Characterization of *Staphylococcus aureus* Isolated from Chronically Infected Dairy Goats

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ABSTRACT

A herd of 88 Alpine goats in Northern Italy was monitored for a complete lactation. Milk samples were taken from each udder half during 8 monthly visits. Goats ($n = 28$) with ≥ 2 consecutive positive tests for *Staphylococcus aureus* in the same udder half were identified as chronically infected, and all of those had ≥ 4 positive tests of the 8 samples. Goats with no infections in either udder half during any visit were considered healthy ($n = 26$). Linear mixed models were used to examine the relationship between chronic infection by *S. aureus* and SCC and production traits. The bacteria isolated from one sample from each infected goat were genotyped on the basis of polymorphism in several genes and evaluated for the presence of genes encoding for enterotoxins. The bacteria isolated from each animal were also subject to a test for β -lactamase production and to minimum inhibitory concentration tests for 11 antimicrobial agents. As expected, SCC ($\log_2$) was significantly higher in infected goats than in healthy goats (7.55 vs. 5.50). Also, mean log SCC from infected udder halves (8.02) was greater than that in uninfected udder halves from the same goats (6.44). No significant differences were observed in milk yield or for fat and protein percentages between infected and healthy goats. No genetic variability was observed among the bacteria isolated, suggesting that all were from the same strain, although isolates did vary in susceptibility to various antimicrobial agents. All *S. aureus* isolates were negative for the β -lactamase production test. The most effective drugs when tested in vitro were benzylpenicillin, amoxicillin plus clavulanic acid, cloxacillin, and cephalosporins.

(**Key words:** goat, *Staphylococcus aureus*, mastitis, antimicrobial susceptibility)

Abbreviation key: MLVA = multiple-locus VNTR analysis, VNTR = variable number of tandem repeats.

INTRODUCTION

For dairy producers, subclinical IMI is an important cause of poor milk quality (bacteriological quality, biochemical characteristics, and SCC). Staphylococci are the bacteria most frequently isolated from IMI in Italian dairy goat farms (Moroni et al., 2005) and, thus, are considered the most important etiological agents for mastitis (Bergonier et al., 2003). Among this group, *Staphylococcus aureus* is considered a major contagious pathogen. Because of its pathogenicity, *S. aureus* must be considered an important caprine mastitis agent because it is responsible for chronic, subclinical IMI and sometimes clinical and gangrenous mastitis, which often leads to the loss of the affected gland. Few data are available in the literature for strains of *S. aureus* isolated from dairy goats. In some early studies, *S. aureus* was isolated in a small number of subclinical IMI cases (Kalogridou-Vassiliadou, 1991; Deinhofer and Pernthaner, 1995; Contreras et al., 1997). However, in another study (da Silva et al., 2004), *S. aureus* was identified in 37% of the subclinical mastitis isolates. Because infected animals may pass on this microorganism in milk, they represent a source of IMI for other animals in the herd and a danger for public health. Production of enterotoxins during the manufacture and ripening of many cheeses has been studied by several researchers (Bachmann and Spahr, 1995).

The objective of this study was to examine SCC and milk quality measures of goats from a Northern Italian dairy farm; the goats were either healthy or chronically infected by *S. aureus*. In addition, the *S. aureus* isolates were characterized genotypically by multiple-locus variable numbers of tandem repeat analysis (MLVA),

Received March 8, 2005.

Accepted June 16, 2005.

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by coagulase gene RFLP analysis, and by detection of staphylococcal enterotoxin genes. These PCR-based methodologies have proven to be useful for molecular typing and epidemiological analysis of *S. aureus* isolated from human carriers (Sabat et al., 2003) and bovine mastitis (Aarestrup et al., 1995; Lange et al., 1999; Akineden et al., 2001; Sommerhäuser et al., 2003). Moreover, recent studies have shown genotypic traits of *S. aureus* strains isolated from goats and sheep (Scherrer et al., 2004). In our study, these techniques allow one to discriminate among different *S. aureus* isolates and provide information about the spread of strains within the herd. Finally, tests of antimicrobial sensitivity were applied, using 11 different antimicrobial agents.

