5 M NaCl, 0.5 M Tris-HCl, pH 7.6 (neutralizing solution). After drying in air for 15–20 min, filters were baked at 80°C for 2 h.

Extraction of DNA for probe preparation

A modified version of the method for DNA purification described above was used to extract chromosomal DNA from a small number of cells that was suitable for digoxigenin labeling. In brief, 10–20 colonies were scraped from the surface of agar plates and resuspended in 557 μl TE (10 mM Tris, pH 7.6, 1 mM EDTA). In addition, 1 μg of

Table 1. List of strains which had target DNAs fixed to nitrocellulose.

		Probe results		
Species	Strain**	Reactions with same species	Reactions with heterologous species	Heterologous reactions
Streptococcus sanguis I	F CO1	1/1	0/37	
Streptococcus sanguis II	F A1	1/1	0/37	
Streptococcus intermedius	F JS26	1/1	1/37	*S. sanguis I* CO1
Peptostreptococcus micros	F JH20	1/1	0/37	*S. intermedius* JS26
Actinomyces naeslundii	A 12104	*	*	
Bacteroides veroralis	A 33779	1/1	0/37	
Bacteroides gracilis	F 402	*	*	
Bacteroides gracilis	F 1083	3/3	3/35	*W. recta* 371,1219, *C. concisus* 484
Bacteroides gracilis	F 406	*	*	
Bacteroides intermedius	F 581	*	*	
Bacteroides intermedius	V 8944	*	*	
Bacteroides intermedius	A 25261	3/3	0/35	
Bacteroides gingivalis	F 381	1/1	0/37	
Bacteroides forsythus	F 338	1/1	0/37	
Wolinella curva	V 9584	3/3	3/5	*W. recta* 371,1219, *C. concisus* 484
Wolinella curva	F. 521	*	*	
Wolinella curva	F 640	*	*	
Wolinella sputigena	IB4	*	*	
Wolinella recta	F 1219	*	*	
Wolinella recta	F 371	2/2	0/36	
Capnocytophaga sputigena	F 4	1/1	2/37	*C. ochracea* 6, 25
Capnocytophaga ochracea	F 6	2/2	1/36	*C. sputigena* 4
Capnocytophaga ochracea	F 25	*	*	
Capnocytophaga gingivalis	F 27	*	*	
Capnocytophaga type IV	SD4	1/1	0/37	
Eikenella corrodens	F 373	1/1	0/37	
Campylobacter concisus	F 484	*	*	
Bacteroides zoogleoformans	A 33285	1/1	2/37	*B. heparinolyticus* 35895, *B. denticola* 33185
Bacteroides heparinolyticus	A 35895	1/1	0/37	
Veillonella parvula	A 10790	1/1	0/37	
Fusobacterium nucleatum	F MG	1/4	0/34	
Fusobacterium nucleatum	F EM48	*	*	
Fusobacterium nucleatum	F EL28	*	*	
Fusobacterium nucleatum	F 364	3/4	0/34	
Actino. actinomycetemcomitans	F Y4	2/2	2/36	*H. aphrophilus* H77, 626
Actino. actinomycetemcomitans	A 33384	2/2	2/36	*H. aphrophilus* H77, 626
Haemophilus aphrophilus	F 626	*	*	
Haemophilus aphrophilus	H77	2/2	2/36	*A. actinomycetemcomitans* Y4, 33384

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calf thymus DNA was added to each suspension (10 μl of 100 μg/ml DNA in TE). Cell lysis and CTAB extractions were performed as described above. After chloroform:isoamyl extraction, centrifugation (12,000 × g, 15 min) and removal of the upper aqueous layers to fresh tubes, DNA was precipitated by the addition of 0.6 volumes of isopropanol. Suspensions were centrifuged (12,000 × g, 5 min) and the precipitates washed twice with 70% ethanol. Precipitates collected by centrifugation (12,000 × g, 5 min) were allowed to dry in air for approximately 20 min, after which 15 μl of a low EDTA-Tris buffer (10 mM Tris, 0.1 mM EDTA, pH 7.6) was added to each tube. Tubes were incubated at 4°C overnight to allow the DNA present in precipitates to dissolve in the buffer.

