JOURNAL OF CLINICAL MICROBIOLOGY, Sept. 1988, p. 1708–1713
0095-1137/88/091708-06$02.00/0

Vol. 26, No. 9

Simple Genetic Method To Identify Viridans Group Streptococci by Colorimetric Dot Hybridization and Fluorometric Hybridization in Microdilution Wells

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Received 16 February 1988/Accepted 6 June 1988

Simple dot hybridization and fluorometric hybridization methods in microdilution wells were designed and established for rapid and routine genetic identification of viridans group streptococci. Reference DNA extracted from each strain of 24 reference *Streptococcus* species was fixed both on a nitrocellulose filter and in a microdilution well. A 1-ml portion of the bacterial suspension which matched the turbidity of McFarland no. 2 standard was prepared when a streptococcal strain was isolated. It was lysed with achromopeptidase, and the DNA was quickly labeled with photobiotin under a sunlamp for 15 min. Dot hybridization and fluorometric hybridization were then carried out between the labeled DNA of the unknown organism and 24 unlabeled reference DNAs. Hybridized fragments on a nitrocellulose filter were detected by using alkaline-phosphatase-conjugated streptavidin and analyzed with a color graphic analyzer. Hybridized fragments in microdilution wells were quantitatively detected by using an enzyme, streptavidin-conjugated β-D-galactosidase, and a fluorogenic substrate, 4-methylumbelliferyl-β-D-galactoside. Strains belonging to each genetically distinct species could be identified by this dot blot hybridization test. However, some clinical strains cross-hybridized with two or more reference species, and then they were difficult to differentiate by dot blot hybridization. In such a case, fluorometric identification provided reliable results because the fluorometric method was more quantitative than dot blot identification. By these methods, it was possible to determine species assignment within the viridans group.

Quantitative DNA hybridization is an indispensable method for determining genetic relatedness among microorganisms. Quantitative DNA-DNA hybridization between atypical strains and the type strains of the most phenotypically related species offers reliable data to determine whether the organism is identical or closely related to the type strain. This method, however, requires radioisotopes and involves more complex procedures. Thus, its application is limited to research and reference laboratories.

Recently, many workers have reevaluated the taxonomy of the genus *Streptococcus* by DNA-DNA hybridization. Using this approach, many new species have been established, and strains which were thought to belong to several different Lancefield serogroups were proved to be a single species (1, 2, 7, 8, 12). These studies demonstrated that conventional phenotypic identification methods were sometimes unable to differentiate established species. Thus, we wished to simplify DNA-DNA hybridization and carry it out as a routine streptococcal identification method. There were two major problems with establishing this method as routine laboratory work. First, lysis of streptococci may be problematic when conventional enzymes are used. Second, a laboratory safety problem is posed by radioisotopes.

The former problem was resolved by the introduction of achromopeptidase (3), which can lyse most gram-positive organisms. The latter one was also solved by the development of a new nonradioactive method for labeling DNA.

Forster et al. (9) have reported a simple DNA-labeling method which uses photobiotin instead of radioisotope and nick translation. Since photobiotin bound DNA under visible light within a few minutes, labeling became a simple and rapid procedure. Visualization of biotinylated DNA was also simplified by the availability of commercial streptavidin-conjugated alkaline phosphatase. Thus, dot hybridization became easy to carry out; however, it is not suitable to handle many samples or to automate the procedures.

To carry out hybridizations in microdilution wells, we used a sensitive fluorogenic substrate, 4-methylumbelliferyl-β-D-galactoside, and a detection enzyme, streptavidin-conjugated β-D-galactosidase. Using these reagents, we have developed a method to identify species of streptococci.

MATERIALS AND METHODS

Immobilization of reference DNAs on nitrocellulose filters and microdilution wells. DNAs of 24 reference strains were prepared by the standard procedure of Marmur (14) with minor modifications (5), and the concentration was adjusted to 110 μg/ml in a 10-fold dilution of saline-trisodium citrate (0.1× SSC) buffer (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate). The DNA solution was then heat denatured, and the buffer concentration was increased to 2× SSC by adding 10 μl of 20× SSC solution. The solution was then serially diluted, and three DNA solutions of different concentrations (100, 25, and 6.25 μg/ml) of each reference strain were prepared. A 2-μl sample of each DNA solution was spotted on a nitrocellulose filter (Millipore HAHY; Nihon Millipore Kogyo K.K., Yonezawa, Japan). The filters, baked at 80°C for 2 h, could be stocked at room temperature in a desiccation chamber for several months.

