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Evaluation of PCR Methods for Rapid Identification and Differentiation of *Streptococcus uberis* and *Streptococcus parauberis*

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***Streptococcus uberis* and *Streptococcus parauberis* reference strains and isolates obtained from routine diagnostics were investigated by PCR with oligonucleotide primers designed according to species-specific parts of the 16S rRNA gene, the 23S rRNA gene, and the 16S-23S rRNA intergenic spacer region of both species. All three primer pairs allowed an identification of 67 isolates as *S. uberis* and 4 isolates as *S. parauberis*.**

The use of PCR as a diagnostic tool for the detection of bacterial pathogens has become more frequent during the past few years. The PCR technique allows the amplification of preselected, species-specific DNA regions which can be used for genotypic identification of bacteria. A molecule most suited for these purposes appears to be the gene encoding the 16S rRNA. According to Bentley et al. (4) and Bentley and Leigh (6), the sequence variability of the V2 region of the 16S rRNA allows a differentiation of 31 species of the genus *Streptococcus*, including the species *S. uberis* and *S. parauberis*. Both species, formerly classified as *S. uberis* types I and II (19), are well known as causative agents of bovine mastitis. According to biochemical and serological characteristics, the two species are almost indistinguishable (19). A molecular identification of both species can be performed by analysis of restriction fragment length polymorphisms (RFLPs) of the 16S rRNA gene (13, 14, 18) or by the use of species-specific oligonucleotide probes (5, 6). A major disadvantage of these procedures is the additional manipulation of the samples subsequent to the PCR and the time-consuming preparation of the gene probes. In the present study, species-specific regions of the 16S and 23S rRNA genes and the 16S-23S rRNA intergenic spacer region of *S. uberis* and *S. parauberis* were used to construct species-specific oligonucleotide primers. These oligonucleotide primers were used to identify and differentiate both species.

A total of 17 *S. uberis* and 2 *S. parauberis* strains were used in this study, all of which were obtained from the Institute's strain collection. The cultures were identified and differentiated into both species as described previously (15, 18). In addition, 51 streptococci obtained from routine mastitis diagnostics and also *S. parauberis* strain 94/16 were included. The latter, kindly obtained from J. F. Fernández-Garayzábal (Facultad de Veterinaria, Universidad Complutense de Madrid, Madrid, Spain), was originally isolated from a diseased turbot (9). The cultures isolated from routine diagnostics were preliminarily identified as *S. uberis* by using conventional methods (15, 16). These methods included carbohydrate fermentation tests for arabinose, fructose, inulin, lactose maltose, mannitol, raffinose, ribose, saccharose, salicin, sorbitol, and trehalose; esculin and sodium hippurate hydrolysis; determination of arginine hydrolysis; and analysis of the enzymes β-D-glucuronidase, pyrrolydonylaminopeptidase, and hyaluronidase. The β-D-glucuronidase and pyrrolydonylaminopeptidase enzyme activities were investigated with diagnostic tablets (Rosco, Hiss Diagnostics, Freiburg, Germany) as substrates, and the enzyme hyaluronidase activity was determined with a plate decapsulation test with cultivation of the isolates in close proximity to a growing mucoid *Streptococcus equi* subsp. *zooepidemicus* strain. The 51 isolates were additionally investigated by RFLP analysis of the 16S rRNA gene, using the restriction enzymes *Rsa*I and *Ava*II (18). For control purposes, streptococci of the species *S. pyogenes* ($n = 4$), *S. agalactiae* ($n = 12$), *S. equi* subsp. *equi* ($n = 2$), *S. equi* subsp. *zooepidemicus* ($n = 3$), *S. dysgalactiae* serogroups C ($n = 4$), G ($n = 4$), and L ($n = 4$), *S. canis* ($n = 12$), and *S. porcinus* ($n = 4$) were included.

Based on the sequence analysis of the V2 region of the 16S rRNA gene reported by Bentley and Leigh (6) and the sequence data of the variable region of the 23S rRNA gene given by Harland et al. (12), species-specific primers were designed using the primer design program OLIGO 4.0.

