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Identification of staphylococcal and streptococcal causes of bovine mastitis using 16S–23S rRNA spacer regions

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Bovine mastitis is caused mainly by certain *Staphylococcus* and *Streptococcus* species. The sequences of the 16S–23S rRNA spacer regions were determined for the nine species which cause mastitis: *Staphylococcus aureus*, *Staphylococcus chromogenes*, *Staphylococcus epidermidis*, *Staphylococcus hyicus*, *Staphylococcus simulans*, *Staphylococcus xylosus*, *Streptococcus agalactiae*, *Streptococcus dysgalactiae* and *Streptococcus uberis*. Significant variation was found between the spacer sequences of different species with the lengths of the spacers varying from 240 to 461 bp. Between genera the spacers shared only short conserved regions (8–9 bp) and within genera the sequence identities varied from 53 to 85%. This variation made it possible to construct specific primer pairs for these species and genera. The specificities of these primers were tested with 25 bacterial species and 51 isolates from cattle with clinical mastitis. The DNA-based identification of the mastitis species was mostly successful.

Keywords: *Staphylococcus*, *Streptococcus*, bovine mastitis, intergenic rRNA spacer

INTRODUCTION

Bovine mastitis is a multifactorial disease and is one of the most difficult to control. It can be caused by many different bacterial species, the most common of which are *Staphylococcus* and *Streptococcus* species. The prevalence of different species varies geographically, temporally and also due to control measures adopted in herds. In addition, different pathogens are typical of different types of mastitis (clinical, subclinical or heifer mastitis).

In many countries the most common bacterial species causing mastitis are *Staphylococcus aureus*, *Streptococcus dysgalactiae* and *Streptococcus uberis* (Aarestrup *et al.*, 1995; Ministry of Agriculture and Forestry Mastitis Committee, 1989; Jonsson *et al.*, 1991). *Streptococcus agalactiae* was previously the most common, but due to the use of antibiotic therapy its prevalence has decreased in many countries (Bramley & Dodd, 1984). The importance of coagulase-negative staphylococci (CNS) as pathogenic agents has been under discussion (Kloos & Bannermann, 1994). CNS are part of normal skin flora and have previously been regarded only as minor pathogens. However, the proportion of CNS mastitis has increased markedly during the last decade (Myllys *et al.*, 1994). The most frequently isolated CNS species include *Staphylococcus hyicus*, *Staphylococcus simulans*, *Staphylococcus epidermidis*, *Staphylococcus chromogenes* and *Staphylococcus xylosus* (Harmon & Langlois, 1989; Jarp, 1991; Todhunter *et al.*, 1993; Aarestrup *et al.*, 1995; Honkanen-Buzalski *et al.*, 1994).

The identification of pathogens causing mastitis is important for disease control and epidemiological studies. In a clinical laboratory this is done using traditional microbiological methods (Klastrup & Schmidt Madsen, 1974). The species determination usually takes at least 24 h. There are no rapid and cheap methods available for the classification of CNS to the species level and therefore CNS species are usually determined only at the group level. There are commercial biochemical kits available for the determination of CNS species (e.g. API-Staph, Staph-Ident, Staph-Trac, Staph-Zym) but they have proved unreliable for the identification of veterinary pathogens (Watts & Yancey, 1994).

The development of molecular biological methods, such as nucleic acid analysis, protein patterns or fatty acid

Abbreviations: ATC-PCR, Air Thermo-Cycler PCR; CNS, coagulase-negative staphylococci.

The GenBank accession numbers for the sequences reported in this paper are U39765–U39773, U39813–U39814 and U90010–U90027.

profiles, has added possibilities for the rapid identification of bacteria (Busse *et al.*, 1996). Species-specific DNA sequences can be used for the identification of bacterial species. The 16S–23S rRNA intergenic spacer of the ribosomal RNA operon (*rrn*) has proven useful for identification of strains and species (Barry *et al.*, 1991; Jensen *et al.*, 1993; Gürtler & Stanisich, 1996). The spacer region is considered non-functional and is consequently argued to be under minimal selective pressure (Barry *et al.*, 1991). Its evolutionary rate is 10 times greater than that of 16S rDNA (Leblond-Bourget *et al.*, 1996), the sequence of which is routinely used for phylogenetic studies of bacteria (Ludwig & Schleifer, 1994). The high evolutionary rate makes it possible to distinguish closely related bacterial species.

The aims of this study were to determine the sequences of the 16S–23S rRNA intergenic spacers from the most common staphylococcal and streptococcal bovine mastitis pathogens and to investigate the possibility of designing species-specific oligonucleotide primers from the spacer sequences for the rapid identification of these bacterial species.

METHODS

Bacterial strains and culture conditions. All bacterial species and strains used in this study (Table 1) were grown aerobically in Tryptic Soy Broth (Difco) at 37 °C. Most of the specificity control species (Table 2) were obtained as cultures on agar plates or as DNA. The rest of the control species (e.g. all *Lactobacillus* species) were grown as recommended in the ATCC catalogue. Mastitic milk samples were obtained from the Food and Environmental Laboratory, Oulu, Finland and from the National Veterinary and Food Research Institute (EELA), Helsinki, Finland. Milk samples were cultivated overnight on M17 (Difco) agar plates at 37 °C. The total bacterial mass was harvested for DNA isolation. For PCR sensitivity tests, *Sta. aureus* DNA was isolated directly from mastitic milk according to the method described by Hynes *et al.* (1992).