A 100-μl portion of a 25-μg/ml concentration of each denatured reference DNA solution in phosphate-buffered saline (PBS; 8 mM Na_2PO_4, 1.5 mM KH_2PO_4 [pH 7.2], 137

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mM NaCl, 2.7 mM KCl) containing 0.1 M $MgCl_2$ (15) was incubated for 1 h at 37°C in a microdilution well (MicroFluor "B" Plate; Dynatech Laboratories, Inc., Alexandria, Va.), and then the solution was removed. The microdilution plate was irradiated under a UV transilluminator (model TS-36; Ultra-Violet Products Inc., San Gabriel, Calif.) with 1.5 kJ/m^2 to immobilize the DNA.

Small-scale extraction of DNA from clinical strains. Organisms were harvested from 1 to 3 ml of brucella HK broth (brucella broth supplemented with hemin and vitamin K_1; Kyokutou Corp., Tokyo, Japan) or Gifu Anaerobic Medium (Nissui Corp., Tokyo, Japan) and suspended in 1 ml of 50 mM EDTA buffer, pH 8.0. The bacterial suspension was adjusted to the turbidity of a McFarland no. 2 standard. The suspension was then transferred to an Eppendorf tube and centrifuged at 15,000 × *g* for 30 s in a microcentrifuge (KM-15200; Kubota Corp., Tokyo, Japan). Sedimented bacteria were suspended in 200 μl of 5 mM EDTA buffer (pH 8.0) and vortexed for a few seconds. The suspension was mixed with 40 μl of achromopeptidase (Wako Pure Chemical Corp., Osaka, Japan) solution (5 mg/ml) and incubated at 37°C for 30 min. Then, 50 μl of 20% sodium dodecyl sulfate (SDS) solution was added, and the reaction mixture was immediately placed in a 60°C water bath and incubated for 10 min. After the addition of 500 μl of phenolchloroform-isoamylalcohol (25:24:1, vol/vol/vol), the mixture was vortexed for 20 s. It was then centrifuged at 15,000 rpm for 5 min, and supernatant was transferred into a new Eppendorf tube. After the addition of 20 μl of 5 M NaCl, 2 volumes of ethanol were mixed with the supernatant to precipitate the DNA. After centrifugation, the sedimented crude DNA was rinsed with 70% ethanol and dried out. DNAs were then suspended in 100 μl of 0.1 M Tris hydrochloride (pH 7.5) and treated with 10 μl of RNase A (2 mg/ml; Sigma Chemical Co., St. Louis, Mo.) for 30 min at 37°C. DNA solutions were then treated with chloroform-isoamylalcohol (24:1, vol/vol) and then sedimented with ethanol. The DNAs were then suspended in 40 μl of distilled water. The amount of DNA prepared by the above method was determined by the modified diphenylamine method (4, 10).

Briefly, 30 μl of DNA solution was placed in a microtest tube, 30 μl of 20% perchloric acid was added, and then 60 μl of glacial acetic acid containing 4% diphenylamine was added to the tubes. A solution of acetaldehyde (10 μl; 0.16%) was added to the tube and heated for 10 min at 100°C. A_{595} and A_{700} were read by an enzyme immunoassay reader (model 2550; Bio-Rad Laboratories, Richmond, Calif.), and the value was compared with pure standard DNA (salmon sperm DNA; Sigma Chemical Co.).

Photobiotinylation of DNA. Photobiotin (2 μl; Vector Laboratories, Inc., Burlingame, Calif.) and an equal volume of denatured DNA solution (0.5 to 5 μg of DNA) in distilled water were mixed in an Eppendorf tube, cooled in an ice-water bath, and then irradiated with a sunlamp (500 W) at a distance of 10 cm for 15 min. After labeling, the total volume of the reaction mixture was increased to up to 100 μl by adding 0.1 M Tris hydrochloride buffer, pH 9.0. Then, 100 μl of 2-butanol was added to the tube. The mixture was thoroughly mixed and centrifuged, and the upper phase was discarded. This 2-butanol extraction procedure was repeated twice to remove free photobiotin. This labeled DNA preparation was immediately used for hybridization experiments.

