Intramammary infections caused by coagulase-negative staphylococci and the effect on somatic cell counts in dairy goats

Karen Aulrich[1] and Kerstin Barth[1]

Abstract

The aim of the present study was the monitoring of the dairy goat herd of the Institute of Organic Farming for intramammary infections (IMI) caused by coagulase-negative staphylococci (CNS) over an entire lactation. The effect of IMI on somatic cell count (SCC) of goat's milk was investigated. A total of 64 German Improved Fawn goats were sampled. Foremilk samples were taken three times during lactation, at the beginning, in the early stage and at the end of lactation. The udder halve samples (n = 342) were analysed for somatic cell count (SCC) and for species identification by PCR. Therefore PCR methods based on species-specific 16S - 23S ribosomal RNA spacer sequences were used as well as internal transcribed spacer PCR (ITS-PCR). DNA was directly isolated from milk samples. The prevalence of IMI caused by CNS decreased during lactation from 33 % of infected animals to 15 % at the end of lactation. The most prevalent specific pathogen observed in the herd was *Staphylococcus epidermidis*. Other identified bacteria were *S. xylosus*, *S. simulans* and *S. caprae*. The prevalence of CNS infections in both half udders were similar. SCC increased with stage of lactation. The parity had no significant effect on SCC in the presented investigation. Infected udder halves had significant higher levels of SCC than uninfected halves (2.35 vs. 3.02, $P < 0.01$). It was observed that the udder halves respond not independent. If one half was infected with CNS the SCC of the non-infected udder half was influenced and tended to increase. This fact should be also taken into account as well as all other known factors influencing the SCC in goat's milk in the establishment of criteria for evaluation of goat's milk quality.

Keywords: goats, mastitis, coagulase-negative staphylococci, somatic cell count, Organic Farming

Zusammenfassung

Euterinfektionen durch koagulase-negative Staphylokokken und ihre Auswirkungen auf die Zellzahl von Ziegenmilch

Das Ziel der vorgestellten Studie ist das Monitoring intramammärer Infektionen (IMI), hervorgerufen durch koagulase-negative Staphylokokken, der Milchziegenherde des Instituts für Ökologischen Landbau über eine Laktationsperiode. Der Effekt intramammärer Infektionen auf die somatische Zellzahl (sZZ) von Ziegenmilch wurde untersucht. Es standen 64 Ziegen der Rasse Bunte Deutsche Edelziege zur Verfügung. Während der Laktation wurden dreimal Vorgemelksproben entnommen, zu Beginn, im frühen Stadium der Laktation und am Ende der Laktation. In den Proben aus den Euterhälften (n = 342) wurden die sZZ bestimmt und die Bakterienspezies mittels PCR identifiziert. Hierfür kamen Methoden zum Einsatz, die auf spezies-spezifischen ribosomalen Spacersequenzen des16S-23S RNA-Genes beruhen wie auch Methoden der ITS-PCR. Die DNA wurde direkt aus der Milch isoliert. Die Prevalenz durch CNS hervorgerufener IMI verringerte sich während der Laktation von 33 % infizierter Tiere auf 15 % infizierter Tiere am Ende der Laktation. Der am häufigsten vorkommende spezifische Erreger in der Herde war *Staphylococcus epidermidis*. Als weitere Erreger wurden *S. xylosus*, *S. simulans* and *S. caprae* identifiziert. Die Prevalenz von CNS Infektionen in beiden Euterhälften war ähnlich. Die sZZ stieg während der Laktation an. Die Laktationsnummer hatte keinen signifikanten Einfluss auf die sZZ. Infizierte Euterhälften hatten signifikant höhere sZZ als nichtinfizierte (2.35 vs. 3.03, $P < 0.01$). Es wurde festgestellt, dass die Euterhälften bei einer Infektion nicht unabhängig voneinander reagieren. Wenn eine Hälfte mit CNS infiziert ist, so ist die sZZ der anderen, nichtinfizierten Hälfte beeinflusst und zeigt tendenziell höhere Werte. Diese Feststellung und alle weiteren bekannten Faktoren, die die sZZ in Ziegenmilch beeinflussen, müssen in Betracht gezogen werden, wenn es um die Festlegung von Kriterien zur Bewertung der Qualität von Ziegenmilch geht.