Labelling of isolated DNA

A random primer technique (2) was employed to construct digoxigenin-labelled probes from DNA contained in the CTAB preparations, using reagents supplied by Boehringer Mannheim (Indianapolis, IN). DNA was denatured, by heating at 95°C for 10 min, and incubated at 37°C for 60 min with 2 μl of a random hexanucleotide mixture, 2 μl of a dNTP mixture containing dCTP, dATP, dGTP (1 mmol/l each), dTTP (0.65 mmol/l) and digoxigenin-dUTP (0.35 mmol/l), sterile distilled water upto 19 μl, and 1 μl Klenow fragment. The reaction was stopped by the addition of 2 μl 0.2 M EDTA, and DNA was precipitated by adding 2 μl 4 M lithium chloride and 60 μl ice-cold 95% ethanol to the mixtures, which were subsequently kept at −20°C for 2 h, or −70°C for 30 min. DNA was collected by centrifugation (12,000 × g, 2 min), washed with ice-cold 70% ethanol, dried under vacuum and dissolved in 50 μl TE (10 mM Tris, pH 7.6, 1 mM EDTA). Hybridization reagents and conditions employed for digoxigenin-labelled probes were as recommended by Boehringer Mannheim. Briefly, filters were prehybridized in sealed plastic bags for 60 min at 68°C in 20 ml hybridization solution per 100 cm^2 filter. The hybridization solution, which consisted of 5 × SSC, 1% (w/v) blocking reagent, 0.1% (w/v) sodium salt of N-lauroylsarcosine, 0.02% (w/v) SDS, was allowed to dissolve at 50−70°C for at least 60 fresh hybridization solution (2.5 ml per 100 cm^2 of filter) containing a denatured digoxigenin-labelled probe (the entire labelled DNA preparation from 10–20 colonies). After overnight incubation at 68°C, filters were washed to remove unbound probe as follows: 2 × 5 min in 2 × SSC, 0.1% (w/v) SDS at room temperature, then 2 × 15 min in 0.1 × SSC, 0.1% (w/v) SDS at 68°C. To detect hybrids, filters were washed briefly in a 100 mM Tris-HCl, 150 mM NaCl buffer (pH 7.5), and incubated at room temperature with an anti-digoxigenin antibody – alkaline phosphatase conjugate, diluted 1:5000 in the same buffer for 30 min. Unbound conjugate was removed by 2 × 15 min washes in buffer only at room temperature. Filters were equilibrated in 100 mM Tris-HCl, 100 mM NaCl, 50 mM $MgCl_2$ (pH 9.5) and incubated with 10 ml of this buffer containing 34 μg/ml nitroblue tetrazolium (NBT) and 18 μg/ml bromo-chloro-indolyl phosphate (BCIP). Development proceeded in the dark for 60 min–24 h and the reaction was terminated by soaking the filters in 10 mM Tris-HCl, 1 mM EDTA, pH 8.0.

Results

Table 1 lists 38 strains from which DNA was purified and fixed to nitrocellulose filters. Twenty-three of these strains were selected for digoxigenin-labelled DNA probe preparation and the results of their hybridizations are summarized in Table 1. Of 23 probes, 21 detected all members of the homologous species present on the filters. The 2 exceptions were a probe to *F. nucleatum* strain MG, which detected only its homologous DNA, and a probe to *F. nucleatum* strain 364, which detected the 3 remaining strains of this species. Overall, the labelled probes detected the DNAs of the same species in 36 of the 40 instances when such detection should have occurred.

Thirteen of the 23 digoxigenin-labelled DNA probes showed no false-positive reactions with target DNAs from heterologous species. For example, Fig. 1 shows 2, 38-strain nitrocellulose filters after hybridization and development. The top filter was hybridized with a probe to *Bacteroides gingivalis* 381; a single spot indicates the site of probe-