Dot hybridization. Hybridization reactions were carried out as described by Leary et al. (13) with minor modifications (4). Briefly, the labeled and denatured DNA (0.5 to 5 μg) was prepared for one dot hybridization experiment with a filter size of 100 cm^2. The filter was completely soaked in 10 ml of 10× Denhardt solution in 2× SSC buffer, kept for 1 h at 45°C, and then soaked in prehybridization mixture (4× Denhardt solution, 2× SSC, 0.1× SDS, 200 μg of denatured salmon sperm DNA per ml, 50% formamide) for 20 min at 45°C. Hybridization was carried out for 6 h at 45°C in 10 ml of hybridization solution (2× Denhardt solution, 2× SSC, 0.1× SDS, 50 μg of denatured salmon sperm DNA per ml, and 50% formamide) containing variable amounts of labeled DNA (from 3 to 25 μg). After hybridization, the filter was washed at room temperature, twice with 2× SSC buffer containing 0.1% SDS and three times with 0.2× SSC buffer containing 0.1% SDS.

Detection of biotinylated DNA on nitrocellulose filters. The washed filter was dipped in buffer 1 (0.1 M Tris hydrochloride, 0.1 M NaCl, 2 mM $MgCl_2$, 0.05% Triton X-100 [pH 7.5]) with 3% bovine serum albumin at 42°C for 20 min. The filter was transferred into 5 ml of buffer 1 with 5 U of streptavidin-alkaline phosphatase (ZYMED, San Francisco, Calif.) and incubated at 37°C for 10 min. The filter was washed three times with buffer 1 and twice with buffer 2 (0.1 M Tris hydrochloride, 0.1 M NaCl, 50 mM $MgCl_2$ [pH 9.5]). The filter was incubated in 10 ml of buffer 2 with substrates (2.5 mg of Nitro Blue Tetrazolium and 1.25 mg of 5-bromo-4-chloro-3-indolylphosphate) until hybridized spots became visible (from 15 min to 2 h). When several reference DNA spots became visible, their color intensities were measured with a color graphic analyzer (TEF Corp., Tokyo, Japan). The color intensity of control salmon sperm DNA spot was calculated as background, and the most strongly hybridized spot was determined.

Dot hybridization among viridans streptococci with radioisotope. [^{32}P]thymidine (Amersham-Japan, Tokyo, Japan)-labeled DNAs of nine type strains were prepared by the nick translation method described previously (5). Reference DNA filters were prepared by the method described above. Hybridization and filter washing were also carried out under the same experimental conditions as the nonradioisotope method described above. X-ray films for autoradiography (Fuji X-ray film; Fuji Photofilm Corp. Ltd., Asikaga, Japan) were exposed overnight at −80°C.

Fluorometric identification of viridans group streptococci in microdilution wells. Prehybridization solution (200 μl; 20 mM phosphate buffer [pH 6.5], 2× SSC, 5× Denhardt solution, 50% formamide) containing 200 μg of denatured salmon sperm DNA per ml was added to each microdilution well, which was already coated with reference DNA, and incubated at 45°C for 1 h. The prehybridization solution was discarded and replaced with 100 μl of hybridization solution (20 mM phosphate buffer [pH 6.5], 2× SSC, 2× Denhardt solution, 50% formamide) containing 50 μg of denatured salmon sperm DNA per ml. DNA extracted from a clinical strain by a small-scale method was labeled with photobiotin, and the photobiotinylated DNA was added to the hybridization mixture. Hybridization was carried out overnight at 45°C.

The microdilution well was then washed three times with 200 μl of PBS containing 0.1% Triton X-100. PBS buffer (200 μl) containing 2% bovine serum albumin and 0.1% Triton X-100 was added, and the mixture was incubated at 37°C for 10 min. The solution was discarded, and 100 μl of 0.3 U of streptavidin-conjugated β-D-galactosidase (ZYMED Laboratories) per ml in PBS buffer with 2% bovine serum albumin and 0.1% Triton X-100 was added to each well and incubated at 37°C for 30 min. After incubation, the well was washed three times with PBS buffer containing 0.1% Triton X-100.

