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Development of PCR-Based Hybridization Protocol for Identification of Streptococcal Species

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16S rRNA of *Streptococcus agalactiae*, *S. uberis*, and *S. parauberis* was bound to streptavidin-coated magnetic beads by using a biotinylated oligonucleotide probe complementary to a highly conserved region of the molecule. In-solution hybridization of radiolabelled oligonucleotide probes to immobilized 16S rRNA allowed the specific identification of *S. agalactiae* and *S. parauberis* but not *S. uberis*. PCR was used to amplify a species-specific region of the 16S rRNA gene from these species. One of the PCR primers was biotinylated at the 5′ end to allow purification of the amplified product on streptavidin-coated magnetic beads and subsequent denaturation to yield immobilized single-stranded DNA. Radiolabelled oligonucleotide probes were hybridized in solution to the single-stranded target molecule and enabled species-specific identification of the target organism. This protocol overcame problems associated with hybridization of the *S. uberis*-specific probe to 16S rRNA in solution. A similar procedure may enable the specific detection of other streptococci which exhibit a species-specific sequence in this region of the gene.

The identification of streptococci currently relies heavily on serological grouping by use of Lancefield antisera (14) and analysis of biochemical phenotypes. As a result, streptococci are frequently referred to as belonging to a Lancefield group, group A (*Streptococcus pyogenes*) or group B (*S. agalactiae*), for example. In the two cases cited, the procedure works well, but for other, more heterogeneous groups of streptococci, serogrouping can be misleading. For example, *S. uberis* is regarded as Lancefield group E, but in some studies it has been shown that approximately 50% of isolates are serologically ungroupable (16) and the remaining isolates react with either Lancefield group E, C, D, P, or U antiserum. Further confusion may arise from the observation that different species may express the same Lancefield group antigen. Hence, streptococci classed as Lancefield group C may belong to the species *S. dysgalactiae*, *S. equi*, *S. equisimilis*, or *S. zooepidemicus* (10).

The development and application of nucleic acid-based diagnostic techniques for the identification and classification of streptococci and other genera of bacteria has been described in the literature with increasing frequency (2, 6, 9, 13, 20). These methods avoid the subjectivity associated with the interpretation of results obtained with conventional test schemes and, by referring to natural taxonomic groupings, remove some of the confusion arising from cross-reactions of Lancefield antisera.

One of the molecules most suited to these purposes is small-subunit (16S) rRNA. 16S rRNA is present in actively growing cells at a high copy number (25). Also, sequence comparisons of 16S rRNAs have shown that some segments of the molecule are highly conserved while others vary (12). The overall variability of rRNA sequences is sufficiently stable to allow investigation of phylogenetic relationships (27), which can be calculated from sequence differences. In many instances, there are enough stable sequence differences in variable regions to allow the design of species-specific oligonucleotide probes which can be used for diagnostic purposes (3, 8, 24). Previous work described the 16S rRNA sequences of 31 species of *Streptococcus* (2) and the identification of a highly variable diagnostic region of sequence in the V2 area of the molecule (3, 26). In this paper, we describe the use of a technique to hybridize oligonucleotide probes to 16S rRNA or PCR-amplified rRNA genes (rDNA) of *S. agalactiae*, *S. uberis*, and *S. parauberis* in solution.

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MATERIALS AND METHODS

Bacterial strains and culture. *S. uberis* ST10, isolated from a clinical case of bovine mastitis, was obtained from the culture collection at the Institute for Animal Health (Compton, United Kingdom). Strain 8 of *S. agalactiae* was obtained from the Veterinary Investigation Centre (Weybridge, United Kingdom), and *S. parauberis* NCDO 651 was obtained from the National Collection of Food Bacteria (Reading, United Kingdom). All strains were grown in Todd-Hewitt broth (THB) (Oxoid) at 37°C overnight and stored in THB containing 50% glycerol (vol/vol) at −70°C.

Extraction of bacterial nucleic acids. Total cellular RNA was extracted as described by Embley et al. (7), and solutions at a concentration of 4 mg/ml were stored at −70°C.