Bacterial DNA isolation and amplification of spacer region. Genomic DNA was isolated by the method of Anderson & McKay (1983) with modifications described by Steenson & Klaenhammer (1985). Oligonucleotide primers used for amplifying the 16S–23S rRNA intergenic spacer region were selected from the conserved regions at the 3′ end of the 16S rRNA and the 5′ end of the 23S rRNA genes. The sequences of the primers were 5′ GTCGGAATCGCTAGTAATCG 3′ for 16-1A (bases 1333–1352 in the *Escherichia coli* 16S rDNA sequence, A14565) and 5′ GGGTTCCCCCATTCGGA 3′ for 23-1B (bases 130–114 in the *E. coli* 23S rDNA sequence, A14566). PCR was performed in a DNA thermal cycler 480 (Perkin-Elmer) with a DyNAzyme DNA Polymerase Kit (Finnzymes). A typical reaction mixture (50 µl) consisted of reaction buffer (10 mM Tris/HCl, pH 8·8, 1·5 mM $MgCl_2$, 50 mM KCl, 0·1% Triton X-100), 200 µM each dNTP, 1 µM 16-1A and 23-1B primers, 5 ng bacterial DNA and 0·5 U DyNAzyme DNA polymerase. The reaction mixtures were overlaid with mineral oil (Sigma), incubated at 92 °C for 2 min, then subjected to 30 cycles of 95 °C for 30 s, 55 °C for 30 s, 72 °C for 30 s. Amplification products were analysed on 1·5% (w/v) agarose gels.

Sequencing methods. PCR products from the spacer regions were sequenced directly by a cycle sequencing method using a CircumVent Thermal Cycle Dideoxy DNA Sequencing Kit (New England Biolabs). Sequencing reactions were performed using 15 cycles of 95 °C for 40 s, 55 °C for 30 s and 72 °C for 2 min. The oligonucleotide fragments produced were separated on polyacrylamide gels (Sequagel-6, National Diagnostics). Both strands of DNA were sequenced. If the sequencing was not performed using the total PCR products, the products were cloned into a TA-cloning vector (Original TA Cloning Kit, Invitrogen). Ten positive clones were selected from each species, and plasmid DNA was isolated by alkaline lysis–PEG precipitation (Sambrook *et al.*, 1989). Inserts were sequenced using a CircumVent Thermal Cycle Dideoxy DNA Sequencing Kit or DNA Sequencing Kit for the ABI Prism automatic sequencer (Perkin Elmer).

Analysis of sequence data. The sequences were analysed with DNASIS version 7.0 (Hitachi), and with GCG version 6.0 (Devereux *et al.*, 1984).

Table 1. Bacterial strains used for determination of 16S–23S rRNA spacer sequences

Species	Strain*	Length of spacer (bp)	tRNA in spacer	GenBank accession no.
Str. agalactiae	ATCC 27956	279	Ala	U39765
Str. dysgalactiae	ATCC 27957	285	Ala	U39767
Str. uberis	ATCC 27958	340	Ala	U39768
Sta. aureus	ATCC 25923	458	Ile	U39769
Sta. chromogenes	ATCC 43764	279	–	U39770
Sta. epidermidis	ATCC 12228	261–263	–	U39771, U90018–U90021
Sta. hyicus	KNS 264/92	375–380	–	U39772, U90010–U90015
		461	Ile	U90016
Sta. simulans	ATCC 11631	240–242	–	U39813, U90022–U90027
		333	Ile	U39814
Sta. xylosus	ATCC 12162	283	–	U39773, U90017

* The ATCC strains were obtained from the American Type Culture Collection, Rockville, MD, USA and the KNS strain was obtained from EELA, National Veterinary and Food Research Institute, Helsinki, Finland.

Table 2. Bacterial strains used for assessing the specificity of primers

Species	Strain	PCR products*
Actinomyces pyogenes	Isolated from mastitic milk‡	STAX (150 bp–2·8 kb)
Bacillus cereus	Isolated from mastitic milk‡	–
Corynebacterium bovis	EMO-M/18-I-17‡	STRA (1·5 kb); STAS (2·4 kb)
Enterobacter cloacae	Isolated from mastitic milk‡	–
Enterococcus sp.	Isolated from mastitic milk‡	STR
Escherichia coli	Isolated from mastitic milk‡	STAX (200 bp–1·6 kb)
Klebsiella oxytoca	Isolated from mastitic milk‡	–
Klebsiella pneumoniae	Isolated from mastitic milk‡	–
Lactobacillus acidophilus	ATCC 4356	–
Lactobacillus casei	ATCC 27092	–
Lactobacillus delbrueckii	ATCC 15808	–
Lactobacillus helveticus	ATCC 15009	–
Lactococcus lactis	F7/2 industrial starter strain	–
Leptospira borgpetersenii serovar *hardjo* subtype *hardjobovis*†	Clinical isolate	–
Micrococcus varians	Isolated from mastitic milk‡	STAX (500 bp–1 kb)
Mycoplasma bovis	Donetta	STAH (240 bp)
Nocardia brasiliensis	HAMBI 1960	–
Peptococcus indolicus	AHC 14990‡	–
Propionibacterium freudenreichii	ATCC 6207	–
Pseudomonas aeruginosa	Isolated from mastitic milk‡	–
Str. bovis	ATCC 27960	STR
Str. thermophilus	ATCC 19987	STR
Str. zooepidemicus	EMO-M/18-III-63‡	STR

* The DNA of these strains were tested with STAA, STAC, STAE, STAH, STAS, STAX, STRA, STRD, STRU, STA and STR primer pairs (Table 4). Primer pairs which yielded a PCR product are indicated.