MATERIALS AND METHODS

Animals and Herd

A total of 88 Alpine goats from a commercial dairy farm in Italy were monitored for an entire lactation (February to September 2004). The farm was free of brucellosis, tuberculosis, mycoplasmosis, and caprine arthritis-encephalitis virus after a voluntary eradication program. The farm practiced seasonal milking, and the does kidded between January and February 2004. The herd grazed on natural pastures for 6 h/d and was supplemented with concentrates in the milking parlor (1.53 Mcal of NE_I/kg, 16% CP, as fed) at a flat rate of 0.5 to 1 kg/d per doe according to lactation stage and with 0.5 kg of alfalfa per doe and 0.5 kg of alfalfa pellets per doe in the shelter. Water was available at all times. After weaning, all goats were hand-milked 2×/d without teat preparation. Neither teat dipping nor dry therapy was practiced. No clinical signs of IMI were observed for any of the goats throughout the study; thus, no mastitis therapy was administered.

Milk Sampling

Foremilk samples were obtained monthly (February to September 2004) during the usual quality test day program performed by the technicians of the Provincial Breeding Association of Asti (A.P.A. Asti). Numbered collars were used to identify each goat in the herd, and none of the animals studied had evidence of clinical mastitis at the time of sampling. Teat ends were cleaned with chlorhexidine before sampling. The first streams of foremilk were discharged, and then 10 mL of milk was collected aseptically from each udder half into sterile vials. Samples were kept at 4°C until bacteriological assays and SCC tests were performed. Samples of pooled milk (across udder halves) were tested for fat and protein concentrations (%). Milk yield (kg) was re-

corded on the same day as the samples were collected by using recording jars in the milking parlor.

Bacteriological Procedures

Ten microliters of each milk sample was spread on blood agar plates (5% defibrinated sheep blood). The plates were incubated aerobically at 37°C and examined after 24 h. The colonies were provisionally identified on the basis of Gram stain, morphology, and hemolysis patterns. The number of each colony type was recorded. The representative colonies were then subcultured on blood agar plates and incubated aerobically at 37°C for 24 h to obtain pure cultures. Catalase and coagulase production were tested for gram-positive cocci. Specific identification of staphylococci was made using commercial micromethods (API Staph, BioMérieux, Italy). The infection status of milk samples was defined according to the procedures recommended by the National Mastitis Council (1999), and IMI was diagnosed when >500 cfu/mL were present and when 1 to 3 colony types were isolated. Milk samples from which >3 colony types or ≤500 cfu/mL colonies of any microorganism were isolated were regarded as contaminated or uninfected, respectively, for most bacterial species. In the case of *S. aureus*, an IMI was diagnosed when 1 colony was isolated (≥100 cfu/mL).

After monitoring the whole herd for an entire lactation, all goats (26 animals) with healthy half udders throughout the lactation and all goats (28 animals) chronically infected with *S. aureus* were chosen for statistical analyses. An animal was defined as healthy when all milk samples collected during lactation were bacteriological negative for both half udders, whereas animals chronically infected by *S. aureus* were those that had at least 1 half udder with at least 2 consecutive milk samples infected by *S. aureus*. In fact, all of the chronically infected animals had ≥4 positive tests for *S. aureus* across the 8 possible sampling days in at least 1 of the udder halves. Because the precise objective of this work was to compare healthy goats to goats that were chronically infected by *S. aureus*, the data from the other 34 goats that did not fit into either chronically infected or healthy groups (as defined previously) were not included in subsequent analyses.

Determination of SCC and Percentages of Protein and Fat

For each milk sample, SCC was determined by an automated fluorescent microscopic somatic cell counter (Bentley Somacount 150, Bentley Instrument). Ethidium bromide dye was used for specific binding to the DNA in the cell nuclei. For statistical analyses, the

SCC were converted to SCS using the standard $\log_2$ transformation of Ali and Shook (1980). Percentages of milk fat and protein were determined on composite milk samples and assayed by an automated Fourier transformed infrared absorption spectrophotometric analyzer (Milkoscan, Foss, Hillerød, Denmark).

Genotypic Characterization

Twenty-eight *S. aureus* isolates from different animals with chronic infection throughout the lactation were frozen and stored at -70°C in a nutrient broth enriched with 15% glycerol until DNA extraction could be performed. Briefly, for the molecular assay, the nucleic acids were extracted from 1 mL of bacterial suspension using the PUREGENE DNA Purification Kit (Gentra Systems, Minneapolis, MN) according to the recommendation of the manufacturer for gram-positive bacteria. The isolates were identified as *S. aureus* by amplification of a *S. aureus*-specific section of the 23S rRNA gene using conventional, previously described PCR methods (Straub et al., 1999).