Extraction of chromosomal DNA. Chromosomal DNA was isolated by using a modification of the method described by Hill and Leigh (11). Cells from 1.5 ml of overnight culture were washed once in 1.0 ml of 10 mM Tris–5 mM EDTA at a final pH of 7.8 and resuspended in 350 μl of the same buffer. After the addition of 25 μl of mutanolysin (5,000 U/ml; Sigma) to *S. uberis* and *S. parauberis* cell suspensions or 20 μl of lysozyme (50 mg/ml) to *S. agalactiae* and *S. dysgalactiae* cell suspensions, bacteria were incubated at 37°C for 30 min and lysed by the addition of 20 μl of sodium dodecyl sulfate (SDS) (20% [wt/vol] in 50 mM Tris–20 mM EDTA [pH 7.8]) and then 3 μl of proteinase K (20 mg/ml; Sigma). Bacteria were then incubated for a further 1 h at 37°C. Protein was precipitated by the addition of 200 μl of saturated sodium chloride (approximately 6.0 M) followed by agitation for 15 s and removed by centrifugation (7,000 × *g*) for 10 min. The supernatant was removed and mixed with 200 μl of Tris (0.1 M, pH 8.0)-equilibrated phenol and 200 μl of chloroform-isoamyl alcohol (49:1 [vol/vol]). The mixture was centrifuged (7,000 × *g*) for 10 min, the upper aqueous phase was removed, and the DNA from this was precipitated by the addition of 2.5 volumes of ethanol and 30 μl of sodium acetate (1 M) and incubation at −20°C for 2 h. The resulting precipitate was collected by centrifugation at 7,000 × *g* for 5 min, dried under a vacuum, and rehydrated in 30 μl of Tris-EDTA buffer (10 mM Tris, 1.0 mM EDTA [pH 7.5]). DNA concentration was determined by measuring A_{260} with a Shimadzu UV-160A spectrophotometer.

Oligonucleotide probes used for hybridizations. The oligonucleotide probes were supplied in aqueous solution by British Biotechnology Products Ltd. (Abingdon, United Kingdom). Probe sequences are shown in Table 1. Probe BB9966 was complementary to the highly conserved nucleotide positions 260 to 307 (5) of the 16S rRNA molecule and was supplied biotinylated at the 5′ end to allow capture on streptavidin-coated magnetic beads (Dynabeads M-280 Streptavidin).

Probe BB9971 was complementary to the highly conserved nucleotide positions 395 to 415 of 16S rRNA and was used as a positive control to identify the presence of captured 16S rRNA and 16S rDNA. The species-specific probes for

TABLE 1. Oligonucleotide probes used for hybridization in solution

Probe	Specificity	Sequence (5′ to 3′)
BB9966	Capture probe	CCGTGTCTCAGTCCCAGTGTGGCCGATCACCCTCTCAGGTCGGCTATG
BB9967	*S. agalactiae*	ACTAACATGTGTTAATTACTCTTA
BB9969	*S. parauberis*	AGTACATGAGTACTTAATTGTCA
BB9970	*S. uberis*	GGTACATGTGTACCCTATTGTC
BB9971	Universal	TTCTTCACTCACGCGGCGTTG

S. agalactiae, *S. parauberis*, and *S. uberis* were complementary to nucleotide positions 179 to 202, 179 to 201, and 179 to 203 (5), respectively.

Labelling of oligonucleotide probes with ^{32}P. A single 10-μl labelling reaction mixture was made by mixing 3.5 pmol of the oligonucleotide, 50 μCi (5 μl) of [γ-^{32}P]dCTP, 1.0 μl of 10× PNK reaction buffer (500 mM Tris-HCl [pH 7.6], 100 mM $MgCl_2$, 50 mM dithiothreitol, 1 mM spermidine, 1 mM EDTA), and 2 U of T4 polynucleotide kinase (Boehringer, Mannheim, Germany). The reaction mixture was incubated at 37°C for 45 min, and the reaction was terminated by the addition of 15 μl of TNES (0.14 M NaCl, 20 mM Tris-HCl [pH 7.5], 5 mM EDTA, 0.1% SDS). The labelled probe was removed from unincorporated nucleotides by centrifugation through a Sephadex G-25 spin column. Labelled probe was stored at −20°C and used within 2 weeks. The specific activity of oligonucleotide probes was determined by methods described by Sambrook et al. (19).