† The subtype was determined by restriction endonuclease digestion of genomic DNA.

‡ Strains isolated from mastitic milk were obtained from EELA, National Veterinary and Food Research Institute, Helsinki, Finland where the species was determined.

PCR with species-specific primers. Based on the comparison of the nucleotide sequences of the spacers, specific primers were designed for each of the species. Genus-specific primers for the *Staphylococcus* and *Streptococcus* species included in this study were also constructed. The PCR reactions were carried out as above using optimized $MgCl_2$ concentrations for each primer pair (see Table 4). A rapid new generation thermal cycler, Air Thermo-Cycler (ATC, Idaho Technology), was also tested for amplification with the specific primer pairs. ATC-PCR uses glass capillary tubes instead of microcentrifuge tubes, which are incubated at 94 °C for 15 s, then 30 cycles of 94 °C for 0 s, 55 °C for 0 s and 72 °C for 15 s. This program takes a total of 15 min instead of 2 h using a conventional thermocycler. The reaction buffers included 0·25 μg BSA ml^{-1}. The $MgCl_2$ concentrations used in ATC-PCR are listed in Table 4.

RESULTS AND DISCUSSION

Amplification of the spacer region for sequencing

Primers complementary to conserved sequences near the 3′ end of the 16S and the 5′ end of the 23S rRNA genes were used to amplify the 16S–23S spacer from nine species which commonly cause mastitis. In staphylococcal species more than one PCR product was detected, although in all cases one band predominated. It is known that the bacterial genome can contain several *rrn* operons, e.g. *Sta. aureus* has nine operons (Gürtler & Barrie, 1995). The size of the main band differed between species. The weaker bands may also represent heteroduplex molecules resulting from cross-hybridization of amplification products from different kinds of operons (Jensen & Straus, 1993).

Sequencing the spacer regions from total PCR products

The 16S–23S spacer region from streptococcal species and *Sta. chromogenes* could be sequenced directly from the PCR products. This indicated that there was a low level of sequence variation in the *rrn* operons within these species. Only the spacer sequence of *Sta. chromogenes* contained a variable nucleotide site (marked S at position 331 in Fig. 1a), with both G and C detected at this position. The previously published spacer sequence

of *Str. agalactiae* (GenBank accession no. L31412; Hall *et al.*, 1995) was compared to our *Str. agalactiae* sequence and only one insertion/deletion and one substitution were found (positions 98 and 351 in Fig. 1a). Sequencing of the spacer region from *Sta. aureus*, *Sta. epidermidis*, *Sta. hyicus*, *Sta. simulans* and *Sta. xylosus* gave multiple sequences that could not be read, suggesting the presence of insertions and/or deletions in the 16S–23S spacer regions of different *rrn* operons. The purification of the main PCR product from agarose gels for sequencing did not improve the readability of sequences, possibly because the insertions and/or deletions were too small to cause sufficient length variation in the spacers. The aligned nucleotide sequences of the spacer regions of streptococcal species are shown in Fig. 1(b) and the spacer region of *Sta. chromogenes* is shown in Fig. 1(a).

Sequencing the spacer regions from plasmid clones

The PCR products from the spacer regions of *Sta. aureus*, *Sta. epidermidis*, *Sta. hyicus*, *Sta. simulans* and *Sta. xylosus* were cloned into TA-cloning vector. Ten clones from different PCR amplifications were sequenced from *Sta. epidermidis*, *Sta. hyicus*, *Sta. simulans* and *Sta. xylosus*. Several different spacer sequences were obtained: five from *Sta. epidermidis*, seven from *Sta. hyicus*, eight from *Sta. simulans* and two from *Sta. xylosus* (Fig. 1a, clone types A–H). Most of the differences between the sequences from a single strain were single base insertions/deletions and substitutions. Given that the rate of artefactual misincorporation during PCR is relatively low and random, it is likely that these differences represent mutations present in the genome. The first sequence shown for each species (Fig. 1a) was obtained from clones from two independent amplifications. Some substitutions and insertions/deletions of tRNA genes were also found. All the streptococcal species had a tRNA-Ala gene in the spacer and *Sta. aureus*, *Sta. hyicus* and *Sta. simulans* had a tRNA-Ile gene in some of their *rrn*-operons (Fig. 1, Table 1). In general, however, the sequence variation within species was very low. From *Sta. aureus* only one clone was sequenced, since the sequences of all *Sta. aureus rrn* operons have been published previously by Gürtler & Barrie (1995). Our cloned *Sta. aureus* spacer sequence was 99% homologous to the allele *rrnF* (GenBank accession no. U11779; Gürtler & Barrie, 1995), with only three single base insertions/deletions and four mismatches.