The amplification of variable number of tandem repeats (VNTR) loci, including *clfA*, *clfB*, *sdr*, *spa*, and *ssp* genes, was carried out by a PCR assay using the protocol described by Sabat et al. (2003) with slight modifications. Briefly, we used the following conditions: a 5-min precycle at 94°C , then 30 times the following sequence (30 s at 94°C , 30 s at 55°C , and 30 s at 72°C), followed by final extension incubation at 72°C for 7 min. The PCR products were analyzed on a 4% agarose gel (GellyPhor, Euroclone, Milan, Italy) stained with ethidium bromide ($0.05\text{ }\mu\text{g}/\mu\text{L}$; Sigma Aldrich, Milan, Italy).

To detect the polymorphic region of the coagulase gene (*coa*), a PCR assay was performed as described by Hookey et al. (1998). For the restriction endonuclease analysis, approximately 300 ng ($12\text{ }\mu\text{L}$) of PCR products were digested at 37°C for 2 h with 1 U of the restriction endonuclease *AluI* (New England BioLabs) according to the manufacturer's instructions. All of the digested PCR products were analyzed on a 4% agarose gel (GellyPhor) stained with ethidium bromide ($0.05\text{ }\mu\text{g}/\mu\text{L}$). The reference strain ATCC 25923 was included in both PCR assays as positive control.

The PCR products of the *coa* gene of 6 representative isolates were sequenced by CRIBI Services (CRIBI, Padova, Italy) on an ABI377 sequencer by using the ABI PRISM dye-terminator cycle sequencing ready reaction kit with Amplitaq DNA polymerase (Perkin-Elmer, Boston, MA; Applied Biosystems, Foster City, CA).

The isolates were also analyzed to detect the enterotoxin genes, including *sea*, *sec*, *sed*, *seg*, *seh*, *sei*, *sej*, and *sel*. Staphylococcal enterotoxin genes were detected by multiplex PCR assay as described by Cremonesi et al.

(2005). The PCR products were analyzed on a 2% agarose gel (GellyPhor) stained with ethidium bromide ($0.05\text{ }\mu\text{g}/\mu\text{L}$). The reference strain ATCC 700699 harboring *sea*, *sec*, *seg*, *sei*, and *sel* genes (Letertre et al., 2003) was included as positive for the multiplex PCR assay.

MIC Tests

The *S. aureus* (28 isolates) isolated from different animals with chronic infection throughout the lactation were tested for antimicrobial susceptibility. The antibiotics selected for the study were benzylpenicillin, ampicillin, amoxicillin, amoxicillin plus clavulanic acid, cloxacillin, cephalonium, cephoperazone, oxytetracycline, doxycycline, kanamycin, and lincomycin. They were provided in powder form by the respective manufacturers. The antibiotics were dissolved in suitable solvents to make stock solutions and then diluted in sterile distilled water according to the methods recommended by the National Committee for Clinical Laboratory Standards (NCCLS, 2002). Minimum inhibitory concentration tests were performed according to the microdilution broth method as recommended by the NCCLS (2002) using 96-well microtiter plates. Serial 2-fold dilutions of the antimicrobial agents were prepared starting from the stock solution of each drug. The dilution schemes differed according to the antibiotic. Inoculates were prepared by diluting an overnight Mueller-Hinton broth culture in buffered saline solution to a density of 0.5 on the McFarland turbidity scale and finally diluting again 40-fold before testing. The MIC was defined as the lowest concentration of antibiotic at which bacterial growth was completely inhibited. A reference strain of *S. aureus* (ATCC 29213) was inoculated as a control on each plate. The MIC data were summarized by calculating the MIC value for which 50 and 90% of the isolates were equal to or below (i.e., MIC_{90} = MIC required to inhibit growth for 90% of the isolates tested and MIC_{50} = MIC required to inhibit 50% of the isolates tested), as well as the range of MIC values required to prevent growth of any (minimum) and all (maximum) isolates. Moreover, the *S. aureus* isolates were tested for β -lactamase production by the nitrocefin test (Dry Slide Nitrocefin, Difco Laboratories, Franklin Lakes, NJ).