Hybridization of oligonucleotide probes to 16S rRNA. Magnetic beads precoated with streptavidin (Dynabeads M-280, product no. 112.05; Dynal, Oslo, Norway) were washed twice in an equal volume of BW buffer (5 mM Tris-HCl [pH 7.5], 0.5 mM EDTA, 1 M NaCl) and resuspended at 10 mg/ml in BW buffer. Beads were separated from solutions by using a magnetic stand as recommended by the manufacturers. Extracted RNA (0.2 μg in 2 μl, approximately 20 fmol), biotinylated capture probe BB9966 (200 fmol), and detection probe (approximately 10^6 cpm) were added to 78 μl of hybridization solution (0.25 M sodium phosphate [pH 7.2] containing 7% [wt/vol] SDS and 1% [wt/vol] acetylated bovine serum albumin), and the mixture was heated to 95°C for 5 min. Samples were cooled to a hybridization temperature of 48°C, and 20 μg of prewashed magnetic beads was added. Incubation at 48°C was continued for 30 min with occasional mixing. Following hybridization, beads were washed once in 2× SSC (0.3 M sodium chloride and 0.03 M trisodium citrate) containing 0.1% (wt/vol) SDS for 5 min at room temperature, twice in 1× SSC containing 0.1% SDS for 15 min at the hybridization temperature, and once in 1× SSC containing Triton X-100 (0.05% [vol/vol]) for 5 min at room temperature. Beads were resuspended in 80 μl of 5× SSC and 5 ml of Optiphase Safe scintillation fluid (LKB, St. Albans, United Kingdom). The amount of hybrid complex bound to the beads was measured in the ^{32}P channel of a Tri-Carb 4660 scintillation counter (Canberra-Packard, Pangbourne, United Kingdom). The radioactivity of wash solutions was also measured. The radioactivity retained on the beads was expressed as a percentage of the total radioactivity present in wash solutions plus that retained on the beads.

Determination of 16S rRNA secondary structure. The secondary structure of 16S rRNA in the probe target regions was calculated by using the program FOLD (28) from the University of Wisconsin Molecular Biology package.

Use of PCR to amplify the V2 region of 16S rRNA. The sense primer (AR1) 5′-GAGAGTTTGATCCTGGCTCAGGA-3′, corresponding to nucleotide positions 8 to 30 of the rDNA (5), was supplied by British Biotechnology Products Ltd. and was biotinylated at the 5′ end to allow capture on streptavidin-coated Dynabeads (Dynal). The antisense primer (KK) sequence, 5′-TTACCGCGGC TGCTGGCACGT-3′, corresponded to nucleotide positions 536 to 516 of the 16S rRNA gene and was also supplied by British Biotechnology Products Ltd.

PCR buffer (1 ml) was made by mixing 100 μl of 10× Mg^{2+}-free PCR buffer (Promega), 2 μl of a 100 mM solution of deoxynucleoside triphosphates (Boehringer), 60 μl of a 25 mM solution of $MgCl_2$ (Promega), 500 pmol of oligodeoxynucleotide AR1, 500 pmol of oligodeoxynucleotide KK, and distilled water to a final volume of 1 ml. Aliquots (100 μl) of this mixture were stored at −20°C.

Amplification was carried out by mixing chromosomal DNA (20 ng), 2 U of *Taq* DNA polymerase (Promega), and 100 μl of PCR buffer. The samples were overlaid with 70 μl of mineral oil, and a programmable thermal controller (MJ Research Inc.) was used to subject samples to 30 cycles of denaturation at 92°C for 2 min, reannealing at 55°C for 1 min, and extension at 72°C for 1.5 min. After the final cycle, chain extension was completed by incubating samples at 72°C for 5 min.

Southern hybridization of the amplified product (21) was performed according to the method of Sambrook et al. (19) by using probe BB9966 labelled with digoxigenin according to the manufacturer's protocols (Boehringer).

Separation of ssDNA from double-stranded PCR product by using magnetic particles. Single-stranded DNA (ssDNA) was produced from double-stranded amplification product according to the instructions of the manufacturer of the magnetic particles (Dynal). The method consisted of four steps: bead preparation, PCR product immobilization, DNA duplex melting, and strand separation. Dynabeads (200 μg) were washed twice in 20 μl of 2× BW buffer and resuspended in 40 μl of the same buffer. PCR product (40 μl) was mixed with the prepared bead solution, and the mixture was incubated for 15 min at room temperature. The beads were resuspended occasionally during the incubation by gently tipping the tube. The beads coated with double-stranded DNA were pulled to one side of the tube by using the magnetic stand, and the bead-free solution was removed. Beads were resuspended in 10 μl of NaOH (0.1 M) and incubated for a further 10 min at room temperature to dissociate the nucleic acid strands. Dynabeads were collected on the side of the tube, and the NaOH solution containing the nonbiotinylated single strand was removed. A further 40 μl of NaOH (0.1 M) was mixed with the beads, and the mixture was incubated for 5 min. The NaOH solution was removed as before, and the beads were washed once with 40 μl of BW buffer, finally resuspended in 40 μl of Tris-EDTA buffer, and stored at 4°C.

