Conventional Identification of *Streptococcus uberis* Isolated from Bovine Mastitis in Argentinean Dairy Herds

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ABSTRACT

The objective of this study was to evaluate a conventional scheme for identifying *Streptococcus uberis* strains isolated from bovine mastitis. Seventy-five gram-positive, catalase-negative cocci were collected from cows with mastitis from 19 dairy herds located in the east-central region of Argentina. Five American Type Culture Collection strains and bovine isolates were identified by the API 20 Strep system and by restriction fragment length polymorphism analysis of 16S rDNA. A conventional scheme based on 11 biochemical tests was selected for identification of *Strep. uberis* strains: the Christie–Atkins–Munch–Petersen reaction; hydrolysis of Arg, esculin, and sodium hippurate; growth in inulin, mannitol, raffinose, salicin, and sorbitol; and growth at 45°C and in 6.5% NaCl. Reference strains and 25 bovine isolates were classified accurately to the species level by the conventional scheme in a blind assay. Each reference strain and each bovine isolate were identified as belonging to the same species following these 3 methods. The remaining 50 isolates identified as *Strep. uberis* by the API 20 Strep system and 16S rDNA RFLP were assayed by the conventional scheme. This scheme correctly identified 47 (94%) of 50 isolates as *Strep. uberis* by comparing their biochemical profile with that of the reference strain. Three (6%) of the 50 isolates were classified as *Strep. uberis* by the API 20 Strep system and by 16S rDNA RFLP and were identified as *Enterococcus faecalis* by the conventional scheme. Thirty percent of the *Strep. uberis* strains showed biochemical profiles identical to the *Strep. uberis* American Type Culture Collection 27958 strain. Seventy percent of the *Strep. uberis* strains demonstrated variability compared with the reference strain, resulting in 19 different biochemical profiles. The conventional scheme proposed in this study resulted in a relatively low number of misidentifications and could

biochemically identify not only typical, but also atypical *Strep. uberis* strains. This conventional scheme can be considered an adequate method for identifying *Strep. uberis* strains isolated from bovine mastitis because of its affordable cost in developing countries, and it may contribute to determining the frequency of isolation of *Strep. uberis* strains in Argentinean dairy herds.

Key words: *Streptococcus uberis*, bovine mastitis, conventional identification

INTRODUCTION

Bovine mastitis is the most costly disease among dairy cows. Estimates of losses caused by mastitis range from US\$35 to \$295 per cow per year (DeGraves and Fetrow, 1993). In Argentina milk production losses have been estimated at US\$221 million a year (Asociación Argentina de lucha contra la mastitis, 1983).

Streptococcus uberis is known worldwide as an environmental pathogen responsible for a high proportion of cases of clinical and subclinical mastitis in lactating cows and is also the predominant organism isolated from mammary glands during the nonlactating period (Bradley, 2002; Khan et al., 2003). Accurate and cost-effective methods of identifying mastitis pathogens are important for the diagnosis, surveillance, and control of this economically important disease among dairy cows (McDonald, 1984; Leigh, 1999).

Gram-positive, catalase-negative, esculin-hydrolyzing cocci isolated from cases of clinical and subclinical mastitis are commonly categorized as *Strep. uberis* in routine diagnostic laboratories. The heavy workload of mastitis diagnostic services does not allow more accurate identification. Upon closer examination, however, many of these bacteria may in fact be very different from this major streptococcal pathogen. The differential scheme for mastitis streptococci and enterococci of the US National Mastitis Council (National Mastitis Council, 1990) lists *Strep. uberis*, *Streptococcus bovis*, *Streptococcus equinus*, *Enterococcus faecalis*, *Enterococcus faecium*, and *Enterococcus saccharolyticus* as esculin-hydrolyzing species and *Streptococcus dysgalactiae* and

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Streptococcus equi as esculin-variable species. More recently, 2 new esculin-hydrolyzing streptococcal species, *Streptococcus parauberis* (Williams and Collins, 1990) and *Streptococcus plurianimalium* (Devriese et al., 1999), were isolated from bovine mastitis. However, according to different authors (King, 1981; Calvino et al., 1991; Khan et al., 2003), *Strep. uberis*, *Strep. dysgalactiae*, *Strep. bovis*, *Strep. equinus*, and *E. faecalis* represent the esculin-positive or esculin-variable cocci most frequently isolated from mastitic cows. Farrow and Collins (1984) examined a collection of strains, most of which were nonhuman isolates, and reported that the phenotypically described *Strep. bovis* and *Strep. equinus* type strains belonged to a single DNA group and corresponded to the same species.