Schlüsselwörter: Milchziegen, Mastitis, koagulase-negative Staphylokokken, Zellzahl, Ökologischer Landbau

[1] Johann Heinrich von Thünen-Institut, Federal Research Institute for Rural Areas, Forestry and Fisheries (vTI), Institute of Organic Farming, Trenthorst 32, 23847 Westerau/Germany; Email: karen.aulrich@vti.bund.de

1 Introduction

Intramammary infections (IMI) caused by coagulase-negative staphylococci (CNS) are increasing during the last years. IMI in dairy goats play an important role like in dairy cows. The relevance of IMI in dairy goats is not only an economic issue but also an hygienic and safety issue with respect to the bacteriological quality of milk.

The coagulase-negative staphylococci (CNS) are the most prevalent microorganisms in subclinical mastitis in goats (Contreras et al., 2003) ranging from 25 to 93 % of intramammary infections (Bergonier et al., 2003). Contreras et al. (2007) mentioned a prevalence of subclinical mastitis of 5 - 30 %. Among the CNS in dairy goats, *Staphylococcus caprae* was described as the most prevalent species followed by *S. epidermidis*, *S. xylosus*, *S. chromogenes* and *S. simulans* (Bergonier et al., 2003). But the results are not consistent, however Moroni et al. (2005a; 2005b) found *S. epidermidis* as the most prevalent CNS species in two different investigations altogether including seven dairy farms in Italy. A previous study (Contreras et al., 1999) also reported high prevalence of *S. epidermidis* in a commercial dairy goat herd. The pathogenic importance of CNS in goats is still uncertain and the differences among the various CNS species are unclear.

The somatic cell count (SCC) represents a sensitive marker of udder health in dairy cattle and is a useful parameter to evaluate the relationship between IMI and changes in milk characteristics. SCC is widely used for evaluating milk quality. In the European Union (EU) a legal limit for SCC in cows bulk milk was established with 400.000 ml^{-1} (EU, 1992). For goats no legal limit exists in the EU, in contrast the Food and Drug Administration in the United States defines 1.000.000 ml^{-1} for goats. Nevertheless, the necessity for evaluation of milk quality exists also for goat milk. The transmission of findings from dairy cattle to small ruminants leads to errors in the diagnosis of subclinical IMI (Raynal-Ljutovac et al., 2007) and in the evaluation of goat`s milk quality. SCC was controversial discussed in the literature. Attention should be paid to the fact that the geometric mean of SCC for healthy udder halves in goats was considerably higher than values obtained for other dairy ruminants (Luengo et al., 2004). The main aspects influencing SCC in goats were summarized recently (Contreras et al., 2007; Paape et al., 2007; Raynal-Ljutovac et al., 2007). However, because of important differences among dairy ruminants, mastitis control should be carefully made with a specific point of view to small ruminants (Contreras et al., 2007).

The objectives of the present study were to determine the occurrence of CNS in the dairy goat herd of the Institute of Organic Farming, to identify the CNS species by PCR based methods and to investigate the prevalence of the species over an entire lactation. The influence of SCC on IMI caused by CNS should be examined.

2 Material and methods

The investigations were carried out on about 60 German Improved Fawn goats from the organically managed institute owned farm. Milk samples were taken during the first five days *post partum* (in March 2005, sampling day 0), in the early stage of lactation (sampling day 1) and at the end of lactation in December (sampling day 2). The milk samples from each udder half were collected aseptically after premilking and cleaning of the teat ends according to the standards of DVG (2000). Samples were kept at 4 °C until SCC analysis and at -20 °C until molecular biological analysis were performed.

SCC was determined in milk samples from sampling day 1 and 2 by the fluoro-opto-electronic method of the International Dairy Federation (IDF Standard 148A:1995) using the Fossomatic unit (FM 500 and FM 369, Foss Electric, Denmark). Samples from day 0 were excluded because of the special characteristics of colostrum. For statistical analysis, the SCC readings were normalized using the standard log10 transformation.