Hybridization to ssDNA. Approximately 250 fmol of target ssDNA attached to 200 μg of magnetic beads and 10^6 cpm of ^{32}P-labelled detection probe were added to 78 μl of hybridization buffer, after which the mixture was heated to 60°C for 5 min, cooled slowly to 45°C, and allowed to hybridize for 1 h at 45°C with occasional mixing by tipping the tube. Hybridization, washing, and detection were carried out as described above for hybridization of 16S rRNA.

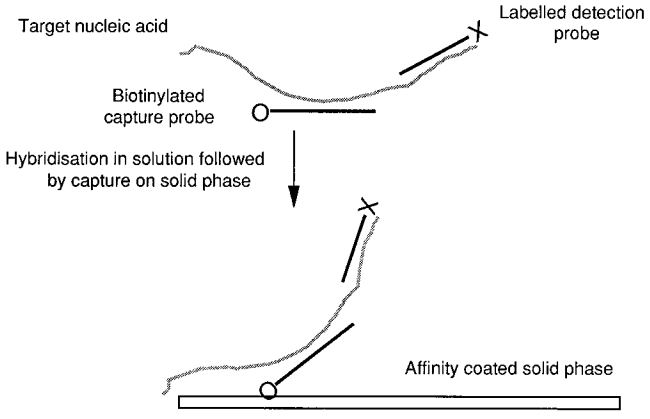

FIG. 1. Solution phase sandwich hybridization.

RESULTS AND DISCUSSION

Hybridization of probes to 16S rRNA in solution. Previous work has described the 16S rRNA sequences of 31 species of *Streptococcus* (2) and the identification of a highly variable diagnostic region of sequence in the V2 area of the molecule

TABLE 2. Hybridization in solution with ssDNA and rRNA as target molecules

Probe	Result for indicated species and nucleic acid target[a]					
	S. agalactiae		*S. parauberis*		*S. uberis*	
	DNA	RNA	DNA	RNA	DNA	RNA
BB9967	12.92	0.53	<0.01	<0.01	<0.01	<0.01
BB9969	<0.01	<0.01	12.32	1.03	0.06	<0.01
BB9970	0.03	<0.01	0.03	<0.01	2.54	<0.01
BB9971	7.91	0.50	8.61	1.02	8.43	0.84

[a] Figures are percent total radioactivity bound to beads, taking into account labelling efficiency.

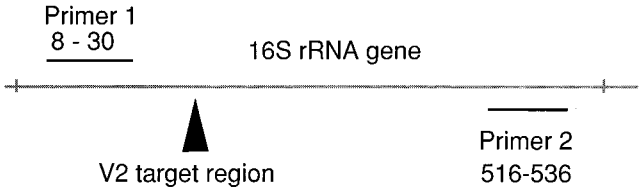

FIG. 2. Probe and primer sites within 16S rDNA. Numbers refer to nucleotide positions.

(1, 26). Oligonucleotide probes complementary to this region of sequence were used for hybridization to nucleic acids of *S. agalactiae*, *S. parauberis*, and *S. uberis* in solution.

Solution phase sandwich hybridization relies on hybridization of both the capture and detection probes to the target nucleic acid in solution. Hybrids are subsequently captured onto a solid phase via an affinity label on the capture probe (Fig. 1) (22). Unbound capture probe also binds to the affinity label, but only those capture probes that have bound target nucleic acids will have detection probes attached and generate a signal. The capture probe used in the present investigation (BB9966) was complementary to a highly conserved 16S rRNA sequence and was added to the hybridization mixture along with the rRNA target and radiolabelled species-specific detection probe prior to heat denaturation. The magnetic beads were added to capture probe-RNA hybrids once the mixture had cooled. In this way, all nucleic acid interactions occurred in solution, the most efficient format for hybridization (4).

The use of 16S rRNA as the target molecule for hybridization was successful for the specific detection of nucleic acids from *S. agalactiae* and *S. parauberis*, where 0.53 and 1.03%, respectively, of the total label added was bound (Table 2). However, the method failed to detect rRNA from *S. uberis*, where <0.01% of the total labelled probe was immobilized. The universal probe BB9971 hybridized to rRNA from all species tested, giving signals of 0.5% of total label added for *S. agalactiae*, 1.02% for *S. parauberis*, and 0.84% for *S. uberis* (Table 2).

