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# Development and Use of Species-Specific Oligonucleotide Probes for Differentiation of *Streptococcus uberis* and *Streptococcus parauberis*

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**Oligonucleotide probes specific for 16S rRNA and capable of differentiating *Streptococcus uberis* and *S. parauberis* from each other and other esculin-hydrolyzing streptococci were developed. Use of a mini-RNA extraction technique for gram-positive cocci associated with bovine mastitis has allowed the probes to be used for identification of esculin-hydrolyzing streptococci from two dairy herds at the Institute for Animal Health, Compton, United Kingdom. One hundred seventy-nine of 206 isolates were identified as *S. uberis*, 3 were identified as *S. parauberis*, and 24 were not identified. Isolates not identified by the probes were tested biochemically and found to be mainly *Enterococcus faecium*, *E. faecalis*, or *S. bovis*.**

*Streptococcus uberis* is a common cause of bovine mastitis and accounts for approximately 20% of all clinical cases of this disease in the United Kingdom (3).

*S. uberis* is serologically and biochemically heterogeneous (10). Serological tests are of little value in the identification of *S. uberis*, since 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. Rapid biochemical tests have been evaluated (6, 12, 15) but are also inadequate for reliable identification. A more rapid method to differentiate streptococci isolated from cows with mastitis was based on the production of the species-specific enzymes β-glucuronidase, *N*-acetyl-β-D-glucosaminidase, and β-D-mannosidase and agglutination with *Dolichos biflorus* lectin (17), but this method has yet to be widely tested.

Two genotypes (designated type I and type II) of *S. uberis* have been demonstrated on the basis of chromosomal DNA hybridizations (7). Comparison of 16S rRNA sequences has confirmed this distinction, and type II strains have now been assigned to a separate species, *S. parauberis* (20). Biochemical and serological differentiation of *S. parauberis* from *S. uberis* is not possible at present. Methods to differentiate the two species based on Southern hybridization analysis of chromosomal digests (21) or polymerase chain reaction amplification of 16S ribosomal DNA followed by analysis of restriction fragment length polymorphisms (11) have been described. However, these techniques have not been applied to a large number of isolates, and the relative incidence of mastitis caused by *S. uberis* and *S. parauberis* remains unknown.

Small-subunit rRNA is being used increasingly for probe design because of natural amplification of the target (9, 19) and the presence of hypervariable regions which facilitate the design of highly specific oligonucleotide probes (8). The 16S rRNA sequences of *S. uberis* and *S. parauberis* have been published (20), and the design and use of complementary species-specific probes constitute a logical extension of this work. In this article, we describe the development of species-specific probes based on 16S rRNA sequences from *S. uberis* and *S. parauberis*. We have used these to screen a number of esculin-hydrolyzing gram-positive cocci isolated from clinical and subclinical cases of mastitis and from the environment in order to determine the relative incidence of these species.

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## MATERIALS AND METHODS

**Bacterial strains.** Reference strains of *S. parauberis* (NCDO 2020, NCDO 2018, NCDO 651, NCDO 649, and NCDO 648), *S. agalactiae* (NCDO 1348), *S. dysgalactiae* (NCDO 2023), *S. equinus* (NCDO 1037), *S. bovis* (NCDO 597), and *S. salivarius* (NCDO 1779) were obtained from the National Collection of Food Bacteria (Reading, United Kingdom). *S. anginosus* (NCTC 10713) and *S. downei* (NCTC 11391) were obtained from the National Collection of Type Cultures (London, United Kingdom). *S. uberis* strains were obtained from a collection of isolates at the AFRC Institute for Animal Health (Compton, United Kingdom) and were classed as clinical (118 isolates from mammary glands with overt signs of infection), subclinical (48 isolates from mammary glands lacking overt clinical signs), or environmental (40 isolates [11 from bedding, 11 from skin, 11 from rectal swabs, and 7 from vulval swabs]). Isolates were stored at −20°C in 50% glycerol–50% Todd-Hewitt broth (Oxoid, Basingstoke, United Kingdom).

