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Evaluation of tRNA intergenic spacer length polymorphism analysis as a molecular method for species identification of streptococcal isolates from bovine mastitis

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Sixty-nine bovine mastitis streptococci belonging to the species *Str. agalactiae* (*n*=13), *Str. dysgalactiae* (*n*=16), *Str. canis* (*n*=22), *Str. uberis* (*n*=20) and *Str. parauberis* (*n*=4) and six reference strains of the five streptococcal species were examined for their tRNA gene intergenic length polymorphism (tDNA-ILP) fingerprint pattern. Epidemiologically unrelated isolates from bovine mastitis cases were selected by macrorestriction analysis with pulsed-field gel electrophoresis (PFGE). Their results were compared with those obtained from biochemical and serological studies and with those obtained by PCR-mediated identification amplifying species-specific gene segments of the five streptococcal species. According to the present results tDNA-ILP allowed a correct identification of all *Str. agalactiae, Str. uberis* and *Str. parauberis* strains investigated also including the reference strains of each species showing species-specific banding pattern. However, all *Str. dysgalactiae* ssp. *dysgalactiae* and all *Str. canis* strains appeared with an undistinguishable pattern which did not allow an identification of the species.

Keywords: *Streptococcus* species, bovine mastitis, identification, tDNA-ILP-PCR.

Streptococci are recognized worldwide as important aetiological agents of infections of the bovine mammary gland (Lämmler & Hahn, 1994). The prevalence of different species varies geographically, temporally and due to control measurements adopted in herds. In most countries the common streptococcal species causing mastitis are *Streptococcus agalactiae, Str. dysgalactiae* ssp. *dysgalactiae* and *Str. uberis* (Lämmler & Hahn, 1994). However, owing to antibiotic therapy, the prevalence of *Str. agalactiae* has decreased (Kloppert et al. 2000; Wolter et al. 2000). A correct identification to species level of the bacterial pathogens causing bovine mastitis is of clinical and epidemiological interest. This knowledge helps in the development of preventive strategies. In routine bacteriological laboratories, mastitis-causing bacteria are usually identified by conventional methods using serological and biochemical identification systems, which are more time-consuming than newer molecular biological methods. However the inability to identify 1–10% of isolates because of atypical test results is reported by numerous authors (McDonald & McDonald, 1976; Poutrel & Ryniewicz, 1984; Watts, 1988; Watts, 1989; Jayarao et al. 1991).

In the last decade, molecular methods like polymerase-chain-reaction (PCR) have been increasingly used in microbiological diagnostics. This is performed with highly conserved sequences such as rRNA or tRNA genes which can be used to design species-specific oligonucleotide primers. tRNA genes contain sequence motifs that are highly conserved among eubacteria. These genes proved to be an interesting target to investigate tRNA gene intergenic lenth polymorphism (tDNA-ILP) between various bacterial species (Welsh & McClelland, 1991).

The present study was designed to evaluate a PCR-mediated tRNA-ILP system for identification and differentiation of streptococci isolated from bovine intramammary infections.

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Materials and Methods

Bacterial strains and phenotypic characterization

The following type strains of the genus *Streptococcus* ($n=6$) were used: *Str. agalactiae* (ATCC 13813 and ATCC 27956), *Str. dysgalactiae* ssp. *dysgalactiae* (ATCC 9926), *Str. canis* (ATCC 43496), *Str. uberis* (ATCC 19436) and *Str. parauberis* (DSM 6631). All these strains were provided by the German Collection of Microorganisms and Cell Cultures (DSM).

Clinical isolates collected after cultivation of quarter-milk samples (IDF, 1982) from cows suffering from subclinical or clinical mastitis (somatic cell count [Fossomatic] >100 000 cells/ml) or macroscopic signs of mastitis including *Str. agalactiae* ($n=11$), *Str. dysgalactiae* ssp. *dysgalactiae* ($n=15$), *Str. canis* ($n=21$), *Str. uberis* ($n=19$) and *Str. parauberis* ($n=3$). Selection of clinical strains was according to their epidemiological and genetic relatedness. Only epidemiologically unrelated strains from different flocks and cows were selected for further investigations. Additionally, genetic relatedness was based on the results of DNA macrorestriction analysis with pulsed-field gel electrophoresis (PFGE; Soedarmato & Lämmler, 1996) and defined as published by Tenover et al. (1995). Only genetically unrelated isolates were used in this study. Strains were classified as genetically unrelated if they showed a six band or greater difference and a Dice coefficient of correlation of 60% or less. Banding patterns were compared visually by two independent observers and by calculating the Dice coefficient (number of shared fragments $\times 2 \times 100$/total number of fragments in the two samples) with the Gel Compare Software (Applied Maths, Kortrijk, Belgium).