Inhibition of hybridization due to secondary structure of 16S rRNA. The lack of hybridization between the *S. uberis*-specific probe and *S. uberis* rRNA was unlikely to have been due to degradation of the target nucleic acid, since the universal probe, the target for which is further from the capture sequence than the species-specific sequences are (Fig. 2), was capable of 16S rRNA detection, indicating target integrity. A more plausible cause of the failure of the species-specific probe to detect *S. uberis* RNA may be the secondary structure of the 16S rRNA molecule, which has been predicted to be a very complex mixture of hairpin and loop structures (15). The secondary structure of 16S rRNA in the species-specific probe regions was calculated for *S. agalactiae*, *S. parauberis*, and *S. uberis*. A very tight secondary structure for *S. uberis* (Fig. 3) compared with those for *S. agalactiae* (Fig. 4) and *S. parauberis* (Fig. 5) was predicted. It is possible that the tight hairpin structure of the *S. uberis* probe region prevents the species-specific probe from binding. Alternatively, if the probe does bind (after the RNA structure has been opened by heat denaturation), it could be displaced by reannealing RNA-RNA duplex, which is predicted to be more thermodynamically stable than RNA-DNA hybrids (17). The theoretical determination of secondary structure may not fully represent the state of the target molecule in solution, as it does not take into account the three-dimensional structure of the 16S rRNA, which might also exert an influence on target site availability and probe hybridization.

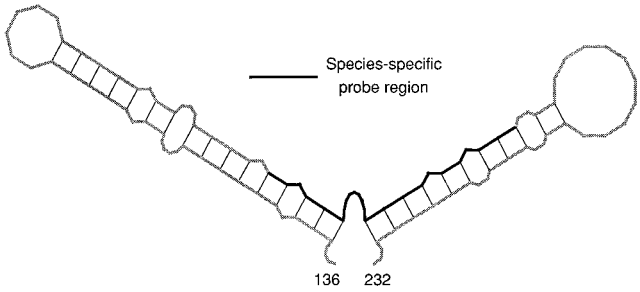

FIG. 4. Secondary structure of nucleotide positions 136 to 232 of *S. agalactiae* 16S rRNA.

PCR amplification of 16S rDNA. The PCR (18) was used to selectively amplify a 529-bp fragment of the 16S rDNA from *S. uberis*, *S. agalactiae*, and *S. parauberis* (Fig. 6). The identity of this product was confirmed by Southern hybridization (21) to the universal 16S rRNA probe BB9966 (Fig. 6). The sense primer used in the PCR was biotinylated to allow capture of the amplified product on streptavidin-coated magnetic beads and subsequent production of ssDNA containing the target sequence but lacking the secondary structure of 16S rRNA.

Hybridization of probes to 16S rDNA in solution. ssDNA from *S. uberis*, *S. parauberis*, and *S. agalactiae* was hybridized to species-specific probes in solution (Table 2). Hybridization of

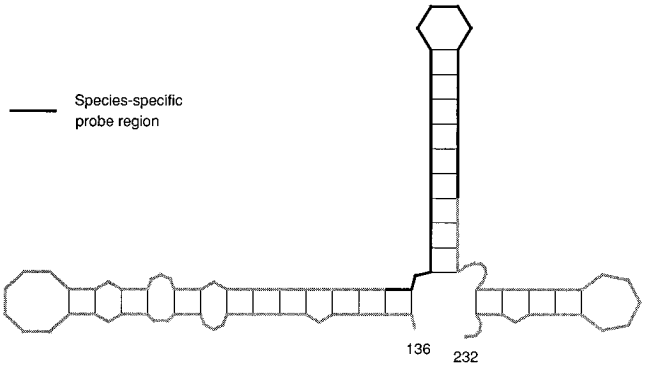

FIG. 3. Secondary structure of nucleotide positions 136 to 232 of *S. uberis* 16S rRNA.

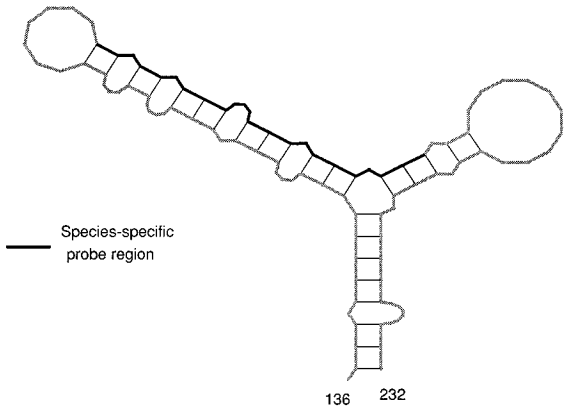

FIG. 5. Secondary structure of nucleotide positions 136 to 232 of *S. parauberis* 16S rRNA.