TABLE 1. Sensitivity to achromopeptidase and yield of extracted DNA from 24 reference strains of streptococci

Reference strain	Sensitivity to achromopeptidase[a]	Amt of DNA extracted (μg)[b]
S. anginosus NCTC 10713	+++	10–15
S. intermedius ATCC 27335	+++	7–15
S. constellatus ATCC 27823	+++	8–13
S. salivarius ATCC 7073	+++	7–10
S. morbillorum ATCC 27824	+++	15–18
S. mitis NCTC 3165	++	6–10
S. sanguis ATCC 10556	+	5–7
S. oralis NCTC 11427	++	5–9
S. pneumoniae NCTC 7465	+++	15–18
S. mutans ATCC 25175	+++	9–13
S. bovis NCDO 597	++	5–8
S. acidominimus NCDO 2025	++	8–10
S. uberis NCTC 3858	+++	8–15
S. parvulus ATCC 33793	+++	10–15
S. pleomorphus ATCC 29734	+++	13–15
S. hansenii ATCC 27752	+++	11–15
S. pyogenes ATCC 12344	+++	10–15
S. agalactiae NCTC 8181	+++	10–16
"*S. equisimilis*" NCTC 8543	+++	13–15
S. canis ATCC 43496	+++	11–17
S. equi NCTC 9682	+++	13–15
S. porcinus NCTC 10228	+++	9–15
S. iniae ATCC 29178	++	7–10
S. suis NCTC 10237	+++	7–15

[a] Final concentration of achromopeptidase was 1,000 U/ml. +++, Turbidity of a bacterial suspension (optical density at 600 nm) was decreased by 70% or more in 30 min; ++, turbidity was decreased by 50 to 70% in 30 min; +, turbidity was decreased by 30 to 50% in 30 min.

[b] DNA was recovered from 1 ml of a bacterial suspension which matched the turbidity of McFarland no. 2 standard. Values were obtained from three independent experiments.

4-Methylumbelliferyl-β-D-galactoside (50 μl; 3×10^{-4} M; Koch-light Ltd., Haverhill, Suffolk, England) in PBS buffer containing 2% bovine serum albumin and 0.1% Triton X-100 was added, and the plates were incubated at 30°C for 60 min. The fluorescence intensity was measured with a MicroFluor reader (Dynatech) at wavelengths of 360 nm for excitation and 450 nm for emission. Fluorescence intensity of the well of salmon sperm DNA was calculated as 0, and the intensity of the well which emitted strongest fluorescence was calculated as 100%.

RESULTS

Lysis of streptococci with achromopeptidase and small-scale DNA extraction. The 24 reference strains listed in Table 1 were lysed well with achromopeptidase, and their DNAs were sufficiently extracted from 1 ml of each bacterial suspension which matched the turbidity of McFarland no. 2 standard. Total amounts of crude DNA thus obtained were between 5 and 18 μg (Table 1). DNAs from clinical strains were also sufficiently extracted by this method, and the amount of DNA ranged from 3 to 20 μg (data not shown).

Differentiation of reference strains of viridans group streptococci by colorimetric dot blot and fluorometric hybridization. The amount of labeled DNA and incubation time for color detection variably influenced the color strength of hybridized spot. At first, we used 5 to 20 ng of labeled DNA per ml for one dot hybridization experiment with a 100-cm^2 filter size. With these amounts of labeled DNA, the number of cross-hybridized spots was decreased but color intensity was not clear enough to analyze the results by a graphic analyzer. Therefore, we increased the labeled DNA concentration to 200 ng/ml, and consequently, the degrees of cross-hybridization increased. However, results of analysis by the color graphic analyzer showed a difference in color intensity between homologous DNA and DNA from a different species. This is seen as a difference in intensity in peak area (see Fig. 2 for calculation).