Type strains and clinical isolates were investigated for cultural properties biochemically using the API ID 32_{Strep} system (BioMerieux, Nürtingen, Germany) according to the manufacturer's instructions by use of the APILAB software (version 2.0) and serologically with a commercial coagglutination test (Phadebact Streptococcus test, Innogenetics, Heiden, Germany). The bacteria were additionally tested for CAMP reactivities (Lämmler & Hahn, 1994).

Genotypic characterization

PCR with species specific primers. For genomic DNA preparation, 5–10 colonies of the bacteria were suspended in 100 µl TE-buffer (10 mM-Tris–HCl, 1mM-EDTA, pH 8·0, containing mutanolysin (50 U), Sigma, Deisenhofen, Germany) for 90 min at 37 °C and 10 µl proteinase K (14·8 mg/ml, Boehringer, Mannheim, Germany) for 120 min at 56 °C. After boiling for 10 min at 100 °C the suspension was centrifuged at 10 000 ***g*** for 5 s and subsequently cooled before use. The reference strains and the streptococci isolated from routine diagnostics were subsequently identified genotypically by a PCR-mediated detection of species-specific segments of 16S RNA V2-region using single primers as described recently (Lämmler et al. 1998; Abdulmawjood & Lämmler, 1999; Abdulmawjood & Lämmler, 2000; Hassan et al. 2003a)

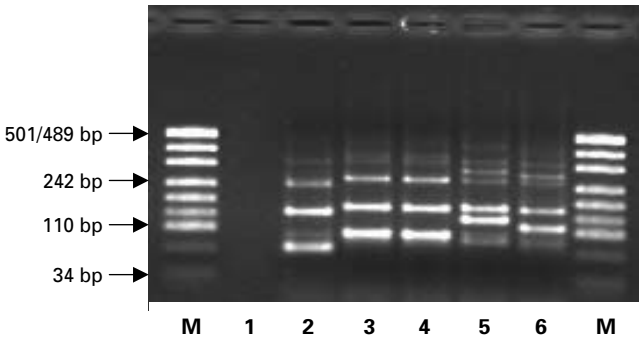

Fig. 1. Typical tDNA-ILP banding patterns of the reference strains *Str. agalactiae* ATCC 13813(2), *Str. dysgalactiae* ssp. *dysgalactiae* ATCC 9926 (3), *Str. canis* ATCC 43496 (4), *Str. uberis* ATCC 19436 (5), *Str. parauberis* DSM 6631(6), representing patterns I (2), II (3,4), III (5) and IV (6), respectively. Lane (1) represents a negative control (destilled water) lanes (M) the size marker pUC 19 DNA/AspI.

tDNA-ILP-PCR. PCR was carried out with the outwardly directed tRNA gene (tDNA) consensus primers T5A (5′-AGT CCG GTG CTC TAA CCA ACT GAG-3′) and T3B (5′-AGG TCG CGG GTT CGA ATC C-3′) described by Welsh & McClelland (1991). Oligonucleotide primers were synthesized by MWG Biotech (Ebersberg, Germany). The PCR reaction mixture (50 µl) contained 1·25 U of Taq-polymerase (Sigma), 1× PCR buffer (50 mM-KCl, 10 mm-Tris–HCl, pH 8·3, 1·5 mM-$MgCl_2$, 0·5 µM-(each) primer, 0·2 mM of each of the four desoxynucleoside triphosphates and 5 µl of the bacterial lysate as DNA template. Amplification conditions were as described by Welsh & McClelland (1991). Amplification products were separated by electrophoresis for 3 h and 30 min at 160 V in a 4% metaphore agarose gel (Biozym, Hess. Oldendorf, Germany). DNA Standard pUC 19 DNA/AspI (Fermentas, St Leon-Rot, Germany) containing DNA fragments between 154 and 2176 bp was used as a size marker. After ethidium bromide-staining the amplification product was visualized using Gel Doc 1000 (BioRad, München, Germany). The amplicon patterns were compared and classified with roman numerals.