Identification of *Strep. uberis* is currently based on observation of the cultural and morphological characteristics, determination using biochemical tests, and enzyme activity (Leigh, 1999; Khan et al., 2003). On the other hand, several commercial microbial identification systems have also been used to differentiate *Strep. uberis* from the other streptococci and enterococci isolated from bovine mastitis (Watts, 1989; Freney et al., 1992), and more recently, molecular tools such as PCR-based protocols have been proposed to provide an accurate identification of *Strep. uberis* isolates (Hassan et al., 2001; Schlegel et al., 2003; Kawata et al., 2004). Among these, RFLP analysis of 16S rDNA was proposed as a general method for bacterial identification and typing (Jayarao et al., 1992). Perhaps the greatest disadvantage of identification techniques based on commercial rapid systems and molecular tools is that these methods can be expensive for most laboratories that wish to offer analyses at an affordable cost. The objective of this study was to evaluate a conventional scheme based on 11 biochemical tests for identification of *Strep. uberis* strains collected from the mammary glands of cows with mastitis from 19 dairy herds located in the east-central region of Argentina.

MATERIALS AND METHODS

Bacteria

Five American Type Culture Collection (ATCC) strains, sent by M. Gottschalk and B. Jayarao, including *Streptococcus agalactiae* ATCC 27956, *Strep. dysgalactiae* ATCC 27957, *Strep. uberis* ATCC 27958, *Strep. equinus* ATCC 27960, and *E. faecalis* ATCC 19433, were used in this study. In addition, over a period of 12 mo, 75 bacterial cultures were collected and presumptively identified as streptococci or enterococci by colony appearance, gram stain reaction, and catalase test (Hogan et al., 1999). The collected isolates were obtained from mammary glands of cows with mastitis from 19 dairy

herds located in the east-central region of Argentina. Four isolates per dairy herd were stored at -20°C in Todd-Hewitt broth (Sigma-Aldrich Co., St. Louis, MO) with 20% glycerol. All ATCC strains and bovine isolates were subcultured from storage media onto 5% sheep blood agar plates and were identified by the API 20 Strep system (bioMérieux, Inc., Durham, NC; Poutrel and Ryniewicz, 1984) and by 16S rDNA RFLP, as previously described (Jayarao et al., 1992).

Biochemical Tests

Eleven biochemical tests were selected for identification of *Strep. uberis* strains: the Christie-Atkins-Munch-Petersen (CAMP) reaction (Hogan et al., 1999); esculin hydrolysis (Hogan et al., 1999); sodium hippurate hydrolysis (Baron et al., 1994a); Arg hydrolysis (McDonald and McDonald, 1976); growth in inulin, mannitol, raffinose, salicin, and sorbitol (McDonald and McDonald, 1976); and growth at 45°C and in 6.5% NaCl (Baron et al., 1994b). The Todd-Hewitt broth was used as a basal medium for temperature- and salt-tolerance tests. Five reference strains and 25 bovine isolates previously identified by the API 20 Strep system and by 16S rDNA RFLP, were classified to the species level by biochemical tests in a blind assay. Five representative colonies, obtained by streaking 5 μL of Todd-Hewitt broth-glycerol onto 5% sheep blood agar plates, were picked from each plate and used in each biochemical test. The remaining 50 isolates of bovine origin, identified by the API 20 Strep system and by 16S rDNA RFLP, were subjected to the biochemical tests, and the results were compared with biochemical profiles obtained from reference strains. Each isolate was identified to the species level when 8 of the 11 biochemical tests were identical to one of the reference strains and at least 2 were different from each of the other reference strains.

RESULTS

Reference strains were identified to the species level by the API 20 Strep system and by 16S rDNA RFLP. Biochemical tests of reference strains were determined by using the conventional scheme proposed for *Strep. uberis* identification. Reference strains were classified accurately to the species level in a blind assay by comparing their biochemical profiles with previously reported results (McDonald and McDonald, 1976; Garvie and Bramley, 1979; Watts, 1988; Lämmle, 1991; Devriese et al., 1999; Facklam, 2002; National Mastitis Council, 2002; Fortin et al., 2003; Khan et al., 2003). Table 1 shows the identification of the reference strains by the conventional scheme obtained after each biochemical test was repeated 3 times for each isolate.