**Large-scale extraction of rRNA.** Strains were grown in 1 liter of Todd-Hewitt broth at 37°C and harvested in the late exponential phase by centrifugation. Total cellular RNA was extracted as described by Chomczynski and Sacchi (4), except that initial cell lysis was achieved by passing the cells through an X-press pressure cell (Life Science Laboratories, Luton, United Kingdom).

**Small-scale extraction of rRNA.** Volumes of 1.5 ml of cultures of bacteria grown at 37°C for 18 h were harvested by centrifugation and resuspended in 300 μl of Tris-HCl (50 mM, pH 8.0). Cell walls were digested by the addition of 25 μl of mutanolysin (5,000 U/ml) followed by incubation at

37°C for 30 min, and RNA was extracted as described by Chomczynski and Sacchi (4).

**Sequencing rRNA with reverse transcriptase.** Partial 16S rRNA nucleotide sequences spanning the variable region V2 (see reference 12 for nomenclature), which was identified by Williams and Collins (20) as a signature region for *S. uberis* and *S. parauberis*, were determined with avian myeloblastosis virus reverse transcriptase (Northumbria Biologicals Ltd., Cramlington, United Kingdom) (13) and a conserved DNA primer, 5′-TCACCCTCTCAGGTCGGCTA-3′ (complementary to positions 287 to 306 [*Escherichia coli* numbering system]). The products of the sequencing reactions were separated on 55-cm-long wedge-shaped (0.2- to 0.6-mm-thick) 6% (wt/vol) polyacrylamide–7 M urea denaturing gels at 55°C with an LKB Macrophor 2010 sequencing unit operated at 50 W per gel.

**Probe synthesis and labelling.** Oligonucleotides were synthesized by the phosphoramidite method (1) with a model 318A DNA synthesizer (Applied Biosystems, Warrington, United Kingdom). Three probes were synthesized: the conserved oligomer described above, an *S. uberis*-specific probe complementary to nucleotide positions 179 to 203 (5′-AGGG TACATGTGTACCCTATTGTCA-3′), and an *S. parauberis*-specific probe complementary to nucleotide positions 179 to 201 (5′-AGTACATGAGTACTTAATTGTCA-3′) (20). The probes were labelled at the 3′ end as previously described (18) and stored at −20°C.

**Immobilization of rRNA for hybridization.** Northern (RNA) blotting was performed as described by Maniatis et al. (14) with a formaldehyde-denaturing agarose gel (1.5% agarose). RNA (1 μg) was denatured by being heated in a formaldehyde-formamide (1/3 [vol/vol]) solution at 65°C for 5 min, chilled on ice, and electrophoresed at 80 V for 3 h. Standard 16S and 23S rRNAs extracted from *E. coli* (Boehringer, Mannheim, Germany) were loaded on the same gel as size markers. The RNA was transferred to a nylon membrane (Hybond $N^+$; Amersham International) in 1.5 M sodium chloride–0.15 M sodium citrate (pH 7.0) (10× SSC) for 3 h.

A dot blot assay performed by the mini-RNA extraction method was used to screen isolates. Briefly, the RNA solution from a 1.5-ml 18-h culture or an equivalent amount of RNA from a large-scale extraction was denatured in 90 μl of formaldehyde-formamide solution at 65°C for 5 min and chilled, and 120 μl of 20× SSC was added. Approximately 0.1 μg of RNA (40 μl) of this solution was applied per dot onto a Hybond $N^+$ membrane by using a standard dot blot manifold. In all cases, RNA was cross-linked to the membrane by exposure to UV light on a transilluminator (Genetic Research Instrumentation, Dunmow, United Kingdom) at 302 nm for 3 min.