Statistical Analyses

Two types of statistical analyses were applied, both by using the procedure MIXED of SAS (Cary, NC). In the first analysis, the relationship between infection status and SCS was examined. For this study, SCS was available for each udder half, and the data set for analysis included 841 records, 404 from healthy animals and 437 from animals with chronic IMI of *S.*

aureus. Each of the 54 animals had either 7 or 8 records. The model equation for the analysis was

$$y_{ijklm} = \text{day}_i + \text{par}_j + \text{inf}_k + \text{day} \times \text{inf}_{ik} + \text{goat}_{kl} + \text{half}_{lm} + e_{ijklm} \quad [1]$$

where y_{ijklm} is the observed value for the SCS obtained on sample day i from udder half m of goat l with infection status k , d_i is the fixed effect of sample day i ($i = 1$ to 8), par_j is the fixed effect of parity j ($j = 1$ to 6), inf_k is the fixed effect of infection status ($k = 1$ for healthy or 2 for chronically infected with *S. aureus*), $\text{day} \times \text{inf}_{ik}$ is the fixed effect of the interaction between infection status and sample date, goat_{kl} is the random effect of goat l nested within infection status k , half_{lm} is the random effect of udder half m of goat l (goat and udder half effects were included to account for repeated observations), and e_{ijklm} is the random residual.

The infection status was defined in 2 ways, and a separate analysis was applied for each. The first definition was made on an animal basis (i.e., healthy or chronically infected) and applied to all observations from both udder halves for a given goat. The second definition was on the basis of the individual udder halves at the time of the sample and included 4 categories. One category simply included all records from goats that remained uninfected throughout the study (i.e., $n = 404$), whereas 3 new categories were established for records from the chronically infected goats. The first of these groups consisted of records from the udder halves (of infected goats) that had been free of IMI up to the sample date ($n = 71$). These data included all records from the healthy udder half of 8 infected (in the other udder half) goats that remained uninfected throughout the study, plus the early lactation records from udder halves of 10 goats that became infected (in that other half) on a later sample date. The second group consisted of records from udder halves that were uninfected at the time of the sample, but had been infected at some other previous sample date ($n = 97$), and the third category consisted of records from quarters that were infected at the time of sampling ($n = 269$). The goals of this second definition were to test whether IMI by *S. aureus* in one udder half of a given goat was associated with increased SCS in the other uninfected udder half and if the presence of *S. aureus* in an udder half at a given point in a lactation was associated with increased SCS during later times when *S. aureus* was no longer detectable. Least squares means were obtained for each category of infection status in the 2 analyses and compared (within analysis) for significant differences among categories.

The second analysis tested the relationship between chronic IMI by *S. aureus* and the recorded milk produc-

tion traits (yields of milk, fat, and protein along with fat and protein percentages). Only one observation was available per goat per sample day for these traits, and the data included 416 observations (202 from healthy and 214 from infected animals) for each trait. The model for this set of analyses was simpler than for that for SCS (equation [1]). First, production traits were based on total production of both udder halves, so the effect of udder half was not included in the model. Second, the interaction of sample day and infection status was not included because preliminary analyses indicated that the effect had low significance. Third, only the single animal-based definition of infection status was testable. The effect of infection status was of primary interest in this analysis, and least squares means were calculated for healthy and infected animals and tested for significant differences. Infection status was defined 2 ways, and separate analyses were run for each. The first analysis was to test for the general effect of infection by *S. aureus* on production, and animals were considered healthy or infected. The second analysis considered whether animals were infected in one or both udder halves. For this analysis, 3 groups were defined: 1) healthy ($n = 202$), 2) unilaterally infected ($n = 122$), and 3) bilaterally infected ($n = 92$).

RESULTS AND DISCUSSION

Bacteriological Analyses

A total of 1367 aseptic foremilk samples were collected from both half udders of 88 lactating goats. The prevalence of infection was 50.2% ($n = 686$) of all samples examined. Among goats with IMI, 52.3% had unilateral infection; the others had both udder halves infected. The most prevalent mastitis agents were coagulase-negative staphylococci at 23.4% ($n = 320$ samples). Environmental pathogens (*Streptococcus* spp. and coliforms) were isolated from 82 milk samples; none had a prevalence of >4%. Neither *Salmonella* spp. nor *Listeria monocytogenes* were detected.

Staphylococcus aureus was in 20.8% ($n = 284$) of milk samples and was the second most common pathogen isolated. Twenty-eight goats were chronically infected throughout lactation with at least 2 consecutive isolations of *S. aureus* (Table 1).

Staphylococcus aureus and SCS and Production Traits

All factors included in equation [1] had significant ($P < 0.05$) effects on SCS. Repeatability of SCS from the same udder half across the lactation was 0.52. Of primary interest, however, were the differences in SCS

Table 1. Isolation of *Staphylococcus aureus* (X) from left (L) and right (R) half udders in 28 goats chronically infected during 8 mo of lactation.