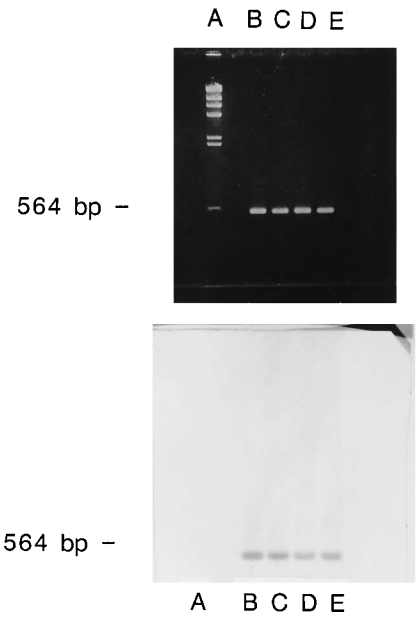

FIG. 6. PCR amplification and Southern hybridization of a 529-bp fragment of 16S rDNA. Lanes A contain λ DNA digested with *Hin*dIII. Lanes B through E contain amplification products from *S. parauberis* NCDO 2020, *S. uberis* C197, *S. agalactiae* 8, and *S. dysgalactiae* A5, respectively. The Southern blot (lower panel) was hybridized to the universal probe P1.

the *S. agalactiae* probe (BB9967) was species specific, giving a strong hybridization signal (12.92% of total label added) (Table 2). Nonspecific hybridization of the *S. agalactiae*-specific probe to ssDNA of *S. uberis* and *S. parauberis* was negligible (<0.01%). Hybridization of the *S. parauberis* probe (BB9969) was also species specific, with 12.32% of the total label bound to ssDNA in comparison with nonspecific signals of 0.06% against *S. uberis* ssDNA and <0.01% against *S. agalactiae* ssDNA. Hybridization of the *S. uberis*-specific probe (BB9970) produced a specific signal from *S. uberis* ssDNA (2.54% of the total label). A negligible signal (0.03% or less) was obtained from *S. agalactiae* and *S. parauberis* ssDNA with the *S. uberis* probe. The universal probe (BB9971) hybridized to ssDNA of all species, giving signals of 7.91% of total label added for *S. agalactiae*, 8.61% for *S. parauberis*, and 8.43% for *S. uberis.*

The technique of hybridization in solution followed by hybrid capture was shown to be feasible with magnetic beads as the solid phase and PCR-amplified ssDNA as the target. Use of 16S rRNA for hybridization in solution revealed that this molecule was not always a reliable target. This may have been due to the secondary structure of the molecule, which was thought to have inhibited specific hybridization of the *S. uberis*-specific probe. The extent to which 16S rRNA secondary structure might affect the signal with probes used in solution hybridization formats for the identification of other bacterial species is unknown, although Van Ness et al. (23) reported success with a format in which 16S rRNA as the target molecule and nylon beads coated with capture probe were used for the specific identification of *Porphyromonas gingivalis.*

Identification of other streptococci with 16S rRNA-derived probes. The hybridization methods discussed above were at-

TABLE 3. Sequence of the highly variable V2 region of 16S rRNA from a number of streptococci