Sequence variation between species

The pairwise sequence identities between 16S–23S spacer sequences of different species were calculated using the DNA Maximum Homology program of DNASIS (Table 3). This program favours short deletions over long ones and may overestimate the influence of a long deletion on the degree of DNA identity. The sequence identity between species within a genus varied from 53 to 85%. The calculated sequence identities between species of different genera were about 50%, but because gaps were used freely, this is an overestimate. The sequences were aligned manually in Fig. 1(a) and (b) to minimize the number of sequence differences. The spacer sequences for *Str. bovis* (GenBank accession no. U39766; this study) and *Str. thermophilus* (U32965; Tilsala-Timisjärvi & Alatossava, 1997) were included in the alignment (Fig. 1b) to obtain more information about the variation of the spacer regions within the *Streptococcus* genus. This knowledge was important for the design of species-specific primers. The amount of sequence difference between staphylococci and streptococci prevented their reliable alignment. The only regions of sequence similarity ($\geqslant 8$ bp) between these two genera have been indicated in the alignments (Fig. 1a and b). Both conserved regions are important for RNA processing (Chiaruttini & Milet, 1993). When comparing the species within genera, the 3′ terminal third of the spacer region was found to be highly variable and difficult to align, especially in staphylococcal species. The 5′ terminus was more conserved (Fig. 1).

Design of primer pairs from the species-specific sequence regions

The variation between the 16S–23S spacer sequences of different mastitis pathogens made it possible to design specific primer pairs for each of the nine species considered in this study. The oligonucleotides were selected near the ends of the spacer region (Fig. 1, Table 4). In addition, primer pairs for *Staphylococcus* and *Streptococcus* were designed (Fig. 1, Table 4). The primer pairs were selected so that the same PCR cycling conditions could be employed for all of them. The optimum $MgCl_2$ concentrations were adjusted for both conventional PCR (Perkin-Elmer 480) and ATC-PCR (Table 4). There was some sequence variation within species at the primer regions. In those cases the primers were selected from the most common spacer type. Species-specific PCR primers have been published previously for some of the species (although not for strains isolated from cases of bovine mastitis) from the 16S rRNA gene, e.g. *Sta. aureus* and *Sta. epidermidis* (Saruta *et al.*, 1995; Gribaldo *et al.*, 1997).

Sensitivity of the primers

Products were detected using as little as 50 pg purified bacterial DNA, with 35 cycles in PCR. The DNA concentrations were measured with a spectrophotometer (Gene Quant II, Pharmacia Biotech) and with a fluorometer (TKO100 Mini-Fluorometer, Hoefer Scientific). When the sensitivity of the primers was estimated by isolating DNA directly without cultivation from mastitic milk samples containing *Sta. aureus*, a 1 μl milk sample containing 40 c.f.u. yielded a PCR product (results not shown).

(a)