**Hybridization of rRNA with oligonucleotide probes.** Membranes were prehybridized for 2 h at 50°C in 5× SSC–0.5% sodium dodecyl sulfate (SDS)–5× Denhardt's reagent (5× Denhardt's reagent is 0.1% [wt/vol] Ficoll, 0.1% [wt/vol] polyvinylpyrrolidone, 0.1% [wt/vol] bovine serum albumin)–50 μg of sheared, denatured salmon sperm DNA per ml. Hybridizations were performed at 55°C for 16 h in the same solution containing the relevant probe (30 ng/ml). Posthybridization washes (two 15-min washes) were carried out at 58°C in 5× SSC containing 0.1% SDS. Immunological detection of the bound probe was carried out as recommended by the manufacturer. The color reaction was typically allowed to proceed for 1 h and stopped by immersing the membrane in Tris-HCl (10 mM)–EDTA (1 mM), pH 8.0, for 5 min and then drying it at 37°C.

## RESULTS

**Species-specific sequence conservation.** Partial sequences spanning the diagnostic V2 region of the 16S rRNA were obtained by reverse transcription from 15 clinical isolates of *S. uberis* and 5 reference strains of *S. parauberis* (NCDO 648, NCDO 649, NCDO 651, NCDO 2018, and NCDO 2020). Within each species, this region showed complete sequence conservation. Oligonucleotide probes complementary to diagnostic regions within the sequence for each species were synthesized and labelled nonradioactively at the 3′ end.

**Theoretical analysis of probe target sequences.** Comparison of the proposed probe target sequences (nucleotide positions 179 to 203 for *S. uberis* and 179 to 201 for *S. parauberis*) with other streptococcal 16S rRNA sequences (2) did not reveal close homology to either species-specific probe region. The species exhibiting the highest homology with the *S. uberis* probe sequence (number of mismatches in parentheses) were *S. iniae* (four), *S. parauberis* (six), *S. dysgalactiae* (eight), *S. anginosus* (nine), and *S. salivarius* (nine); those exhibiting the highest homology with the *S. parauberis* probe sequence were *S. iniae* (four mismatches), *S. uberis* (five), *S. dysgalactiae* (six), and *S. salivarius* (eight). The 16S rRNA sequences of *S. bovis*, *S. equinus*, *S. agalactiae*, and *S. downei* displayed more than 10 mismatches with *S. uberis* and *S. parauberis* probe sequences.

**Specificity of oligonucleotide probes for purified RNA.** Total RNA was extracted from all of the species listed above (including an isolate of *Enterococcus faecalis*) and used in a dot blot assay to determine probe specificity. The universal probe used as a positive control successfully hybridized with RNA from all of the strains tested (Fig. 1). The species-specific probes showed complete specificity toward their respective targets. To confirm that probes hybridized specifically to 16S rRNA, Northern blots of total RNA extracts were probed (Fig. 2). All three probes hybridized specifically to 16S rRNA, and no signal for any other RNA fraction was detected.

**Identification of esculin-hydrolyzing streptococci.** The probes were used in dot blot experiments to screen isolates of esculin-hydrolyzing streptococci from two dairy herds. RNA from a total of 206 isolates from clinical cases of mastitis (118 isolates), subclinical infection (48 isolates), and the environment (40 isolates) was probed. To confirm the accuracy of the probes (Table 1), strains that did not hybridize with both *S. uberis* and *S. parauberis* probes were further examined with the API 20 Rapid Strep system (API Biomerieux, Basingstoke, United Kingdom). A random selection of 23 isolates that hybridized with the *S. uberis* probe and all isolates that hybridized with the *S. parauberis* probe were similarly tested. One hundred seventy-nine (86.9%) hybridized with the *S. uberis* probe, and three (1.5%) hybridized with the *S. parauberis* probe (Table 1). None of the isolates hybridized with both probes. Twenty-four isolates (11.6%) did not hybridize with either probe (Table 1); of these, 10 were identified as *E. faecalis*, 7 as *E. faecium*, 2 as *S. bovis*, 1 as *E. gallinarum*, and 2 as either *Lactococcus lactis* or *E. faecium*, and 2 remained unidentified by the API 20 Strep system. Of 23 isolates which hybridized with the *S. uberis* probe, 21 had API 20 profiles which corresponded to *S. uberis*, but 2 remained unidentified (Table 1). All isolates that hybridized with the *S. parauberis* probe had biochemical profiles corresponding to *S. uberis*.