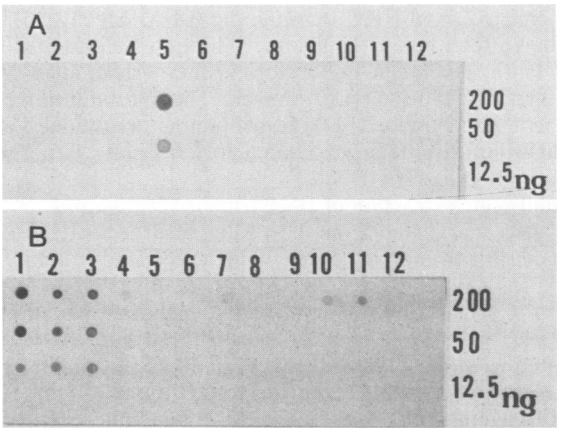

FIG. 1. Dot hybridization of DNA from 12 reference strains of streptococci with biotin-labeled DNA from two species. Hybridization with labeled DNA from type strains *S. morbillorum* ATCC 27824 (A) and *S. anginosus* NCTC 10713 (B). The amount of DNA is indicated on the right. DNAs of strains 1 through 12 are in the order shown in Table 1. Lanes: 1, NCTC 10713; 2, ATCC 27335; 3, ATCC 27823; 4, ATCC 7073; 5, ATCC 27824; 6, NCTC 3165; 7, ATCC 10556; 8, NCTC 11427; 9, NCTC 7465; 10, ATCC 25175; 11, NCDO 597; 12, NCDO 2025.

Genetically distinct organisms such as *S. morbillorum* (Fig. 1A) and *S. agalactiae* did not cross-hybridize with other reference organisms. However, organisms highly cross-hybridized by ^{32}P-labeled dot hybridization (see Fig. 3) also cross-hybridized by this photobiotin-labeled dot hybridization. Cross-hybridization among the species *S. intermedius*, *S. anginosus*, and *S. constellatus* is a good example (Fig. 1B and 2). When *S. anginosus* was labeled, 200 ng of DNA spots of these three species were strongly cross-hybridized and 50 and 12.5 ng of DNA spots were also cross-hybridized, but color intensity of these smaller amounts of DNA spots could be differentiated visually. Differences in their color intensities could be clearly quantitated by a color graphic analyzer (Fig. 2). The incubation time for the enzyme reaction also influenced the color intensities of hybridized spots. Results shown in Fig. 2 were obtained from the colorimetric analysis of 50 ng of DNA spots after a 30-min enzyme reaction.

Fluorescence intensity of each microdilution well was measured at 10-min intervals. Enzyme reaction for fluorometric hybridization was usually stopped after 60 min of incubation; however, the reaction was stopped when fluorescence intensity of the highest count reached 3,000 relative fluorescence units.

To compare nonradioactive dot hybridization with dot blots using radioisotopically labeled DNA, ^{32}P autoradiography was carried out (Fig. 3). *S. anginosus*, *S. constellatus*, and *S. intermedius* could not be differentiated by this method. *S. sanguis*, *S. mitis*, and *S. oralis* also cross-hybridized (Fig. 3).

Identification of clinical strains of streptococci by dot blot and fluorometric hybridizations. DNA from clinical strains was extracted, and dot blot and fluorometric hybridizations