Goat	Side	Month							
		1	2	3	4	5	6	7	8
224	L	X	X	X	X	X	X	X	X
	R		X						
225	L		X						
	R	X	X	X	X	X		X	X
236	L	X	X	X	X	X	X	X	X
	R								
244	L	X	X	X		X	X	X	
	R								
245	L	X		X	X		X	X	X
	R								
250	L	X	X	X	X		X	X	
	R	X	X			X		X	
251	L	X	X	X	X	X	X	X	X
	R	X	X	X	X	X	X	X	X
270	L	X	X	X	X	X	X	X	X
	R	X	X	X	X	X	X	X	X
277	L	X	X		X	X	X	X	
	R								
293	L	X	X	X	X	X	X	X	X
	R		X	X	X	X	X	X	X
296	L		X	X	X	X		X	
	R	X	X				X	X	
297	L	X	X	X	X	X	X	X	X
	R	X	X	X	X			X	
299	L	X	X	X				X	
	R		X		X	X	X	X	X
303	L	X	X		X	X	X	X	X
	R		X	X	X		X	X	X
312	L	X	X	X	X	X	X	X	X
	R	X	X	X	X	X	X	X	X
317	L	X	X	X	X	X	X	X	
	R								
319	L	X	X	X	X	X	X	X	X
	R								
348	L	X	X	X				X	X
	R		X	X					
460	L	X	X	X			X	X	X
	R	X		X	X	X			X
467	L	X	X	X	X	X	X	X	X
	R								
473	L								
	R	X	X	X				X	
479	L	X	X	X				X	
	R	X		X	X				
480	L		X			X			X
	R	X	X	X	X		X	X	X
521	L	X	X	X	X	X	X	X	X
	R	X							
244b	L	X	X	X	X		X		X
	R							X	X
251b	L	X		X	X	X	X	X	X
	R		X	X			X	X	
297b	L	X	X	X	X		X	X	
	R	X	X	X	X			X	
312b	L	X	X	X	X				
	R	X	X	X			X		

according to infection status. Least squares means for the different infection categories are shown in Table 2 for both definitions of infection status. When the comparison was made on an animal basis (analysis 1) the animals with chronic IMI by *S. aureus* had much higher

SCS than did the healthy animals ($P < 0.0001$). The difference between categories was approximately 2 SCS (2.05). Inasmuch as SCS was based on a $\log_2$ transformation of SCC, this result means that the SCC of animals infected by *S. aureus* averaged approximately 4

Table 2. Least squares means of 841 monthly measurements¹ of SCS² in milk from each udder half of 26 healthy goats and 28 goats chronically infected with *Staphylococcus aureus* (Analysis 1) and from udder halves with different infection statuses and infection histories (Analysis 2).

Infection status	n	LSM	SEM
Analysis 1			
Healthy	404	5.50 ^a	0.18
Chronically infected	437	7.55 ^b	0.17
Analysis 2			
Uninfected goat	404	5.48 ^a	0.16
Uninfected udder half, infected goat ³	71	6.40 ^b	0.25
Pathogen-free sample, infected udder half ⁴	97	7.19 ^c	0.23
Infected sample	269	8.02 ^d	0.17

^{a,b,c,d}Least squares means from the same analysis with different superscripts are different ($P < 0.03$).

¹Seven or 8 measurements per udder half across a lactation.

²Log₂ transformation.

³Chronically infected in other udder half.

⁴Infected at some point in the same lactation, but not on the sample date.

times the level of SCC in healthy animals. The difference in SCS among healthy goats and those infected by *S. aureus* observed in this study was much greater than between healthy udder halves and those infected by coagulase-negative staphylococci (SCS = 0.78), as observed in a previous study in Italian goats (Moroni et al., 2005). Similar results were also reported by Leitner et al. (2004), based on a study of goats in Israel. This combination of results suggest that *S. aureus* causes a much greater immune response and could potentially lead to more damage to the udder tissue.

Clear differences between types of *S. aureus* infections were observed when infection status was defined more precisely (analysis 2 of Table 2). The lowest SCS (5.48) was observed among udder halves of completely healthy animals. Infections in one udder half seemed to have systemic effects causing elevated SCS in the other, uninfected udder half, as the SCS in such cases was nearly one SCS greater than that from healthy animals, in agreement with the results of Dulin et al. (1983). This result differs from that observed by Davis et al. (2004) in dairy cattle, where no increase in SCS was observed in healthy quarters of infected cows. However, animals in that study were intentionally infected with *Streptococcus uberis* rather than *S. aureus*. In this study, infections by *S. aureus* seemed to have lingering effects on the immune status of udder halves even after the pathogen was eliminated (or at least undetectable in milk), because the mean SCS from this type of udder half was >1.5 SCS higher than in udder halves that had not been infected in the current lactation. The greatest SCS (8.02) was observed in udder halves with active infections. This value was >2.5 SCS greater than for uninfected goats.