Species and strain	Sequence[a]
S. mutans NCTC 10449^T	UAAUAUUNAUUAUUGCAUGAUNAU
S. pyogenes NCDO 2381^T	UAAGAGAGACUAACGCAUGUNAGUNA
S. parauberis NCDO 651	UGACAAUUAAGUACUCAUGUACU
S. uberis NCDO 643	UGACAAUAGGGUACACAUGUACCCU
S. porcinus NCDO 600^T	UGAAAGUAGAAGACACAUGUCAUCU
S. alactolyticus NCDO 1091^T	UAACAGCUUUUGACACAUGUUAGAAG
S. equinus NCDO 1037^T	UAACAGCAUUUAACACAUGUUAGAUG
S. dysgalactiae NCDO 2023^T	UGACAAUGGAGGACCCAUGUCU
S. acidominimus NCDO 2025^T	UAAUAGUGUUUACUGCAUGGUNAACA
S. iniae NCDO 2722^T	UGACACUAGAGUACACAUGUACUNAA
S. vestibularis NCTC 12166^T	UAACAAUAGGUGACACAUGUCAUUUA
S. thermophilus NCDO 573^T	UAACNAUGGAUGACACAUGUNAUNNN
S. salivarius NCDO 1779^T	UAACAAUGGAUGACACAUGUCAUUUA
S. suis NCTC 10237^T	UAACAGUAUUUACCGCAUGGUAGAUC
S. cricetus NCDO 2720^T	UAAUAGUGAUCAACUCAUGUCAAUNA
S. macacae NCTC 11558^T	UAAUAUCUCUCUAAGCCUUUAGAGGG
S. oralis NCTC 11427^T	UAAGAGUAGAUGUUGCAUGACAUUUA
S. sobrinus DSM 20742^T	UAAGAGGAGUUAACUCAUGUUAACUG
S. intermedius NCTC 11324^T	UAAGAACAUUUACUGCAUNGUAGAUG
S. bovis NCDO 597^T	UAACAGCAUUUAACACAUGUUAGAUG
S. constellatus NCTC 11325^T	UNAGAACAUUUACUGCAUNGUAGAUG
S. pneumoniae NCTC 7465^T	UAAGAGUAGAUGUUGCAUGACAUUUG
S. anginosus NCTC 10713^T	UAACAGUAUGUAACACAUGUUAGAUG
S. agalactiae NCDO 1348^T	UAAGAGUAAUUAACACAUGUUAGU
S. rattus NCDO 2723^T	UAAGAGAGUUNAACACAUGUUAGACG
S. parasanguis NCTC 85-81^T	UAAAAGUCGAUAUCGCAUGAUAUNNA
S. sanguis NCTC 7863^T	UAAAAUUGAUUNUUGCAUGAUNANNN
S. canis DSM 20715^T	UAAAAGUGCUNAACACAUGUUAAGAA
S. hyointestinalis NCTC 20770^T	UAAGAGGUAAUNACACAUGUUNUUNG
S. downei NCTC 11391^T	UGAGAGUGUUUAACACAUGUUAGAGA
S. equi NCDO 2493^T	UAAAAGUGGUUNACCCAUGUUAACCA

[a] N, undetermined nucleotide.

TABLE 4. Number of mismatches in the V2 region between streptococci[a]

Species and strain	No. of mismatches[b] with:																														
	S. acidominimus NCDO 2025^T	*S. agalactiae* NCDO 1348^T	*S. alactolyticus* NCDO 1091^T	*S. anginosus* NCTC 10713^T	*S. bovis* NCDO 597^T	S. canis DSM 20715^T	S. constellatus NCTC 11325^T	*S. cricetus* NCDO 2720^T	*S. downei* NCTC 11391^T	*S. dysgalactiae* NCDO 2023^T	*S. equi* NCDO 2493^T	*S. equinus* NCDO 1037^T	*S. hyointestinalis* DSM 20770^T	*S. iniae* NCDO 2722^T	*S. intermedius* NCTC 11324^T	*S. macacae* NCTC 11558^T	*S. mutans* NCTC 10449^T	*S. oralis* NCTC 11427^T	*S. parasanguis* NCTC 85-81^T	*S. parauberis* NCDO 651	*S. pneumoniae* NCTC 7465^T	*S. porcinus* NCDO 600^T	*S. pyogenes* NCDO 2381^T	*S. rattus NCDO 2723*^{T}	*S. salivarius* NCDO 1779^T	*S. sanguis* NCTC 7863^T	*S. sobrinus* DSM 20742^T	*S. suis* NCTC 10237^T	*S. thermophilus* NCDO 573^T	*S. uberis* NCDO 643	*S. vestibularis* NCTC 12166^T
S. acidominimus NCDO 2025^T	0	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	4	—	—	—	—	—	—	—	—	4	—	—	—	—	—
S. agalactiae NCDO 1348^T		0	—	—	4	—	—	—	4	—	—	4	4	—	—	—	—	—	—	—	—	—	4	4	—	—	—	—	—	—	—
S. alactolyticus NCDO 1091^T			0	—	3	—	—	—	—	—	—	3	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
S. anginosus NCTC 10713^T				0	2	—	—	—	—	—	—	2	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
S. bovis NCDO 597^T					0	—	—	—	—	—	—	0	—	—	—	—	—	—	—	—	—	—	—	4	—	—	—	—	—	—	—
S. canis DSM 20715^T						0	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
S. constellatus NCTC 11325^T							0	—	—	—	—	—	—	—	0	—	—	—	—	—	—	—	—	—	—	—	—	—	3	—	—	—
S. cricetus NCDO 2720^T								0	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
S. downei NCTC 11391^T									0	—	—	—	—	—	—	—	—	—	—	—	—	—	—	4	—	—	—	—	—	—	—
S. dysgalactiae NCDO 2023^T										0	—	—	—	—	—	—	—	—	—	—	—	—	—	—	4	—	—	—	4	—	—
S. equi NCDO 2493^T											0	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
S. equinus NCDO 1037^T												0	—	—	—	—	—	—	—	—	—	—	—	4	—	—	—	—	—	—	—
S. hyointestinalis DSM 20770^T													0	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
S. iniae NCDO 2722^T														0	—	—	—	—	—	4	—	—	—	—	—	—	—	—	—	4	—
S. intermedius NCTC 11324^T															0	—	—	—	—	—	—	—	—	—	—	—	—	4	—	—	—
S macacae NCTC 11558^T																0	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
S. mutans NCTC 10449^T																	0	—	—	—	—	—	—	—	—	1	—	—	—	—	—
S. oralis NCTC 11427^T																		0	—	—	1	—	—	—	—	—	—	—	—	—	—
S. parasanguis NCTC 85-81^T																			0	—	—	—	—	—	—	—	—	—	—	—	—
S. parauberis NCDO 651																				0	—	—	—	—	—	—	—	—	—	—	—
S. pneumoniae NCTC 7465^T																					0	—	—	—	—	—	—	—	—	—	—
S. porcinus NCDO 600^T																						0	—	—	—	—	—	—	—	—	—
S. pyogenes NCDO 2381^T																							0	—	—	—	—	—	—	—	—
S. rattus NCDO 2723^T																								0	—	—	—	—	—	—	—
S. salivarius NCDO 1779^T																									0	—	—	—	0	—	2
S. sanguis NCTC 7863^T																										0	—	—	—	—	—
S. sobrinus DSM 20742^T																											0	—	—	—	—
S. suis NCTC 10237^T																												0	—	—	—
S. thermophilus NCDO 573^T																													0	—	—
S. uberis NCDO 643																														0	—
S. vestibularis NCTC 12166^T																															0