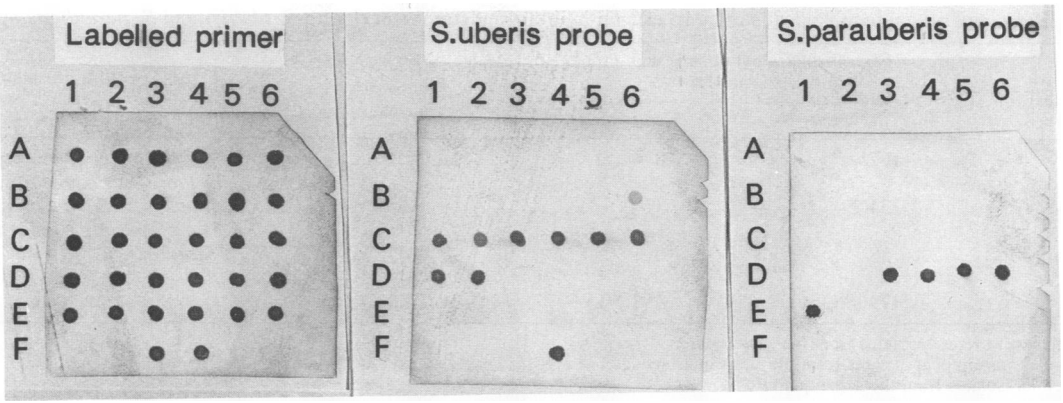


FIG. 1. *S. uberis* and *S. parauberis* probe specificity as shown by dot blots. RNA was applied to the membranes as follows: A1 to A5, *S. agalactiae*; A6 to B5, *S. dysgalactiae*; B6 to D2 and F4, *S. uberis*; D3 to E1, *S. parauberis*; E2, *S. equinus*; E3, *S. bovis*; E4, *S. salivarius*; E5, *S. anginosus*; E6, *S. downei*; F3, *E. faecalis*.

## DISCUSSION

Small-subunit rRNA has become a popular target for probe design because of high target copy number within the cell (19) and the presence of hypervariable regions which facilitate the design of species-specific oligonucleotide probes (8). In an earlier investigation, *S. uberis* and *S. parauberis* were shown to possess quite distinct sequences in the V2 region of their 16S rRNAs (20). In the present study, we have confirmed these differences for a large number of strains of both species. Comparative analysis of these sequences with other streptococcal 16S rRNA sequences (2) facilitated the design of species-specific oligonucleotide probes. It has been reported that oligonucleotides can discriminate nucleic acids containing only one mismatch (5). The number of mismatches between the proposed probe regions for *S. uberis*, *S. parauberis*, and other streptococci was sufficient (four or more mismatches in a 25-mer for *S. uberis* and in a 23-mer for *S. parauberis*) to allow the design and synthesis of species-specific oligonucleotide probes for *S. uberis* and *S. parauberis*. Partial sequence analysis demonstrated the absence of intraspecific variation in the V2 probe region. Empirical testing of probe specificities against RNA from species that had the greatest similarity in the V2 probe region and that were most likely to be isolated from bovine mammary glands showed that the *S. uberis* and *S. parauberis* probes were highly specific (Fig. 1 and 2).