Table 3. Least squares means of 416 monthly measurements¹ of milk production traits for 26 healthy goats and 28 goats chronically infected with *Staphylococcus aureus*. Standard errors of the LSM are presented in parentheses.

	Healthy	Infected	Difference ²
Milk, kg	2.29 (0.10)	2.15 (0.11)	0.14
Fat, kg	0.078 (0.003)	0.075 (0.004)	0.003
Protein, kg	0.071 (0.003)	0.066 (0.003)	0.005
Fat, %	3.64 (0.12)	3.65 (0.12)	-0.01
Protein, %	3.19 (0.04)	3.17 (0.04)	0.02

¹Seven or 8 measurements per goat across a lactation.

²None of the differences were significant ($P > 0.34$).

Table 3 has the least squares means for the production traits for healthy and chronically infected (with *S. aureus*) goats. No effect of *S. aureus* infection was observed, as none of the means for any trait were significantly different between the 2 infection statuses. In addition, no significant differences were observed when lateral or bilateral infection was considered (thus, no table was created). The least squares mean for milk yield was numerically greater for unilaterally infected goats (2.25 kg) than for bilaterally infected goats (2.01 kg), but this difference was not statistically significant. These results seem to suggest that although infection by *S. aureus* has the potential to lead to damage to the secretory tissue of the udder, no effect was observed in the concurrent lactation. Perhaps such effects, assuming they occur, may be manifested in lower production in a subsequent lactation.

Genetic Characterization

The isolates were subtyped on the basis of the PCR-amplicons fragment sizes. Amplification by multiplex PCR of *clfA*, *clfB*, *spa*, and *ssp* genes resulted in a single amplicon with the same size for all 28 *S. aureus* isolates, respectively, of approximately 1013, 930, 90, and 180 bp, and amplification of the *sdr* gene resulted in 2 fragments with the same sizes for all 28 isolates (approximately 550 and 750 bp) (Figure 1A). Amplification of the coagulase gene resulted in a single amplicon (760 bp), and digestion with *AluI* yielded identical restriction profiles for all of the strains examined (Figure 1B). All of the *S. aureus* isolates harbored *sec* and *sel* genes (Figure 1C).

Tenover et al. (1995) defined as “genetically related isolates” (clones) those isolates that are indistinguishable from each other by a variety of genetic tests: identification of clones is based on monitoring of multiple genetic markers of sufficient discriminatory power. As described by Sabat et al. (2003), the MLVA system has proven to have a satisfactory discrimination ability and reproducibility for epidemiological surveys of *S. aureus*.

Figure 1. Agarose gel electrophoresis of PCR products of 8 representative *Staphylococcus aureus* isolates (lanes 1 to 8). A) Multiple-locus variable numbers of tandem repeat analysis patterns: *clfA* (1013 bp), *clfB* (930 bp), *sdr* (750 and 550 bp), *ssp* (180 bp), *spa* (90 bp); B) RFLP patterns of the coagulase gene digested with *AluI*; and C) PCR-amplified toxin genes *sec* (371 bp) and *sel* (240 bp). Lane C is the positive control, and Lane M is the 50-bp DNA ladder (Finnzymes, Espoo, Finland).

and seems to be, at the very least, comparable with any other high-discriminatory power DNA-based method (e.g., PFGE or ribotyping) applied currently for *S. aureus* typing. Moreover, in our study we tested all of the isolates also for PCR RFLP analysis of the coagulase gene, a method previously used for typing *S. aureus* isolates from cattle, goats, and sheep (Aarestrup et al., 1995; Akineden et al., 2001; Scherrer et al., 2004).

According to observed gene polymorphisms, RFLP patterns of the coagulase gene, and toxin patterns, a single bacterial clone seemed to be responsible for the cases of IMI in this herd. Predominance of a single clone is likely to be the result of contagious spread.

The RFLP patterns of 6 isolates, selected on the basis of the different antimicrobial resistance phenotypes (see Table 4: isolate numbers 6, 8, 19, 22, 24, and 27), were substantiated by the analysis of nucleotide sequences. Association between RFLP and sequence analysis of the polymorphic region of *coa* gene allows one to determine whether strains with identical *AluI* patterns have identical nucleotide sequences, thus increasing the discriminatory power (Schwarzkopf and Karch, 1994; Hookey et al., 1998; Shopsis et al., 2000). The sequence analysis confirmed that the *AluI* recognition sites were conserved among isolates and that the sequences of the *coa* polymorphic region were found to have 100% nucleotide identity (data not shown) across all isolates.