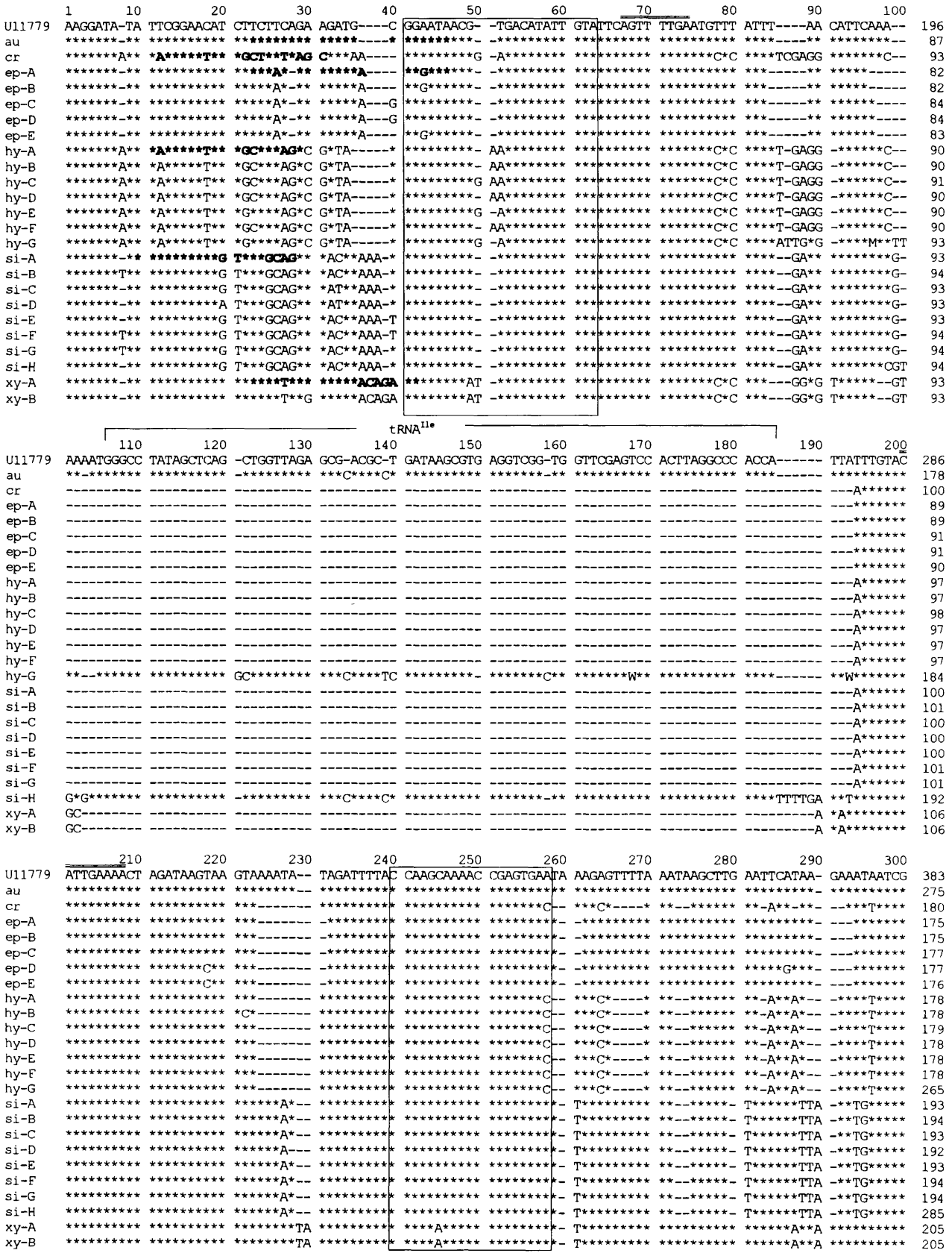

Fig. 1. For legend see page 3497.

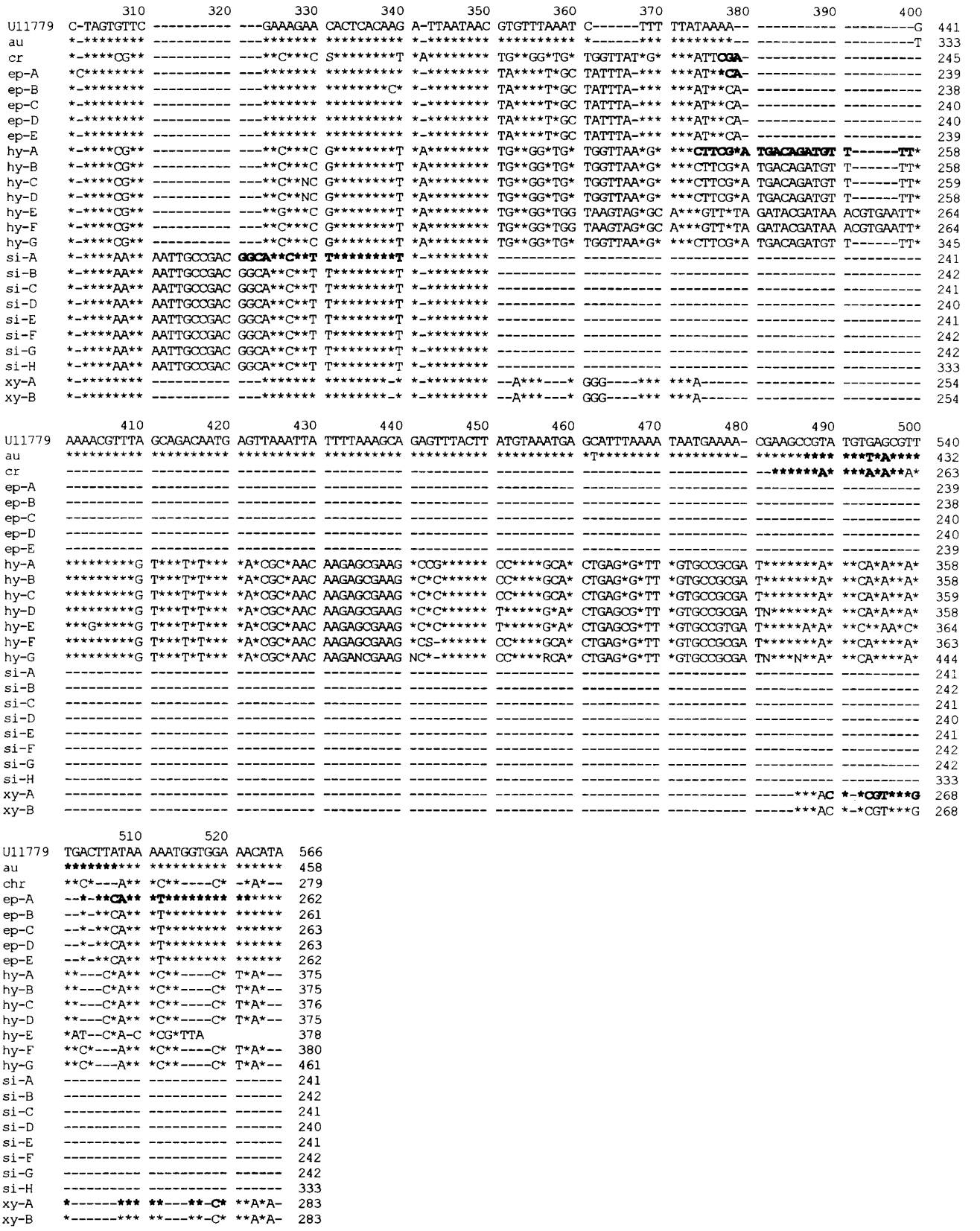

Fig. 1. For legend see facing page.