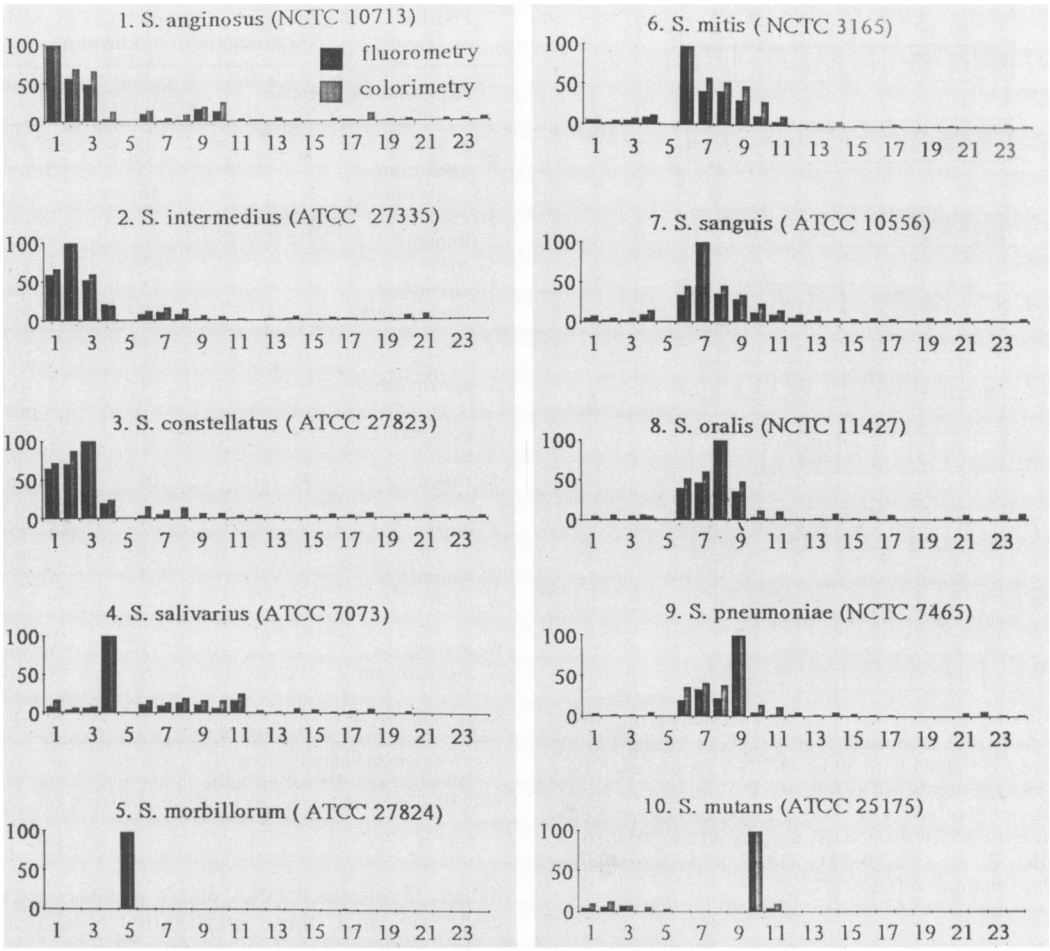

FIG. 2. Colorimetric and fluorometric identification of viridans group streptococci. Ten reference strains of viridans group streptococci were labeled with photobiotin, and both dot blot and fluorometric hybridizations were carried out. Numbers on the left indicate homology values, and strains 1 through 24 (horizontal axis) are in the order shown in Table 1.

were carried out (Table 2). Some strains of viridans group streptococci (*S. mitis*, *S. salivarius*, *S. oralis*, *S. sanguis*, and *S. morbillorum*) identified by conventional biochemical methods (6) did not hybridize with any of the reference strains. Seven clinical strains identified as *S. intermedius* and two strains identified as *S. constellatus* by biochemical tests were genetically identified as *S. anginosus* by dot blot and fluorometric hybridizations.

DISCUSSION

Most clinical strains of streptococci were lysed well with achromopeptidase. Prolongation of bacterial incubation time, however, influenced lytic activity of achromopeptidase. Certain strains of streptococci required more rapid growth under anaerobic conditions.

Labeled DNA concentration and the length of incubation influenced the color intensity of the hybridized spot. Changing the incubation time for the enzyme reaction was the easiest way to control the extent of hybridization. By using the color intensity as a marker, the enzyme reaction was stopped as soon as a spot became visible. However, in ^{32}P autoradiography, proper exposure time was not easily estimated. Genetically closely related strains of three species (*S. anginosus*, *S. intermedius*, and *S. constellatus*) were not differentiated by this radioisotope method.

When a streptococcal strain was isolated, we first carried out dot hybridization and in most cases, in order to identify it, organisms were identified only by dot hybridization. When organisms cross-hybridized strongly with several species, we carried out fluorometric hybridization in microdilution wells to confirm our identification. Because fluorometric hybridization was more quantitative than dot blot hybridization, even closely related organisms could easily be differentiated.

Several clinical strains biochemically identified as *S. intermedius* were genetically identified as *S. anginosus* by both dot hybridization and fluorometric hybridization (Table 2). Thus, the GIFU 7619 strain identified as *S. intermedius* by biochemical tests was confirmed as *S. anginosus* by genetic analysis. The two organisms are different only in hemolytic activity. *S. anginosus* is beta-hemolytic, and *S. intermedius* is not hemolytic.

Coykendall et al. (1) recently proposed to include *S. intermedius* and *S. constellatus* in *S. anginosus*. Though *S. constellatus* is lactose negative, its other biochemical characteristics are identical to those of *S. intermedius* (6).