Table 1. Identification of reference strains by the conventional scheme¹

Reference strains	CAMP reaction	Hydrolysis of			Growth in 6.5% NaCl	Growth at 45°C	Growth in				
		ARG	ESC	HIP			INU	MAN	RAF	SAL	SOR
<i>Streptococcus agalactiae</i> ATCC 27956	+	+	–	+	–	–	–	–	–	+	–
<i>Streptococcus dysgalactiae</i> ATCC 27957	–	+	–	–	–	–	–	–	–	–	–
<i>Streptococcus uberis</i> ATCC 27958	–	+	+	+	–	–	+	+	–	+	+
<i>Streptococcus equinus</i> ATCC 27960	–	–	+	+	–	–	–	–	+	+	–
<i>Enterococcus faecalis</i> ATCC 19433	–	+	+	+	+	+	–	+	–	+	+

¹CAMP = Christie–Atkins–Munch–Petersen; ARG = arginine; ESC = esculin; HIP = sodium hippurate; INU = inulin; MAN = mannitol; RAF = raffinose; SAL = salicin; SOR = sorbitol.

Twenty-five gram-positive, catalase-negative cocci isolated from the mammary glands of cows with mastitis were identified by the API 20 Strep system and by 16S rDNA RFLP as *Strep. agalactiae* (n = 5), *Strep. dysgalactiae* (n = 5), *Strep. uberis* (n = 5), *Strep. equinus* (n = 5), and *E. faecalis* (n = 5). In the blind experiment, these strains were successfully classified to the species level by the conventional scheme. Reference strains and the 25 bovine isolates were identified as belonging to the same species by the 3 methods.

The remaining 50 isolates identified as *Strep. uberis* by the API 20 Strep system and 16S rDNA RFLP were assayed by the conventional scheme. This scheme, based on 11 biochemical tests, correctly identified 47 (94%) of the 50 isolates as *Strep. uberis* by comparing their biochemical profiles with that of the reference strain. All *Strep. uberis* strains were negative for growth in 6.5% NaCl. Most of them (81 to 98%) were positive not only for growth in mannitol, salicin, and sorbitol, but also for hydrolysis of Arg, esculin, and sodium hippurate, and they were negative for the CAMP reaction and for growth at 45°C and in raffinose. However, only 64% of the *Strep. uberis* strains were positive for growth in inulin (Table 2).

Three (6%) of the 50 isolates were classified as *Strep. uberis* by the API 20 Strep system and by 16S rDNA RFLP but were identified as *E. faecalis* by the conventional scheme.

Fourteen (30%) of the 47 *Strep. uberis* strains showed biochemical profiles identical to the *Strep. uberis* ATCC 27958 strain. These strains were positive for growth in inulin, mannitol, and sorbitol, and for hydrolysis of Arg, esculin, and sodium hippurate, whereas they were negative for the CAMP reaction and for growth at 45°C and in 6.5% NaCl and raffinose. The remaining 33 (70%) *Strep. uberis* strains showed some variability with respect to the *Strep. uberis* reference strain, resulting in 19 different biochemical profiles, designated A to S (Table 3).

DISCUSSION

Streptococcal mastitis causes tremendous economic loss in bovine milk production, and *Strep. uberis* has

become one of the most important environmental etiological agents responsible for cases of clinical and subclinical mastitis in lactating and nonlactating cows.

In this study, we proposed an identification scheme for *Strep. uberis* based on 11 biochemical tests. We were able to determine the agreement between the conventional scheme proposed and 2 other methods, the API 20 Strep system and 16S rDNA RFLP. This scheme correctly identified 47 (94%) of 50 isolates as *Strep. uberis* by comparing their biochemical profiles with that of a reference strain. The results showed that 3 of the 50 bovine isolates identified as *E. faecalis* by the conventional scheme were in fact *Strep. uberis* by the API 20 Strep system and 16S rDNA RFLP. The most common mistake was to identify *Strep. uberis* strains as *Enterococcus* spp. (Watts, 1989; Fortin et al., 2003). The conventional scheme proposed in this study resulted in a relatively low number of misidentifications.

In this study, all the strains classified by the conventional scheme as *Strep. uberis* were negative for growth in 6.5% NaCl, which agrees with the results of McDonald and McDonald (1976), Watts (1988), and the Na-

Table 2. Biochemical tests of 47 *Streptococcus uberis* strains isolated from bovine mastitis

Biochemical test	<i>Strep. uberis</i> ATCC 27958	Percentage ¹
CAMP ² reaction	–	81
Arg ³	+	83
Esculin ³	+	89
Sodium hippurate ³	+	96
6.5% NaCl ⁴	–	100
45°C ⁵	–	98
Inulin ⁴	+	64
Mannitol ⁴	+	98
Raffinose ⁴	–	94
Salicin ⁴	+	94
Sorbitol ⁴	+	96

¹Percentage of strains showing reactions in biochemical tests identical to those obtained for the reference strain *Strep. uberis* ATCC 27958.

²CAMP = Christie–Atkins–Munch–Petersen.

³Hydrolysis of.

⁴Growth in.

⁵Growth at.