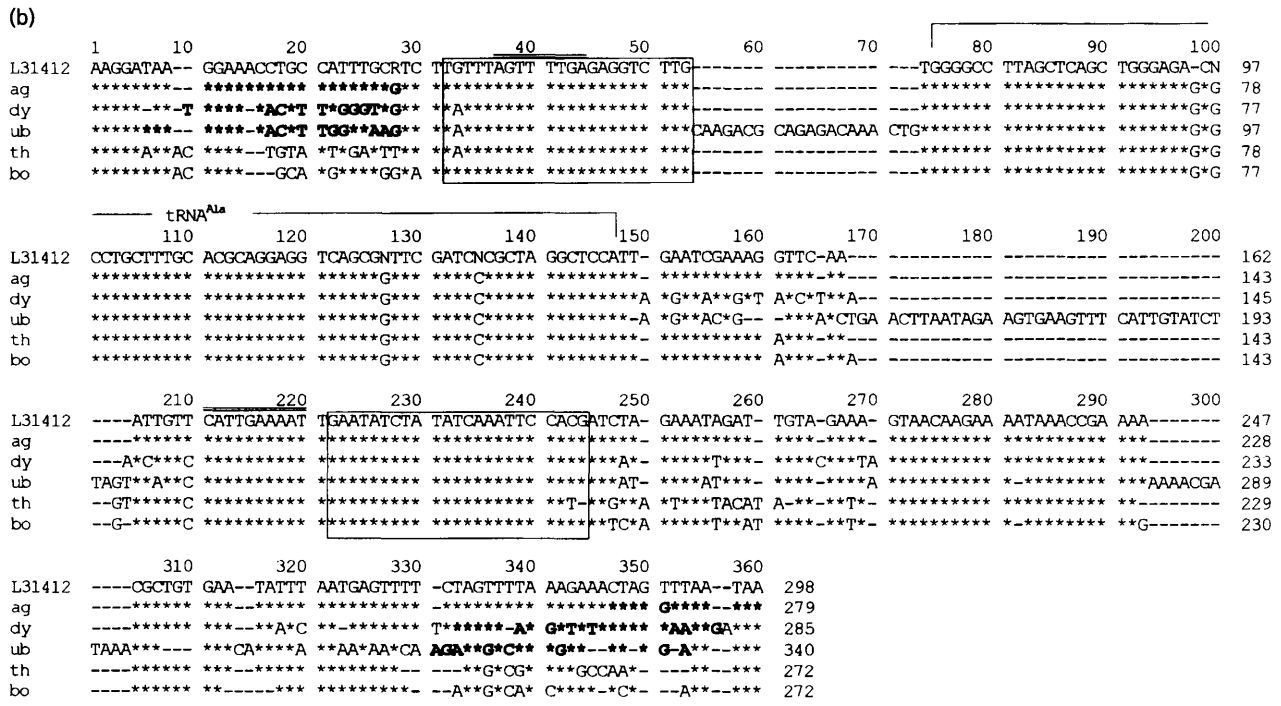

Fig. 1. Alignments of the 16S–23S intergenic spacer sequences from (a) staphylococcal species and (b) streptococcal species. Symbols: au, *Sta. aureus*; cr, *Sta. chromogenes*; ep, *Sta. epidermidis*; hy, *Sta. hyicus*; si, *Sta. simulans*; xy, *Sta. xylosus*; ag, *Str. agalactiae*; dy, *Str. dysgalactiae*; ub, *Str. uberis*; th, *Str. thermophilus* (GenBank accession no. U32965; Tilsala-Timisjärvi & Alatossava, 1997); bo, *Str. bovis* (U39766; this study). The clone types are indicated after the species in capital letters (A–H). U11779 and L31412 are the spacer sequences of *Sta. aureus* and *Str. agalactiae*, respectively (Gürtler & Barrie, 1995; Hall *et al.*, 1995). The species-specific primers are presented in bold, and the genus-specific primers are boxed. The regions (minimum length 8 bp) identical in all staphylococcal and streptococcal species are indicated with double lines. Identical nucleotides are indicated with asterisks, deletions with dashed lines, and transitions and transversions compared to the uppermost sequence of the alignment are indicated with capital letters. The locations of tRNA genes are indicated above the aligned sequences.

Specificity of the primers

Each primer pair was tested at least twice with DNA from 31 different bacterial species (Tables 1 and 2) using 5 ng template DNA in a 20 µl PCR reaction with conventional PCR. Appropriate controls were included in PCR; a positive control for DNA and primers and a negative control for PCR mix. In addition, the primer pairs were tested after raising the concentration of DNA from staphylococcal and streptococcal species up to 100 ng in a 20 µl PCR reaction. The performance of ATC-PCR was tested with nine DNA samples (Table 1). The 'species-specific' primer pairs (Table 4) amplified their targets mainly as expected, amplifying only the corresponding DNA (Table 2, Fig. 2). The unexpected products with STAX, STRA, STAH and STAS primer pairs differed in size from the expected products, and no amplification with the 'genus-specific' primers was observed. If these cases represented true *Streptococcus* or *Staphylococcus* species, a product with the genus-specific primers would have been expected also. The genus-specific primers amplified as expected: the *Staphylococcus* specific primers amplified only staphylococcal DNA, and the *Streptococcus* specific primers amplified streptococcal, lactococcal and enterococcal DNA (Table 2). Lactococci and enterococci are very closely related to streptococci and have similar sequences in the primer regions (Chiaruttini & Milet, 1993; Sechi & Daneo-Moore, 1993; Tilsala-Timisjärvi & Alatossava, 1997; Wood & Holzapfel, 1995).