Group G beta-hemolytic streptococci of human origin hybridized with "*S. equisimilis*" NCTC 8543 (Table 2). "*S. equisimilis*" has also been assigned to beta-hemolytic isolates of group C streptococci of human origin. However, the

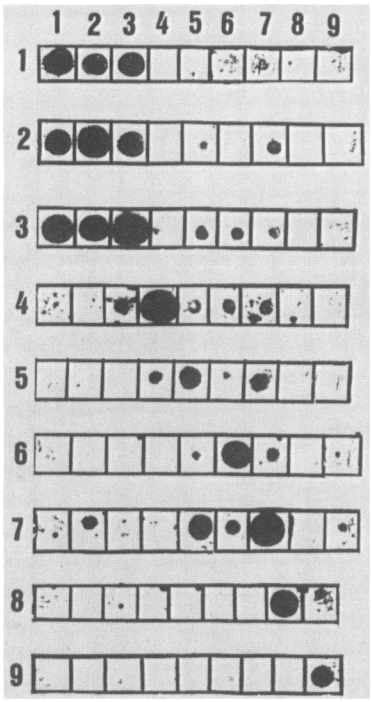

FIG. 3. Autoradiography of dot blots with viridans group streptococci. ^{32}P-labeled DNA from nine type strains of viridans group streptococci were hybridized with 50 ng of DNA spot of each type strain. Organisms were numbered as follows: 1, *S. anginosus* NCTC 10713; 2, *S. intermedius* ATCC 27335; 3, *S. constellatus* ATCC 27823; 4, *S. salivarius* ATCC 7073; 5, *S. sanguis* ATCC 10556; 6, *S. mitis* NCTC 3165; 7, *S. oralis* NCTC 11427; 8, *S. morbillorum* ATCC 27824; 9, *S. mutans* ATCC 25175.

name was not included in the *Approved Lists of Bacterial Names* and thus lost its standing in nomenclature. Farrow (7) suggested that this organism and beta-hemolytic group G streptococci are genetically closely related to *S. dysgalactiae*.

Fourteen clinical isolates identified as *S. intermedius*, *S. salivarius*, *S. mitis*, *S. oralis*, and *S. morbillorum* did not hybridize as expected with the type strain. Thus, the hybridization data confirm the findings of others (8, 12). This might be attributed to the fact that chemotaxonomic reevaluation of current streptococcal taxonomy is not complete. Lancefield serogrouping had been considered a very important test for identifying streptococci, but recently several genetic studies revealed that one species carries many different Lancefield antigens (1, 2, 7). Species assignment using hemolytic activity is also problematic (1). Johnson (11) suggested that a species should be genetically independent and that the homology value among established species and other species should be less than 60% under an optimal condition (11). However, in medical microbiology, there are many cases in which two genetically closely related species are allowed to exist as independent species because one species is pathogenic in humans and the other is not.

We have so far successfully applied the dot hybridization and fluorometric hybridization methods for the identification of both gram-positive and -negative organisms, such as staphylococci, peptostreptococci, nonfermenters, vibrios, legionellae, and campylobacters. If all species were reevaluated and the taxonomy was rearranged by these criteria, identification by hybridization could become well established. If reference DNA plates or filters became commercially available, these quantitative hybridization methods would facilitate rapid and genetic identification of clinically important isolates.

TABLE 2. Identification of streptococci by both dot blot and fluorometric hybridizations

Phenotypic identification	No. of strains used	Genetic identification (no. of strains)[a]
S. pyogenes	7	*S. pyogenes* (7)
S. agalactiae	5	*S. agalactiae* (5)
"*S. equisimilis*"	16	"*S. equisimilis*" (16)
Streptococcus sp. group G (human)	12	"*S. equisimilis*" (12)
S. canis	5	*S. canis* (5)
S. anginosus	15	*S. anginosus* (15)
S. intermedius	17	*S. intermedius* (5); *S. anginosus* (7); no reaction (5)[b]
S. constellatus	4	*S. anginosus* (2); *S. intermedius* (2)
S. porcinus	3	*S. porcinus* (3)
S. suis	3	*S. suis* (3)
S. salivarius	10	*S. salivarius* (8); no reaction (2)
S. mitis	7	*S. mitis* (2); no reaction (5)
S. sanguis	5	*S. sanguis* (5)
S. oralis	5	*S. oralis* (4); no reaction (1)
S. mutans	3	*S. mutans* (3)
S. morbillorum	6	*S. morbillorum* (5); no reaction (1)
S. pneumoniae	7	*S. pneumoniae* (7)

[a] Organism hybridized strongly.

[b] These strains did not hybridize to any type strain. When these strains were labeled, no microdilution well reached 1,000 fluorescence counts within 3 h.

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