Table 3. Atypical biochemical profiles of 33 *Streptococcus uberis* strains isolated from bovine mastitis

Profile	Biochemical test	Atypical biochemical profiles	No. (%) of <i>Strep. uberis</i> strains ¹
A	Sorbitol ²	–	1/33 (3)
B	Sodium hippurate ³	–	2/33 (6)
C	Mannitol ²	–	1/33 (3)
D	Inulin ²	–	5/33 (15)
E	Esculin ³	–	4/33 (12)
F	CAMP reaction	+	2/33 (6)
G	Growth at 45°C	+	1/33 (3)
H	Arg ³	–	2/33 (6)
I	Raffinose ²	+	1/33 (3)
J	Salicin ²	–	1/33 (3)
K	CAMP reaction	+	5/33 (15)
	Inulin ²	–	
L	CAMP reaction	+	1/33 (3)
	Raffinose ²	+	
M	Arg ³	–	1/33 (3)
	Inulin ²	–	
N	Inulin ²	–	1/33 (3)
	Sorbitol ²	–	
O	Esculin ³	–	1/33 (3)
	Inulin ²	–	
	Sorbitol ²	–	
P	CAMP reaction	+	1/33 (3)
	Arg ³	–	
	Inulin ²	–	
Q	Esculin ³	–	1/33 (3)
	Inulin ²	–	
	Salicin ²	–	
R	Esculin ³	–	1/33 (3)
	Inulin ²	–	
	Raffinose ²	+	
S	Arg ³	–	1/33 (3)
	Esculin ³	–	
	Inulin ²	–	

¹Number and percentage of *Strep. uberis* strains with atypical biochemical profiles showing only the tests that differ from those obtained for the reference strain *Strep. uberis* ATCC 27958 [CAMP (Christie–Atkins–Munch–Petersen) reaction –, hydrolysis of Arg +, hydrolysis of esculin +, hydrolysis of sodium hippurate +, growth in 6.5% NaCl –, growth at 45°C –, growth in inulin +, growth in mannitol +, growth in raffinose –, growth in salicin +, growth in sorbitol +].

²Growth in.

³Hydrolysis of.

tional Mastitis Council (2002). However, Fortin et al. (2003) reported that nearly 32% of the *Strep. uberis* strains in that study were positive for growth in 6.5% NaCl.

Nine of the 47 (19%) bovine isolates characterized as *Strep. uberis* were CAMP reaction-positive, although *Strep. uberis* reference strain ATCC 27958 was not. These findings were similar to those of McDonald and McDonald (1976) and Lämmle (1991), but differed from those reported by Watts (1988), the National Mastitis Council (2002), and Khan et al. (2003).

Growth in inulin, which has been recommended as a routine test for identification of *Strep. uberis* (Hogan et al., 1999; Fortin et al., 2003), was observed in only

64% of the isolates classified as *Strep. uberis* in this study. This result agrees with the work of Khan et al. (2003), who reported that 92 (69%) of 132 *Strep. uberis* strains were positive for growth in inulin. In contrast, Watts (1988) reported that 100% (n = 33) of *Strep. uberis* strains were negative for growth in inulin, whereas Lämmle (1991) and Devriese et al. (1999) found that 100 and 98%, respectively, of *Strep. uberis* strains were positive for growth in inulin.

In addition, 83% of the isolates characterized as *Strep. uberis* were positive for hydrolysis of esculin, which agrees with the results of Lämmle (1991), who reported that 83% (n = 36) of *Strep. uberis* strains were positive for esculin hydrolysis. Other authors (Garvie and Bramley, 1979; Khan et al., 2003), however, have reported that all *Strep. uberis* strains evaluated were positive for hydrolysis of esculin.

The CAMP reaction, hydrolysis of esculin, and growth in inulin have been recognized as variable characteristics for *Strep. uberis* (Lämmle, 1991; Fortin et al., 2003; Khan et al., 2003) and can therefore be considered dubious for identification of *Strep. uberis* strains. However, Fortin et al. (2003) proposed a flow chart for the identification of catalase-negative, non- β -hemolytic, gram-positive cocci isolated from milk samples. The authors suggested that the CAMP reaction, the leucine aminopeptidase test, hydrolysis of esculin and of sodium hippurate, and growth in inulin and in raffinose should be routinely done.

The results obtained in this study showed that the conventional scheme proposed could biochemically identify not only typical but also atypical *Strep. uberis* isolates. This conventional scheme may be considered an adequate method to identify *Strep. uberis* strains isolated from bovine mastitis because of its affordable cost in developing countries, and it may contribute to determining the frequency of isolation of the *Strep. uberis* strains in Argentinean dairy herds, especially in herd-monitoring when regular sampling from all animals is performed.

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