Validity of the primers with mastitis samples

The original staphylococcal and streptococcal strains from which the spacer regions were sequenced were mostly ATCC strains (Table 1), not isolated from bovine mastitis. To investigate if there was sequence variation between strains within a species, which could affect the utility of the assays, we performed PCR on 51 isolates from cases of bovine mastitis. The conventional species determination was made in a veterinary clinical microbiology laboratory (Food and Environmental Laboratory, Oulu, Finland or National Veterinary and Food Research Institute Helsinki, Finland). PCR reactions used purified DNA from each cultivated isolate (Table 5). The staphylococcal genus-specific primer pair distinguished the genus with 100% sensitivity and specificity. The streptococcal genus-specific primer pair detected one *Sta. aureus* isolate and all streptococcal isolates.

Table 3. Sequence identity between 16S–23S rDNA spacer sequences of different *Staphylococcus* and *Streptococcus* species

au, *Sta. aureus* clone; cr, *Sta. chromogenes*; ep-A, *Sta. epidermidis* clone type A; hy-A, *Sta. hyicus* clone type A; si-A, *Sta. simulans* clone type A; xy-A, *Sta. xylosus* clone type A; ag, *Str. agalactiae*; dy, *Str. dysgalactiae*; ub, *Str. uberis*.

	DNA identity (%)*							
Spacer	cr	ep-A	hy-A	si-A	xy-A	ag	dy	ub
au	63	68	69	58	65	50	52	53
cr	·	74	71	66	70	52	54	52
ep-A		·	59	71	77	52	52	52
hy-A			·	53	57	51	52	51
si-A				·	71	51	51	50
xy-A					·	53	53	52
ag						·	85	70
dy							·	70

* The tRNA gene region was excluded from the *Sta. aureus* (au) spacer sequence for analysis. The calculations were made by the DNA Maximum Homology program of DNASIS.

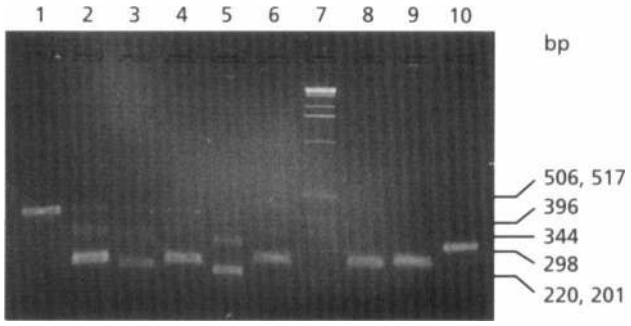

Fig. 2. The PCR products from different streptococcal and staphylococcal species amplified with corresponding species-specific primer pairs. Lanes: 1, *Sta. aureus* ATCC 25923; 2, *Sta. chromogenes* ATCC 43764; 3, *Sta. epidermidis* ATCC 12228; 4, *Sta. hyicus* KNS 264/92; 5, *Sta. simulans* ATCC 11631; 6, *Sta. xylosus* ATCC 12162; 7, 1 kb ladder (Gibco BRL); 8, *Str. agalactiae* ATCC 27956; 9, *Str. dysgalactiae* ATCC 27957; 10, *Str. uberis* ATCC 27958.

Ninety percent of the isolates identified conventionally at the species level (36/40) were recognized with the species-specific primers. The reason for the failure of identification for four isolates may be sequence variation between strains in the primer regions. In addition, three isolates gave a PCR product with a second species-specific primer pair (Table 5). These products may also be due to sequence variation. Alternatively, the milk samples may have contained minor amounts of these other bacterial species. Only five of the CNS isolates were identified to the species level in the clinical

Table 4. Species- and genus-specific oligonucleotide primers from the 16S–23S rRNA intergenic spacer region for bovine mastitis pathogens