The primer design of the species-specific part of the 16S-23S rRNA intergenic spacer region was performed after sequencing of the genes of *S. uberis* and *S. parauberis* reference strains. For the intergenic spacer region of *S. uberis*, the primer sequences recommended by Forsman et al. (10) were used.

For sequencing, the intergenic spacer regions of the *S. parauberis* reference strain NCDO 2020 and the *S. uberis* reference strain NCDO 2038 were amplified with the oligonucleotide primers c (5′-3′, TTGTACACACCGCCCGTCA) and b (5′-3′, GGTACCTTAGTTTCAGTTC) described by Chanter et al. (7). The primers were derived from conserved regions within the 16S rRNA (primer c) and the 23S rRNA (primer b) genes. The oligonucleotide primers were synthesized by ARK-Biosystem (Darmstadt, Germany). The sequencing was per-

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TABLE 1. Oligonucleotide primers and PCR programs for amplification of species-specific parts of the genes encoding the 16S rRNA, 23S rRNA and 16S-23S rRNA intergenic spacer region of *S. uberis* and *S. parauberis*

Species	Target rRNA gene	Oligonucleotide primer	Sequence (5′–3′)	Length (nt)	Size of PCR product (bp)	PCR program[a]
S. uberis	16S	ub-I	CGC ATG ACA ATA GGG TAC A	19	445	1
		ub-II	GCC TTT AAC TTC AGA CTT ATC A	22		
	23S	ub-23S-I	CGT ATT TAA AAT TGA CTT TAG CC	23	451	2
		ub-23S-II	AAT TTC TCC GCT ACC CAC	18		
	16S-23S intergenic spacer	STRU-UbI[b]	TAA GGA ACA CGT TGG TTA AG	20	330	3
		STRU-UbII[b]	TCC AGT CCT TAG ACC TTC T	19		
S. parauberis	16S	paraub-I	CAT GAC AAT TAA GTA CTC ATG TAC TA	26	884	1
		paraub-II	CAC CAC CTG TCA CCT CTG TC	20		
	23S	paraub-23S-I	AAA ATA GTA AAT GAC TCT AGC AGT	24	478	2
		paraub-23S-II	CGG AGA GAA CCA GCT ATC	18		
	16S-23S intergenic spacer	paraub-16S-23S-I	AAA TGG AAG CAC GTT AGG AAA	21	201	4
		paraub-16S-23S-II	GCA AGC CGA ACA TCT CTT TG	20		

[a] 1 = 30× (94°C 60 s, 58°C 90 s, 72°C 90 s); 2 = 30× (94°C 45 s, 64°C 45 s, 72°C 45 s); 3 = 30× (94°C 30 s, 55°C 30 s, 72°C 30 s); 4 = 30× (94°C 10 s, 58°C 10 s, 72°C 10 s).
[b] Forsman et al. (10).

formed with the MegaBACE 1000 DNA sequencing system (Amersham Pharmacia Biotech, Europe, Freiburg, Germany) according to the protocols described by the manufacturer. The sequence data were further studied and analyzed with the computer program MegAlign 1993-97 (DNASTAR Inc., Constance, Germany).