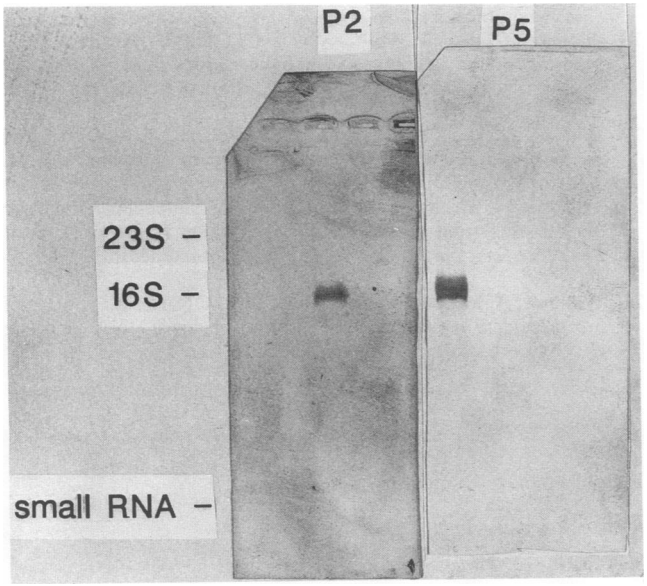


FIG. 2. *S. uberis* and *S. parauberis* probe specificity as shown by Northern blots. P2, *S. uberis*-specific probe against total RNA from *S. uberis*; P5, *S. parauberis*-specific probe against total RNA from *S. parauberis*.

The probes were used in a preliminary epidemiological study of strains from cows with clinical mastitis or subclinical infection and from the environment. The isolates had previously been classified as hydrolyzing esculin (on esculin-blood agar), a property shared by *S. uberis*, *S. parauberis*, and most isolates of *E. faecalis* and *E. faecium*. Previous work has shown that the API Strep system identified a maximum of 95% of *S. uberis* isolates tested (12, 15). The three isolates which were identified as *S. parauberis* by using the probes had biochemical profiles that corresponded to *S. uberis*, confirming current opinion that biochemical differentiation of *S. uberis* from *S. parauberis* is exceedingly difficult. These three isolates were from sequential samples obtained over a 9-week period from the same quarter of a lactating cow and could therefore be considered to be from a persistent subclinical infection. *S. parauberis* has been described as a cause of clinical mastitis (20), but until recently the lack of reliable and routinely applicable methods to differentiate this species from *S. uberis* has meant that very little was known about its epidemiology. Since *S. parauberis* was detected in only one subclinical case and was not found in the environmental group (implying the absence of an infectious reservoir), it is unlikely that this species is a significant cause of mastitis.

Two of the five reference collection strains of *S. parauberis* (NCDO 649 and NCDO 651) failed to hydrolyze esculin when grown on blood agar (although they hydrolyzed esculin when tested with the API 20 Strep system). The use of esculin hydrolysis on blood agar as a biochemical marker for isolates included in this study could therefore have inadvertently led to the exclusion of a number of *S. parauberis* isolates and an underestimate of their contribution to bovine mastitis. A broader epidemiological survey covering more herds and a larger number of isolates is clearly necessary to

TABLE 1. Results of an epidemiological survey of esculin-hydrolyzing streptococci with *S. uberis*- and *S. parauberis*-specific probes

| Source (no. of isolates) | No. of isolates hybridizing with probe specific for: | | No of negative isolates | API identification of probe-negative isolates |
|---|---|---|---|---|
| | *S. uberis* | *S. parauberis* | | |
| Clinical (118)[a] | 107 | 0 | 11 | 5 *E. faecalis*, 2 *E. faecium*, 1 *S. bovis*, 2 *L. lactis* or *E. faecium*, 1 unidentified |
| Subclinical (48)[b] | 34 | 3[c] | 11 | 5 *E. faecalis*, 3 *E. faecium*, 1 *S. bovis*, 1 *E. gallinarum*, 1 unidentified |
| Environmental (40) | 38 | 0 | 2 | 2 *E. faecium* |
| Total (206) | 179 (86.9%) | 3 (1.5%) | 24 (11.6%) | |

[a] Three samples were repeat isolates from the same quarter.
[b] Twelve samples were repeat isolates from the same quarter.
[c] Three sequential isolates from the same quarter.

determine the precise role of *S. parauberis* in bovine mastitis.

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