The size of the polymorphic region of the *coa* gene and the presence of the gene encoding for *sec* in the isolates of *S. aureus* collected from goat's milk are in agreement with previous studies (Scherrer et al., 2004). In other studies, *sec* producers were described as the most prevalent enterotoxin-producing *S. aureus* isolated from goat's milk (Foschino et al., 2002; da Silva et al., 2005), and skin of udder, teats, and milk (Valle et al., 1990). In sheep, goats, and cattle, *sec* was the predominant toxin type detected in *S. aureus* isolated from mastitic milk (Orden et al., 1992a,b). However, this is the first case of identification of an *S. aureus* strain from goat IMI with the *sel* encoding gene.

Antimicrobial Susceptibility

Table 5 shows the antimicrobial susceptibility (as MIC₅₀ and MIC₉₀) of 28 isolates of *S. aureus* collected from different animals throughout the lactation. All *S. aureus* isolates were negative for the β -lactamase production test. The β -lactams (penicillins and cephalosporins) are widely used for intramammary treatment of bovine IMI, and their use is rapidly increasing also for the control of IMI in small ruminants. We observed high efficacy in vitro of benzylpenicillin against *S. aureus* (MIC₅₀ = 0.025 and MIC₉₀ = 0.1 μ g/mL); only 2

Table 4. Distribution panel of antimicrobial resistance (X) among 28 *Staphylococcus aureus* isolates.

Isolate	PEN ¹	AMP	AMX	AMC	CLX	KAN	OT	DOX	CFP	CPH	LIN
1		X	X			X	X	X			X
2		X	X			X	X	X			X
3		X	X				X	X			X
4			X				X	X			
5		X	X				X	X			
6	X	X	X			X	X	X			X
7			X			X	X	X			
8			X				X	X			
9							X	X			X
10			X				X	X			
11		X	X				X	X			X
12		X	X				X				X
13			X				X	X			X
14			X				X	X			
15			X				X	X			
16							X	X			
17		X					X	X			X
18		X	X			X	X	X			X
19		X	X		X		X	X			X
20		X	X			X	X	X			X
21		X					X	X			X
22		X	X				X	X			X
23		X	X				X	X			X
24		X					X	X			X
25		X	X				X	X			X
26	X	X	X			X	X				X
27		X	X		X	X	X				
28		X	X			X	X	X			

¹PEN = Benzylpenicillin, AMP = ampicillin, AMX = amoxicillin, AMC = amoxicillin plus clavulanic acid, CLX = cloxacillin, KAN = kanamycin, OT = oxytetracycline, DOX = doxycycline, CFP = cephaloperazone, CPH = cephalonium, and LIN = lincomycin.

isolates were resistant to this drug. In the penicillin group, cloxacillin showed strong activity, as MIC₉₀ was 0.5 µg/mL. The combination of amoxicillin plus clavulanic acid had similar effects on *S. aureus* (MIC₉₀ = 0.5 µg/mL), and all isolates were susceptible. Ampicillin

Table 5. Antimicrobial susceptibility of strains of *Staphylococcus aureus* isolated from different animals throughout the lactation.

	Breakpoint	MIC ₅₀ ²	MIC ₉₀	Range
		(µg/mL)		
PEN ¹	≥0.25	0.025	0.1	0.0016 to 0.5
AMP	≥0.5	0.5	1	0.1 to 8
AMX	≥0.5	0.5	1	0.25 to 2
AMC	≥8	0.25	0.5	0.25 to 1
CLX	≥4	0.25	0.5	0.1 to 32
CPH	≥32	0.1	0.1	0.05 to 1
CFP	≥64	1	2	0.5 to 8
OT	≥16	>500	>500	250 to >500
DOX	≥16	250	250	0.5 to 250
KAN	≥64	15	>500	8 to >500
LIN	≥4	7.5	31.2	1 to 64

¹PEN = Benzylpenicillin, AMP = ampicillin, AMX = amoxicillin, AMC = amoxicillin plus clavulanic acid, CLX = cloxacillin, CPH = cephalonium, CFP = cephaloperazone, OT = oxytetracycline, DOX = doxycycline, KAN = kanamycin, and LIN = lincomycin.