Results

The species identification of the six reference cultures and the 69 isolates from routine mastitis diagnosis used in the present study could be performed by culture properties, biochemically and serologically using commercial identification systems and genetically by amplification of species specific target genes. Typical species specific amplicons of the five reference cultures are shown in Fig. 1.

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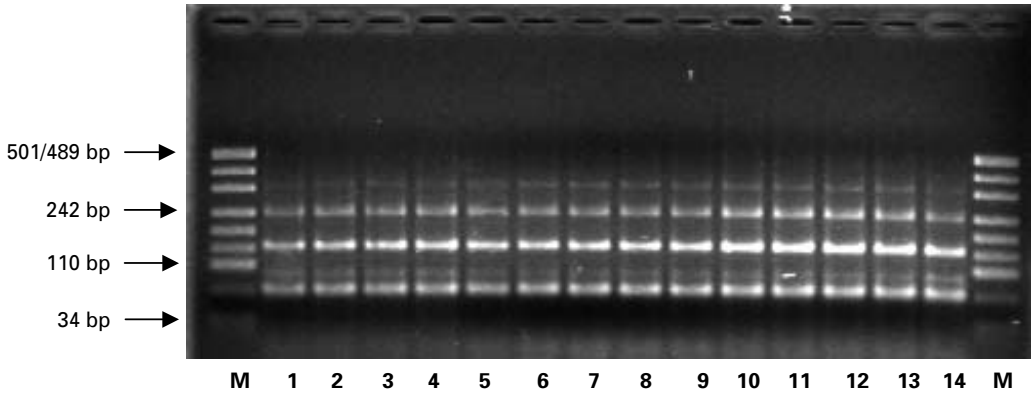

Fig. 2. tDNA-ILP pattern of the *Str. agalactiae* reference strains (1,2) and *Str. agalactiae* strains obtained from bovine mastitis diagnostic (3–14). Lanes M represent the size marker pUC 19 DNA/AspI.

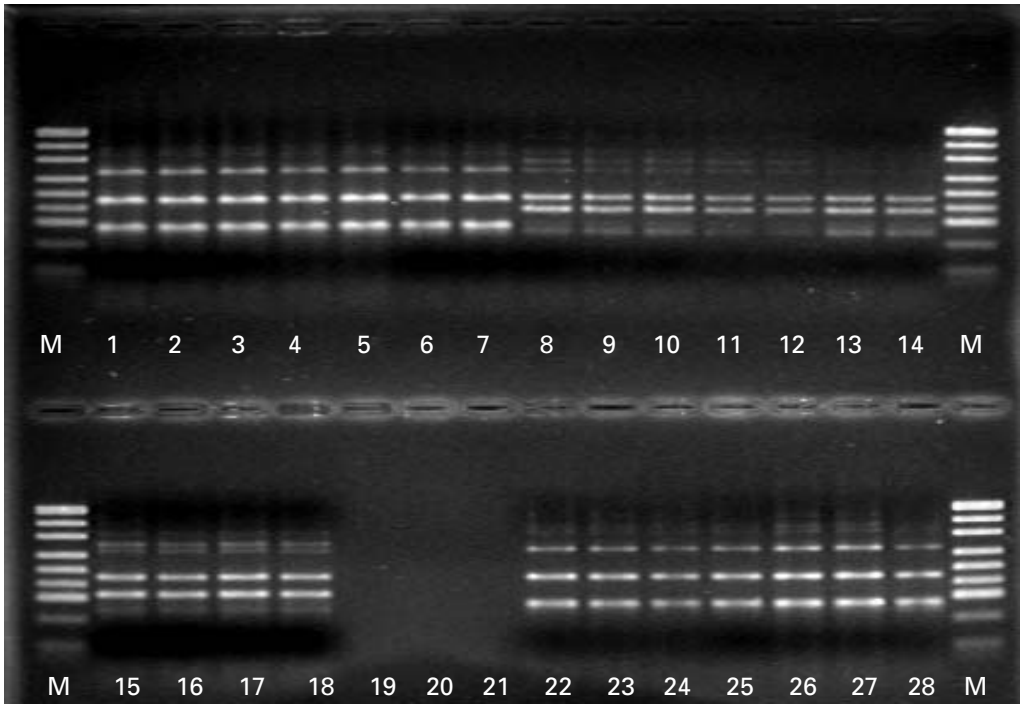

Fig. 3. tDNA-ILP pattern of the reference strains: *Str. dysgalactiae* (1), *Str. uberis* (8), *Str. parauberis* (15), *Str. canis* (22) and strains obtained from bovine mastitis diagnostic: *Str. dysagalactiae* (2–7), *Str. uberis* (9–14), *Str. parauberis* (16–18), *Str. canis* (23–28). Lanes M represent the size marker pUC 19 DNA/AspI. Lanes(19–21) show negative controls (distilled water).