The target genes, the oligonucleotide primers used, and the sizes of the amplicons are summarized in Table 1. For PCR, the reaction mixture (30 μl) contained 1 μl (16S rRNA gene, 16S-23S rRNA spacer region) or 0.8 μl (23S rRNA gene) of primer 1 (10 pmol/liter), 1 μl or 0.8 μl of primer 2 (10 pmol/liter), 0.6 μl of deoxynucleotide triphosphate (10 mmol; MBI Fermentas, St. Leon-Rot, Germany), 3 μl of 10× thermophilic buffer (Promega, Boehringer, Ingelheim, Germany), 1.8 μl (16S rRNA gene, 16S-23S rRNA spacer region) or 1.5 μl (23S rRNA gene) of $MgCl_2$ (25 mmol; Promega), 0.1 μl of *Taq* DNA polymerase (5 U/μl; Promega), and 20.0 μl or 20.7 μl of distilled water. Finally, 2.5 μl of DNA preparation was added to each reaction tube. For DNA preparations, 5 to 10 colonies of the bacteria were first suspended in 100 μl of TE buffer (10 mmol/liter Tris-HCl, 1 mmol/liter EDTA [pH 8.0]) containing 5 μl of mutanolysin (50 U; Sigma, Deisenhofen, Germany) for 60 min at 37°C, and then the bacteria were suspended in 10 μl of proteinase K (14.8 mg/ml; Boehringer) for 120 min at 56°C. After boiling for 10 min at 100°C, the suspension was centrifuged (10,000 × *g*, 5 s) and subsequently cooled before use. The tubes were then placed in a thermal cycler (Techne-Progene; Thermodux, Wertheim, Germany) with the programs summarized in Table 1.

The presence of PCR products was determined by electrophoresis of 12 μl of the reaction product in a 2% agarose gel with Tris acetate-electrophoresis buffer (TAE; 40 mmol of Tris-HCl per liter, 1.14 mol of glacial acetic acid per liter, 1 mmol of EDTA per liter [pH 8.0]) with a 100-bp DNA ladder (Gibco BRL, Eggenstein, Germany) as molecular size markers.

The oligonucleotide primers designed from the species-specific part of the 16S rRNA and 23S rRNA genes allowed a molecular identification of 67 isolates as *S. uberis* and 4 isolates as *S. parauberis*. The latter also included the *S. parauberis* strain 94/16, which was originally isolated from a diseased turbot (9). Both PCR systems produced identical results (Fig. 1). The identity of the *S. uberis* and *S. parauberis* isolates could additionally be confirmed by RFLP analysis (data not shown) and by the use of primer pairs amplifying a species-specific region of the 16S-23S rRNA intergenic spacer region. The consensus sequences of the intergenic spacer regions of both species are given in Fig. 2. All control strains of various species and serogroups were negative throughout. As reported earlier (13, 18), the occurrence of *S. parauberis* as a mastitis-causing pathogen appears to be rare. This species seems to be more frequent among British dairy herds (11). Isolation of *S. parauberis* from diseased fish was described by Doménech et al. (9). According to the results of the present study, the species-specific regions of the described target genes of *S. uberis* and *S. parauberis* did not show any sequence variations. Corresponding to these results, no intraspecies variations were reported in the 16S rRNA genes of *S. agalactiae* (1, 17) and *S. porcinus* (3). However, a variation in the sequence of the 16S rRNA gene was observed for *S. suis* (8) and for *S. equi* subsp. *zooepidemicus* (2).

The PCR amplification of species-specific sequences of *S.*

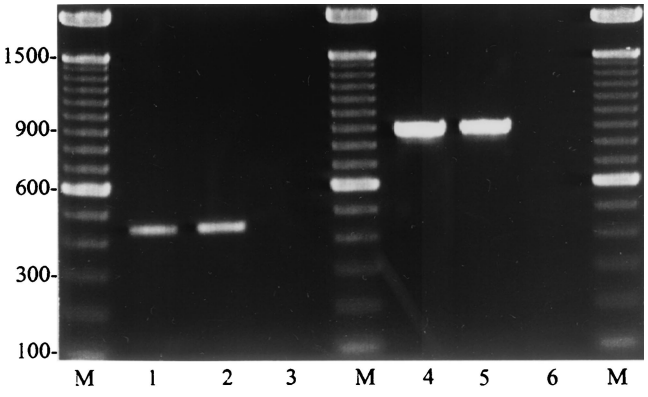

FIG 1. Typical amplicons of *S. uberis* (lanes 1 and 2) with a size of 445 bp and with *S. parauberis* (lane 3) as negative control, using the *S. uberis* 16S rRNA-specific primers ub-I and ub-II, and amplicons of *S. parauberis* (lanes 4 and 5) with a size of 884 bp and *S. uberis* (lane 6) as negative control, using the *S. parauberis* 16S rRNA-specific primers paraub-I and paraub-II. M, 100-bp ladder as size marker.

