Identification and epidemiological characterization of *Streptococcus uberis* isolated from bovine mastitis using conventional and molecular methods

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Introduction

*Streptococcus uberis* is world wide known as an environmental pathogen responsible for a high proportion of cases of clinical, mostly subclinical mastitis in lactating cows and is also the predominant organism isolated from mammary glands during the nonlactating period [37].

* S. uberis differs from other mastitis-causing streptococci in that it can also be isolated from the udder surface, from other sites on the body of cows and also from the cows environment. The most important reservoirs for infections of the mammary gland parenchyma appears to be the skin and the udder surface [35,52]. *S. uberis* can also be isolated from numerous sites including belly, lips, teats, urogenital tract, tonsils, rectum, rumen, nostrils, eye, poll, chest, sacrum, caudal folds and feces [15,18,42,44,57,63]. In addition, *S. uberis* had been isolated in large numbers from the straw bedding of housed cattle usually during the winter housing period and from the pasture grazed by infected cattle [10].

According to Sherman [58] and Slot [61] *S. uberis* showed some similarities to bacteria of genus *Enterococcus*. However, the studies summarized by Schleifer and Kilpper-Bälz [53] and Lämmler and Hahn
[37] revealed that *S. uberis* seems to be more related to the pyogenic group of genus *Streptococcus*. On the basis of chromosomal DNA hybridizations Garvie and Bramley [23] and Collins et al. [14] suggested the existence of two distinct *S. uberis* genotypes, designated as *S. uberis* type I and II. According to a proposal of Williams and Collins [68] type II *S. uberis* were classified as *S. parauberis*.

In the present study *S. uberis* and *S. parauberis* strains isolated during routine diagnostics from bovine milk samples of one region in Germany were investigated together with reference strains of both species for cultural, biochemical, serological and molecular properties. The latter included the detection of various genes by polymerase chain reaction and the determination of epidemiological relationships by pulsed-field gel electrophoresis (PFGE).

**Materials and Methods**

**Collection and cultivation**

For the present study 342 bovine milk samples from 342 quarters of 269 cows from 93 different farms were initially collected within three months from January to March 1999 at different locations in Hesse, Germany. Approximately 0.1 ml milk obtained from clinical as well as subclinical milk samples were initially plated on sheep blood agar (Oxoid, Wesel, Germany), while subclinical samples were subjected to total somatic cell count (SCC) in order to confirm the subclinical status of the collected samples. The determination of cell count was performed with the Fossomatic system (360 N. Foss Electronic A/S, Hamburg, Germany).

All bacteria suspected to belong to genus *Streptococcus* were subsequently cultivated on Columbus esculin blood agar (Merck, Darmstadt, Germany) to determine their cultivating ability. The esculin-hydrolyzing cultures were further cultivated on five different selective growth media specific for enterococci. This included Citrate azide tween carbonate agar (CATC, Merck), Kanamycin esculin azide agar (KAA, Merck), Esculin bile agar (Oxoid), Chromocult enterococci agar (Merck), and Slanetz-Barley media (Oxoid). All media were prepared, used and the results interpreted according to the manufacturers instructions. An *Enterococcus faecalis* strain, obtained from the institutes strain collection (Institute of Milk Science, Giessen University, Giessen, Germany), was used as positive control.

On the basis of the above mentioned cultural ability and growth patterns 131 isolates from 112 cows of 58 different farms affected with subclinical and clinical mastitis were further processed. The isolates were investigated together with the *S. uberis* reference strains NCDO 2038 and NCDO 2086, the *S. parauberis* reference strain NCDO 2020 and the *S. parauberis* strain 94/16. The latter, originally isolated from a diseased turbot, was kindly obtained from J. F. Fernández-Garayzábal (Faculty of Veterinary Medicine, Complutense University, Madrid, Spain) [19].

**Biochemical characterization**

Carbohydrate fermentation tests were determined by using phenol-red broth (Merck) containing 1% arabinose, fructose, glucose, inulin, lactose, maltose, mannitol, raffinose, ribose, saccharose, salicin, sorbitol and trehalose, respectively. Esculin hydrolysis was carried out, using Brain Heart Infusion (BHI, Merck) containing 0.1% esculin and 0.05% iron (III) citrate. For determination of sodium-hippurate hydrolysis the method described by Hwang and Ederer [29] was used. For arginine hydrolysis commercial diagnostic test tablets (Rosco, Hiss Diagnostics, Freiburg, Germany) were used as substrate. The tests were carried out as described by the manufacturer. Commercial diagnostic test tablets (Rosco, Hiss Diagnostics) were also used as substrates for determination of â-D-glucuronidase, and pyrrolidonyl aminopeptidase enzyme activities. In addition, hyaluronidase enzyme activities were investigated by cultivation of the bacteria in close proximity of a mucoid growing *S. equi* subsp. *zoopneumoniae* strain, obtained from the institutes strain collection, as described by Winkle [70].

**Serogrouping**

Serological grouping of the cultures was performed with autoclaved extracts [47] and specific antisera of Lancefield groups A, B, C, E, G, P, U and V. The antisera were obtained from the institutes collection [36].

**Other phenotypic characteristics**

Synergistic CAMP-like hemolytic activities were determined together with a β-toxin producing *S. aureus* on sheep blood agar plates [37], lectin agglutination reactions with the lectin from Helix pomatia (Sigma, Deisenhofen, Germany), on microscopic slides [43]. Self-agglutinating bacterial cultures were pretreated with 5 µl trypsin (1 mg trypsin/ml PBS) for 1 hr at 37°C, washed, resuspended in PBS and subsequently used for lectin agglutination as described [43].

**Genotypic characterization**

The extraction of the DNA of the isolates was performed as described [28]. The gene encoding the 16S rRNA was amplified using the oligonucleotide primer ARI with the sequence 5’ GAGAATTCTGCTCCGAGGA 3’ [8] and the primer AmII with the sequence 5’ CGGCTGTTCAC AAAAACTCTGCGGT 3’ [3]. The oligonucleotide primers were synthesized by MWG-Biotech (Ebersberg, Germany). Restriction fragment length polymorphism