tDNA-ILP analysis of the reference cultures and the isolates from routine diagnosis revealed species-specific banding pattern for *Str. agalactiae*, *Str. uberis* and *Str. parauberis*. The tDNA-ILP pattern of all *Str. agalactiae* consists of four bands with sizes of 80, 150, 250 and an only weak fragment at 350 bp. All *Str. uberis* and all four *Str. parauberis* strains appeared with five bands with sizes of 150, 185, 270, 300, 350 bp and 135, 185, 270, 290, 350 bp, respectively. The reference strains and mastitis isolates of the species *Str. dysgalactiae* ssp. *dysgalactiae* and *Str. canis* showed identical banding patterns with sizes of 115, 180 and 270 bp. Typical tDNA-ILP patterns of the six reference strains and Streptococci mastitis isolates of the present study are shown in Figs 2 and 3, respectively. The determination of patterns of each strain were performed in duplicate showing a complete reproducibility. The results of the tDNA-ILP as compared with biochemical identification and 16S RNA-PCR are summarized in Table 1.

Discussion

Differentiating clinically significant isolates of genus *Streptococcus* from quarter milk samples of cows suffering

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Table 1. Comparison of results of tDNA-ILP analysis, biochemical and serological identification and 16S RNA-PCR for clinical isolates

	Species identification (number of strains)	
ID 32 $_{Strep}$[a]	tDNA-ILP pattern (fragment sizes)	16S (V2-region) RNA
Str. agalactiae (11)	**I** (80, 150, 250, [350] [b] bp)	1265 bp (*Str. agalactiae*)
Str. dysgalactiae ssp. dysgalactiae (15)	**II** (115, 180, [270] bp)	895 bp (*Str. dysgalactiae* ssp. *dysgalactiae*)
Str. canis (21)	**II** (115, 180, [270] bp)	1320 bp (*Str. canis*)
Str. uberis (19)	**III** (150, 185, [270], [300], [350] bp)	445 bp (*Str. uberis*)
Str. parauberis (3)	**IV** (135, 185, [270], [290], [350] bp)	884 bp (*Str. parauberis*)

[a]: phenotypic results (Api ID 32 $_{Strep}$)

	ADH	BGLU	BGAR	BGUR	AGAL	PAL	RIB	MAN	SOR	LAC	TRE	RAF	VP	APPA	BGAL	PYRA
Str. agalactiae	+	–	–	(+)	–	+	+	–	–	–	+	–	+	+	–	–
Str. dysgalactiae	+	–	–	+	–	+	+	–	–	+	+	–	–	+	–	–
Str. uberis	+	+	–	+	–	–	+	+	+	+	+	–	+	+	–	–
Str. parauberis	+	+	–	+	–	+	+	+	+	+	+	–	+	+	–	–
Str. canis	+	–	–	–	+	+	+	–	–	+	–	–	–	+	+	–
	BNAG	GTA	HIP	GLYG	PUL	MAL	MEL	MLZ	SAC	LARA	DARL	MBDG	TAG	BMAN	CDEX	URE
Str. agalactiae	–	–	+	–	+	+	–	–	+	–	–	+	–	–	–	–
Str. dysgalactiae	–	–	–	–	+	+	–	–	+	–	–	–	(+)	–	–	–
Str. uberis	–	–	+	–	–	+	–	–	+	–	–	+	–	–	–	–
Str. parauberis	–	–	+	–	–	+	–	–	+	–	–	+	–	–	–	–
Str. canis	–	–	–	–	+	+	–	–	+	–	–	+	–	–	–	–