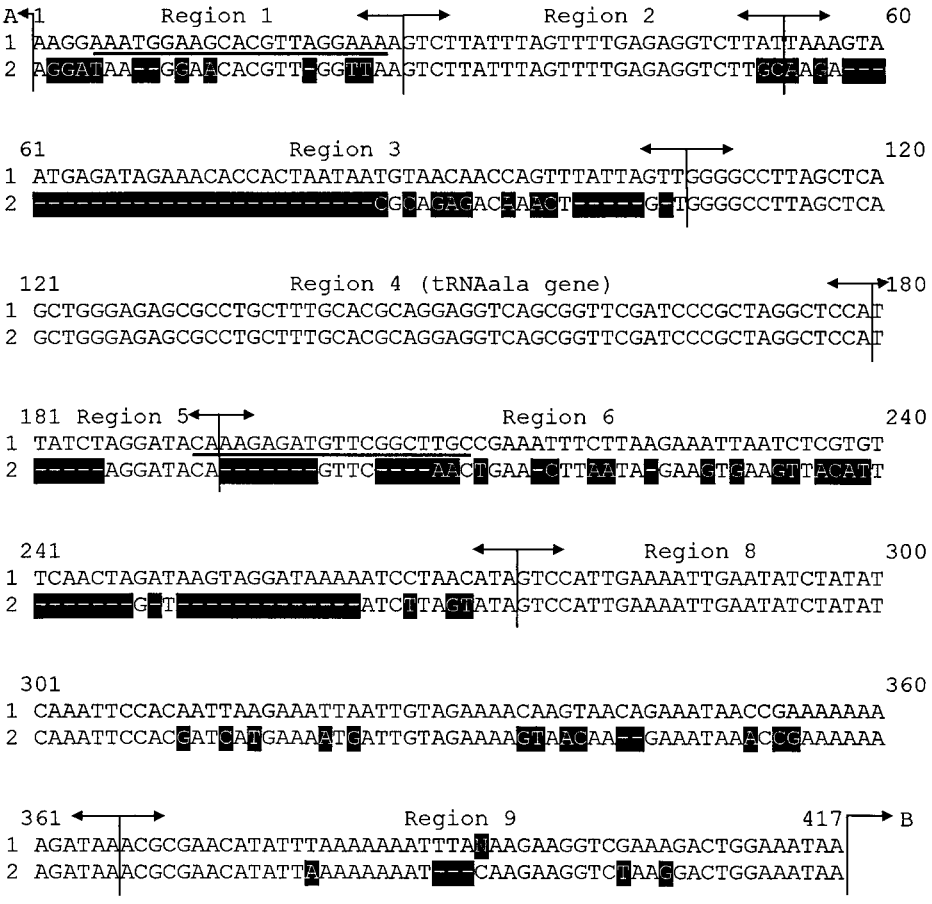

FIG. 2. Alignment of the consensus gene sequences of the 16S-23S rRNA intergenic spacer region of *S. parauberis* NCDO 2020 (417 bp) (accession no. AF255656) and *S. uberis* NCDO 2038 (338 bp) (accession no. AF255657). The selected *S. parauberis*-specific oligonucleotide primers are underlined; the marked areas indicate the differences in nucleotide sequences. The region arrangement was performed according to Chanter et. al. (7); stuffer regions inserted to achieve alignment are indicated by −−−−−. Sequence 1, *S. parauberis* NCDO 2020; sequence 2, *S. uberis* NCDO 2038. A (top), end of 16S rRNA gene; B (bottom right), start of 23S rRNA gene.

uberis and *S. parauberis* in the present study offers a rapid and sensitive method by which to identify both biochemically and serologically almost indistinguishable species. This might help in determining the prevalence of both species in dairy herds.

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