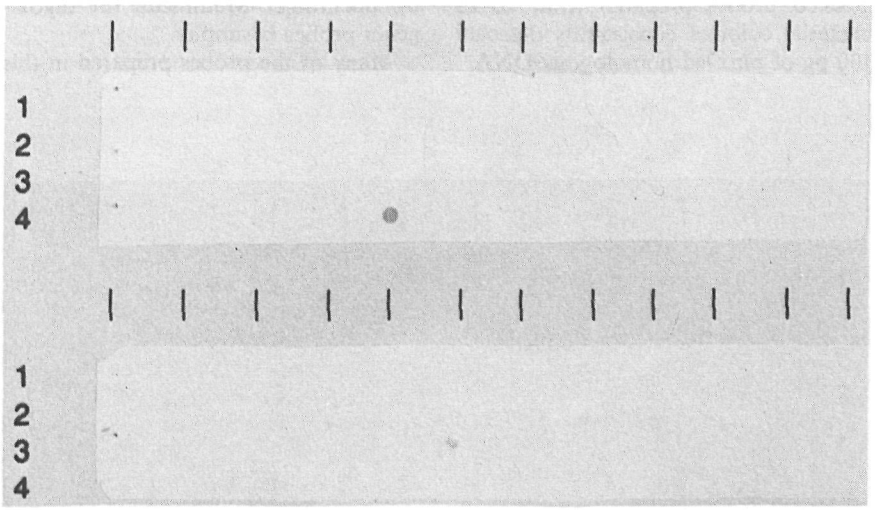

Fig. 1. Nitrocellulose filters carrying 100 ng targets of purified, denatured DNA from 38 strains. Row 1, left to right: *W. recta* 371 and 1219, *C. concisus* 484, *B. gracilis* 1083 and 406, *W. sputigena* IB4, *W. curva* 521, 640 and 9584, *C. concisus* 484 (repeat), *B. gracilis* 402, *E. corrodens* 373. Row 2: *S. sanguis I* CO1, *S. sanguis II* A1, *S. intermedius* JS26, *P. micros* JH20, *A. naeslundii* 12104, blank, *F. nucleatum* 364, MG, EM48 and EL28. Row 3: *A. actinomycetemcomitans* Y4, *H. aphrophilus* H77 and 626, *A. actinomycetemcomitans* 33384, blank, *V. parvula* 10790, *C. ochracea* 25, *C. sputigena* 4, *C. gingivalis* 27, *C. ochracea* 6, *Capnocytophaga* type IV SD4. Row 4: *B. intermedius* 25261, 581 and 8944, *B. forsythus* 338, *B. gingivalis* 381, *B. denticola* 33185, *B. zoogleoformans* 33285, *B. heparinolyticus* 35895, *B. veroralis* 33779. The top filter was hybridized with a digoxigenin-labelled DNA probe prepared with *B. gingivalis* 381 DNA. The lower filter was hybridized with a digoxigenin-labelled DNA

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target DNA reaction. The lower filter was hybridized with a probe to *Veillonella parvula* 10790; this probe also detected DNA from the homologous strain only. The remaining 10 probes displayed cross reactions with DNA from closely related heterologous species present on filters. Labelled DNA from *Actinobacillus actinomycetemcomitans* strains detected *Haemophilus aphrophilus* and vice versa, while *B. gracilis* cross-reacted with *Wolinella recta* and *Campylobacter concisus*. As shown in Fig. 2, a probe to *Bacteroides zoogleoformans* cross-reacted with *Bacteroides heparinolyticus* and *Bacteroides denticola,* whereas the *B. heparinolyticus* probe was specific. Such false-positive reactions could be distinguished by eye, since the signals were clearly weaker than those from homologous probe-target reactions. In all, cross reactions occurred in 15 of 834 possible instances.

Each target "spot" of DNA on the 38-strain nitrocellulose filters contained 100 ng of DNA. In order to determine the limits of sensitivity of each probe, an additional strip of nitrocellulose was included in each hybridization reaction carrying 10 ng, 1 ng, 100 pg and 10 pg spots of the corresponding purified, denatured DNA. Development of these strips revealed that the digoxigenin-labelled probes prepared from 10–20 bacterial colonies consistently detected 100 pg of purified homologous DNA.

Discussion

The purpose of present investigation was to determine the feasibility of a "reverse" hybridization method to identify pure cultures of subgingival species. In this method, DNA was rapidly extracted from small numbers of bacterial cells grown in pure culture, labelled with digoxigenin and hybridized against a range of purified, denatured reference strain DNAs fixed to nitrocellulose. This procedure allowed the identification or grouping of the unknown isolate. The "classical" hybridization technique, in which DNA from unknown microorganisms is fixed to a solid support and probes are constructed from purified reference DNAs, was thus reversed. In the present study, DNA was labelled with digoxigenin, but previous experiments indicated that, using the same relatively simple and rapid procedure described here, DNA could be extracted from 10^8 cells in a form and amount suitable for biotin-labelling with the random primer technique. A wider range of DNA concentration can be incorporated in the digoxigenin-labelling reaction (10 ng–3 μg, as opposed to 0.1–1 μg DNA in the biotin-labelling reaction), and in addition, the hybridization protocol recommended by Boehringer Mannheim for digoxigenin probes is simpler.