[a] For sequences, refer to Table 3.
[b] An undetermined nucleotide opposite a base was treated as a match in homology calculations. —, more than four mismatches.

tempted with nucleic acids from only three species of *Streptococcus*. However, 16S rRNA sequence data from a total of 31 species of *Streptococcus* were available. The PCR primers used to amplify probe target regions from *S. agalactiae*, *S. uberis*, and *S. parauberis* were designed for conserved sequences within the 16S rRNA molecule and should amplify the V2 diagnostic region from all streptococci and possibly members of other gram-positive genera. Comparison of the V2 regions of these species (Table 3) revealed that there were sufficient nucleotide differences, three or more (Table 4), between species to allow the rational design of oligonucleotide probes that could be species specific if hybridized at high stringency. Greisen et al. (9) described oligonucleotide probes that differed at a single nucleotide position but were capable of discriminating the species *S. agalactiae* and *S. pneumoniae*. The rRNA sequences shown in Table 3 were obtained from reference strains of the species named, and it is not known to what extent intraspecific variation of the V2 region might occur, particularly in heterogeneous species. In these cases, the probe-based identification system may need further refinement to achieve the specificity required of a diagnostic test. Intraspecific sequence variation of the V2 region was not seen for 15 isolates of *S. uberis*, 5 isolates of *S. parauberis*, or 7 isolates of *S. agalactiae* (1).

Conventional schemes for the identification of streptococci have relied on the use of serological or biochemical markers. However, these often rely on the expression of a variable phenotype which may not always be expressed under test conditions. A serological group may not correspond to a species, as several species may belong to a single Lancefield group or several Lancefield groups may occur in a single species. Relying on the less variable genotype for identification would inherently lead to greater test stability and yield more accurate epidemiological information. The number of biochemical characteristics screened by conventional test schemes may be insufficient to allow discrimination of species. For example, the reliable serological or biochemical differentiation of *S. uberis* from *S. parauberis* is extremely difficult and unreliable. The nucleic acid-based scheme described in this work would overcome many of these problems and allow precise identification

in less time than is required for current biochemical test systems.

In this communication, we have shown how species-specific oligonucleotide probes may be used for hybridization in solution to identify the species *S. agalactiae*, *S. uberis*, and *S. parauberis*. Problems associated with hybridization of oligonucleotide probes to the 16S rRNA of *S. uberis* were probably related to the secondary structure of this molecule and were circumvented by the use of PCR-generated rDNA. This identification system could also be extended to incorporate many other species of streptococci.

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