ADH Argine dehydrogenase; BGLU β-Glucosidase; BGAR β-Galactosidase; BGUR β-Glucuronate, AGAL α-Galactosidase; PAL alkaline phosphatase; RIB Ribose; MAN Mannitol; SOR Sorbitol; LAC Lactose; TRE Trehalose; RAF Raffinose; SAC Saccharose; LARA L-Arabinose; DARL D-Arabitol; CDEX Cyclodextrin; VP Acetoin Production; APPA Alanyl Phenylalanyl-Prolin-Arylamidase; BGAL β-Galactosidase; PYRA Pyroglutamine Acid Arylamidase; BNAG N-Acetyl-β-Glucosaminidase; GTA Glycyl-Tryptophan Arylamidase, HIP Hydrolysis of Hippurate; GLYG Glycogene; PUL Pullulan, MAL Maltose; MEL Melibiose; MLZ Melezitose; MBDG Methyl-β-Dglucopyranoside; TAG Tagatose; BMAN β-Mannosidase; URE Urease

[b]:parentheses [ ] show only weak framents

from mastitis to species level is a daily issue in routine diagnostic laboratories. Identification to the species level is a fundamental step in the appropriate investigation of, and subsequent control of a mastitis problem in a herd. Phenotypic methods often do not allow for the correct identification of all clinical isolates of streptococci. The use of phenotypic methods, also including commercial identification systems, may not always allow a correct identification of streptococci isolated from animal origin. The accuracy for the identification of animal isolates depends on the accuracy of the reference schemes, the ability of the tests to distinguish between species and the number and diversity of veterinary strains included in the data base (Poutrel & Ryniewicz, 1984; Watts, 1989; Jayarao et al. 1991). Traditionally β-haemolytic streptococci are classified by serogrouping the cell wall carbohydrate antigens according to the Lancefield scheme. However, this system does not classify all streptococci correctly, because group-specific antigens are not always present, identical antigens may occur in different species and more than one antigen may occur in a single species (Lämmler & Hahn 1994).

However, in recent studies a number of molecular-based approaches for identification of species of clinically important bacteria had been described (Hassan et al. 2003a; Hassan et al. 2003b). These mostly PCR-mediated detections of bacterial pathogens appear to be highly sensitive and allow an identification of the bacteria even from clinical samples. t-DNA-ILP analysis was initially used for identification of a limited number of species of genus S*taphylococcus* (Welsh & McClelland, 1991) and *Streptococcus* (McClelland et al. 1992). The present study was performed to evaluate the usefulness of the identification of streptococci isolated from bovine mastitis. As a result of macrorestriction fragment analysis the clinical isolates were selected as being epidemiologically non related (different PFGE-types). Because of the interspecies diversity and a remarkable level of conservation within a species tDNA-ILP appeared to be a useful tool for rapid and reliable identification of *Str. agalactiae, Str. uberis* and *Str. parauberis* isolated from bovine mastitis. The tDNA- ILP results confirmed the species identification obtained by biochemical and serological tests and by PCR amplification of species specific gene segments. tDNA-ILP-PCR had already been successfully used for identification of bacteria of genus *Staphylococcus* species (Maes et al. 1992), *Acinetobacter* species (Ehrenstein et al. 1996) and viridans streptococci (De Gheldre et al. 1999). However, because of identical banding patterns tDNA-ILP-PCR could not be used for differentiation of *Str. dysgalactiae*

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ssp. *dysgalactiae* and *Str. canis*. The lack of discrimination between these two species could possibly be explained by a high degree of sequence homology in this gene segment.

According to previous studies *Str. canis* could be identified by PCR-mediated analysis of species-specific parts of 16S rRNA-Gene, 16S–23S rRNA intergenic spacer region and by PCR amplification of CAMP gene *cfg* (Hassan et al. 2003a). Comparable to these findings *Str. dysgalactiae* ssp. *dysgalactiae* could be detected by PCR amplification of species-specific segments of 16S–23S rRNA intergenic spacer region (Hassan et al. 2003b). However, these two streptococcal species are discriminated simply by their morphological behaviour on blood-containing agar plates. As an advantage of tDNA-ILP only one primer pair and one run protocol is necessary for diagnosis of all these streptococcal species.

A future combination of different PCR systems combining the advantage of tDNA-ILP analysis described in the present study and target gene detection such as those used previously might help to discriminate between *Str. dysgalactiae* ssp. *dysgalactiae* and *Str. canis* and in parallel help to identify *Str. agalactiae, Str. uberis* and *Str. parauberis*.

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