Many of the probes prepared in this study were species-specific. Cross reactions between closely related strains were anticipated, since whole genomic DNA was used for probe construction. As previously reported (5, 7, 9), heterologous probe-target reactions generate weaker signals than true-positive reactions present on the same filters, and therefore can be easily distinguished. The failure of the 2 *F. nucleatum* probes to detect all members of this species represented on filters reinforces the known heterogeneity of this "species" (1, 4, 6). It is of interest that *F. nucleatum* strain MG has been shown to differ from the 3 other test strains by SDS-PAGE (1), suggesting that DNA probe analysis may become a useful method to distinguish subgroups within this "species".

The reverse hybridization protocol has a number of attractions. Once pure cultures are available on agar media, probe construction, hybridizations and identification of unknown isolates can be accomplished within 2–3 days. Many cultures can be processed simultaneously. For example, one individual can conveniently extract DNA for probe construction from 48 cultures on one day, label the DNA and begin hybridizations the following day, and detect the spots the next morning. If necessary, labelled DNA preparations can be stored at −20°C for prolonged periods of time prior to hybridization. Nitrocellulose filters carrying reference strain target DNA can be prepared in bulk and stored until required, either in sealed bags at −20°C, or in a dessicator in a cold room. The variety of strains represented on filters can be "tailor-made", according to the investigator's interest, e.g. limited to suspected periodontopathic species, or expanded to encompass dozens or even the full range of species commonly found subgingivally. In terms of time and materials, the cost or this identification procedure is considerably lower than predominant cultivable techniques.

Whole chromosomal DNA probes have been successfully used to dinstinguish closely related species within the genus *Bacteroides* (5, 8). However, the potential difficulty in distinguishing closely related species is recognized. If the range of cross reactions for each reference strain on a filter is known, it should be possible to place unknown isolates into groups, which would allow their specific identification via ad-

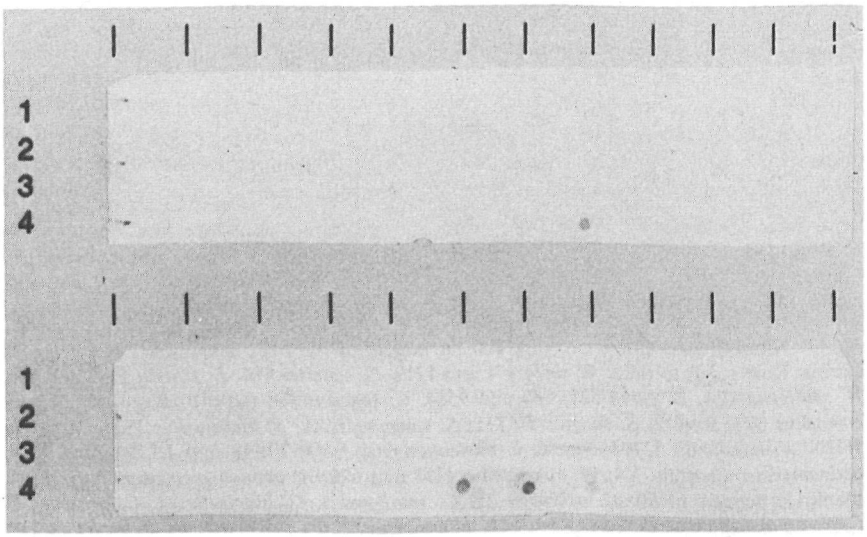

Fig. 2. Nitrocellulose filters as described in Fig. 1 legend. The top filter was hybridized with a digoxigenin-labelled DNA probe prepared with *B. heparinolyticus* 35895 DNA. The lower filter was hybridized with a digoxigenin-labelled DNA probe prepared with *B. zoogleoformans*

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cloned or oligonucleotide probes. Alternatively, it may be possible to avoid such cross reactions if species-specific cloned DNA fragments or specific oligonucleotide sequences were bound to filters and the reverse methodology employed.

There are a number of technical advantages to the reverse hybridization procedure. A major drawback of "classical" hybridization procedures employing biotin- or digoxigenin-labelled probes for strain identification is the need to remove cellular macromolecules, such as RNA and protein, from filters. Failure in this regard results in non-specific binding of the detection complexes used for these probes. Fixation of purified DNA to nitrocellulose for the reverse hybridization procedure eliminates the necessity of treating filters with enzymes or organic reagents. In addition, it appears that relatively small amounts of labelled DNA in a "sample" probe can find and attach to larger amounts of purified target DNA on the filter. This suggests that the method might be adapted to the identification of a range of species within one sample, thus avoiding the problems associated with aliquotting samples to several filters. The procedures described here are being investigated further to assess their potential for the direct identification of suspected pathogens and beneficial species in subgingival plaque samples.

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