²MIC₅₀ and MIC₉₀ are the minimum concentrations of the various antimicrobial agents required to inhibit growth of 50 and 90% of the isolates tested, respectively.

and amoxicillin alone showed lower efficacy against *S. aureus*, as MIC₅₀ and MIC₉₀ were greater than the breakpoint value; only 8 and 5 isolates, respectively, were susceptible to these drugs. However, although the MIC₉₀ value for ampicillin was greater than the 0.5 µg/mL level that is used to categorize staphylococci as being resistant to this compound (NCCLS, 2002), this value was much lower than the MIC₉₀ value found for *S. aureus* isolated from bovine IMI that were positive for β-lactamase (Watts and Salmon, 1997). In that study, the ampicillin MIC₉₀ values for *S. aureus* isolates that were negative and positive for β-lactamase were 0.5 and 4.0 µg/mL, respectively.

The resistance displayed by most of the isolates against ampicillin and amoxicillin, despite the negative β-lactamases test, could be explained by different mechanisms of resistance induced by misuse of these drugs. It has been demonstrated (de Oliveira et al., 2000) that some *S. aureus* strains produce low amounts of β-lactamases but exhibit an inducible expression in the presence of antibiotics.

The cephalosporins are usually classified into different generations on the basis of their antimicrobial spectrum. Globally, there are intramammary formulations of 5 first-generation cephalosporins (cefazolin, cepha-

lexin, cephalotin, cephalonium, and cephapirin), 1 s-generation cephalosporin (cefuroxime), and 1 third-generation cephalosporin (cephoperazone) to treat mastitis in dairy ruminants. In the present study, cephalonium and cephaloperazone were included as first- and third-generation drugs, respectively. Against the *S. aureus* isolated, cephalonium and cephaloperazone showed good activity ($MIC_{90} = 0.1 \mu\text{g/mL}$ and $MIC_{90} = 2 \mu\text{g/mL}$, respectively), and all isolates were susceptible to these drugs. Lincomycin, kanamycin, and oxytetracycline (selected as representative drugs of the macrolide, aminoglycoside, and tetracycline groups, respectively) showed poor activity against *S. aureus* tested in the present study, as MIC_{90} were greater than breakpoint values.

According to the molecular typing results, all isolates seemed to belong to one clone of *S. aureus*, but differences in phenotypic resistance patterns were found (Table 5). Interestingly, some studies have reported that antimicrobial resistance did not appear to be a marker that always spreads clonally through a herd (Goni et al., 2004; Sabour et al., 2004), and this observation was also confirmed in the present study. The resistance genes identified in *S. aureus* are located on plasmids or mobile genetic elements (Kuroda et al., 2001). Acquisition of resistance involves transfer of resistance genes by some form of genetic exchange (conjugation, transduction, or transformation) from another organism, including bacteria of the same or different staphylococcal species and other gram-positive bacteria (Werckenthin et al., 2001).

CONCLUSIONS

Subclinical IMI by *S. aureus* in goats was associated with a significant increase in SCS relative to uninfected animals (7.55 vs. 5.50). Uninfected udder halves of infected goats had lower SCS than infected halves of the same goat, but higher SCS than that from goats that were free from infection throughout the study. No significant difference in milk yield or percentages of fat and protein were observed between healthy and infected goats. Based on these results, one cannot justify treatment of chronic subclinical IMI infections by *S. aureus* during the lactation, at least for the prevention of lost production. Dry treatment may be a better strategy to minimize the amount of milk that would be discarded because of antibiotic residues and to decrease the risk of antibiotic resistance from overuse of antimicrobial agents. The finding that antimicrobial resistance was different for *S. aureus* isolates and drugs tested, point out that one or a few isolates taken from a given site or animal will not necessarily represent the total antimicrobial resistance present in the herd. An investigation into the type and distribution of plas-

mids encoding for antimicrobial resistance within both *S. aureus* and the general bacterial populations could determine the mode of inheritance of resistance and the relatedness of different molecular types. However, based on these observations, goat *S. aureus* isolated from this herd can still be treated effectively with the usual set of antibiotics available for intramammary therapy. Benzylpenicillin, amoxicillin plus clavulanic acid, cloxacillin, cephalonium, and cephaloperazone can be considered the most suitable antimicrobial agents for therapeutic applications to eradicate, or at least reduce, *S. aureus* prevalence.

ACKNOWLEDGMENTS

We are thankful to the Provincial Breeding Association of Asti for technical assistance and R. Zadoks, M. J. Paape, and T. Grant for technical reading. This work was supported by the Fondazione Cariplo (Contract no. 159 2003.1824/10.8441) and by Italian FIRST 2004 (P. Moroni).

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