Species	Oligonucleotide*	Sequence (5′–3′)	Length (nt)	$MgCl_2$ in PCR (mM)†	$MgCl_2$ in ATC-PCR (mM)†	Size of the main PCR product (bp)
Str. agalactiae	STRA-AgI	GGAAACCTGCCATTGCG	18	3·0	5·0	280
	STRA-AgII	TAACTTAACCTTATTAACCTAG	22			
Str. dysgalactiae	STRD-DyI	TGGAACACGTTAGGGTCG	18	3·0	3·0	270
	STRD-DyII	CTTTTACTAGTATATCTTAACTA	23			
Str. uberis	STRU-UbI	TAAGGAACACGTTGGTTAAG	20	1·5	3·0	330
	STRU-UbII	TCCAGTCCTTAGACCTTCT	19			
Sta. aureus	STAA-AuI	TCTTCAGAAGATGCGGAATA	20	2·0	3·0	420
	STAA-AuII	TAAGTCAAACGTTAACATACG	21			
Sta. chromogenes	STAC-ChrI	ACGGAATATCGCTTTTAAGC	20	1·5	3·0	250
	STAC-ChrII	CGTTTACATTCGGCTTTCG	19			
Sta. epidermidis	STAE-EpI	TCTACGAAGATGAGGGATA	19	3·0	3·0	240
	STAE-EpII	TTTCCACCATATTTTGAATTGT	22			
Sta. hyicus	STAH-HyI	TACGGAATATCGCCTTAGG	19	1·5	4·0	250
	STAH-HyII	AAAACATCTGTCATCCGAAG	20			
Sta. simulans	STAS-SiI	ATTCGGAACAGTTTCGCAG	19	1·5	4·0	220
	STAS-SiII	ATTGTGAGTAATCGTTTGCC	20			
Sta. xylosus	STAX-XyI	TCTTTAGAAGATGACAGAGG	20	1·5	3·0	260
	STAX-XyII	TGACTTTTAACACGACGAAG	20			
Streptococcus genus	STR I	TGTTTAGTTTTGAGAGGTCTTG	22	1·5	5·0	150–210
	STR II	CGTGGAATTTGATATAGATATTC	23			
Staphylococcus genus	STA I	GGAATAACGTGACATATTGTA	21	1·5	3·0	100–200
	STA II	TTCACTCGGTTTTGCTTGG	19			

* Primer I in each primer pair is from the 5′ terminus of the spacer and primer II is from the 3′ terminus of the spacer.

† Optimal $MgCl_2$ concentrations in PCR reactions.

Table 5. Specificity of the primer pairs with DNA of 51 isolates from mastitic milk

Bacterial sample	No. of samples	Identification with PCR
Sta. aureus	19	*Staphylococcus*, *Sta. aureus*
Sta. aureus	2	*Staphylococcus*
Sta. aureus	1	*Staphylococcus*, *Sta. aureus*, *Streptococcus*
Sta. aureus	2	*Staphylococcus*, *Sta. aureus*, *Str. agalactiae*
Sta. aureus	1	*Staphylococcus*, *Sta. aureus*, *Sta. hyicus*
*Sta. chromogenes**	1	*Staphylococcus*, *Sta. chromogenes*
*Sta. epidermidis**	1	*Staphylococcus*, *Sta. epidermidis*
*Sta. hyicus**	1	*Staphylococcus*, *Sta. hyicus*
*Sta. simulans**	1	*Staphylococcus*, *Sta. simulans*
*Sta. xylosus**	1	*Staphylococcus*
CNS	1	*Staphylococcus*, *Sta. chromogenes*
CNS	3	*Staphylococcus*, *Sta. simulans*
CNS	4	*Staphylococcus*, *Sta. xylosus*
CNS	3	*Staphylococcus*
Str. uberis	5	*Streptococcus*, *Str. uberis*
Str. uberis	1	*Streptococcus*, *Str. uberis*, *Str. agalactiae*
Str. agalactiae	2	*Streptococcus*, *Str. agalactiae*
Str. dysgalactiae	1	*Streptococcus*, *Str. dysgalactiae*
Str. dysgalactiae	1	*Streptococcus*

* CNS samples identified at the species level.

laboratories (Table 5). Eight out of eleven CNS samples (73%) were detected by PCR with the species-specific primers. With the API-Staph system we identified one of the remaining CNS samples as *Staphylococcus warneri*, which could not be detected with our PCR primers. Two other CNS samples remain unidentified.

Applicability of the results

The important aims of this study were to investigate the level of sequence variation in the 16S–23S spacer region between different staphylococcal and streptococcal mastitis pathogens and the potential use of this variation for the rapid identification of these pathogens to species level. The conventional microbiological and biochemical methods for identifying mastitis pathogens are time-consuming. In addition, CNS species are still difficult to identify, and thus species identification is not done routinely, in spite of their increased prevalence in mastitis cases (White *et al.*, 1989; Myllys *et al.*, 1994). The advantages of PCR compared to other diagnostic methods are rapidity and facility. Furthermore, screening for antibiotic resistance could be combined with PCR-based species identification. In practice, the PCR identification could be done in two stages. First, performing the tests for the genus (STA, STR), *Sta. aureus*, the streptococcal species and antibiotic resistance (e.g. *blaZ* for β-lactamase gene). The second PCR would be the identification of CNS species if necessary. The main disadvantage of a PCR identification method might be excessive sensitivity, with minor contaminants in samples resulting in misdiagnosis. The multiple copy number of *rrn* operons in a bacterial genome is an advantage, increasing the sensitivity of PCR, but the potential for variation within a species may reduce the sensitivity for all strains. The current primer pairs work reasonably well, but not yet with 100% of isolates. Improvement in both primer design and PCR conditions is still needed. The primer pairs designed in this study proved to be specific and appropriately sensitive, such that the normal bacterial flora in milk did not give PCR products. However, a more extensive study of sequence variation in strains of these species isolated from cases of bovine mastitis is still needed to critically assess specificity. The traditional species identification would have to be confirmed by sequencing part(s) of the 16S rRNA gene, since even biochemical identification systems are not 100% reliable. Our future work will focus on detecting mastitic pathogens by PCR directly from milk.

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