Bacteria DNA for PCR analysis was directly isolated from the milk samples in the following way: 1 ml milk was centrifuged at 8.000 x g for 5 min. The supernatant was removed and the pellet was washed twice with phosphate buffered saline and centrifuged at 12.000 x g for 5 min. After each centrifugation the supernatant was removed. The remaining pellet from the second centrifugation was subjected to lysis with a combination of Mutanolysin (Sigma, Germany) and Lysostaphin (Sigma, Germany) at 37 °C for 2 h. After centrifugation (12.000 x g, 5 min) the samples were incubated with Proteinase K for 16 h at 56 °C and further treated with the Tissue Kit (Macherey-Nagel, Germany) according to the technical instructions. The remaining preparations were used as PCR templates.

The PCR reactions were performed using primer systems based on species specific 16S - 23S ribosomal RNA spacer sequences (Forsman et al., 1997) and developed for the detection of the main bovine mastitis pathogens (Tilsala-Timisjarvi et al., 2000). Table 1 shows in extracts the oligonucleotide primers for the detection of *S. epidermidis*, *S. simulans* and *S. xylosus*.

The amplifications were carried out in an iCycler (Bio-Rad Laboratories Inc., USA). The thermal cycling conditions were a hot start (using Hot Start Polymerase, Qiagen, Germany) at the beginning at 95 °C for 15 min followed by 40 cycles of denaturation at 95 °C for 30 s, annealing at 55 °C for 30 s, and extension at 72 °C for 30 s. After the final cycle the preparation was kept at 72 °C for 10 min to complete the reaction.

Table 1:
Species-specific oligonucleotide primers from 16S-23S rRNA intergenic spacer region

Species	Primer	Sequence (5'-3')	Size of the main PCR product (bp)
S. epidermidis	Sta-Ep-F	TCT ACG AAG ATG AGG GAT A	240
	Sta-Ep-R	TTT CCA CCA TAT TTT GAA TTG T	
S. simulans	Sta-Si-F	ATT CGG AAC AGT TTC GCA G	220
	Sta-Si-R	ATT GTG AGT AAT CGT TTG CC	
S. xylosus	Sta-Xy-F	CTC ATT GGA GTA TTC AGT GC	350
	Sta-Xy-R	CTT ACA GCT CCC CAA AGC AT	

The analysis of other *Staphylococcus* spp., especially *S. caprae* occurred by internal transcribed spacer PCR (ITS-PCR) according to Couto et al. (2001) using primers G1 (5'-GAAGTCGTAACAAGG-3') and L1 (5'-CAAGGCATC-CACCGT-3'). Amplification reaction was performed on a iCycler (Bio-Rad Laboratories Inc., USA). The program consisted of an hot start at 95 °C for 15 min and 30 cycles of denaturation at 94 °C for 1 min, annealing at 55 °C for 7 min, and extension at 72 °C for 2 min, followed by an additional extension step at 72 °C for 10 min (Jensen et al., 1993).

The PCR products were analysed by electrophoresis on 2 % agarose gels, however in case of the ITS-PCR products on 3 % gels, stained with ethidium bromide. The molecular size marker (marker VIII, Fermentas, Germany) was running concurrently. Gels were visualized under UV illumination (GelDoc2000, Bio-Rad Laboratories Inc., USA), photographed and saved.

3 Statistical analyses

Statistical analyses were carried out using SPSS 11.0 for Windows®. Univariate and stepwise backward analysis of variance was used to describe the influence of the infection status of udder halves and the parities on SCC. The animal was included as a random effect and the stage of lactation (DIM) as a covariate. The variable remained in the model if $p \leq 0.25$. The least square means were compared by the Bonferroni procedure.

4 Results and discussion

The numbers of sampled goats at different sampling days are shown in Table 2. The animals in the investigated herd were distributed over four parities.

The investigation of the whole dairy goat herd on CNS by PCR based methods showed a decrease of the prevalence of CNS during lactation. Whereas the prevalence within the first days *pp* averaged 33 % related to the sum of infected animals, the prevalence declined to 18 % after 57 days in milk (DIM) and to 15 % at the end of lactation after 263 DIM. Among the 342 udder halves analysed, bacteria were detected in 51 samples. Across stage of lactation, 23 - 9.6 % of udder halves were infected from March to December. Higher rates of IMI caused by CNS were observed during the first days of lactation with 29 infected halves (Table 3). IMI's decreased to 13 infected halves in the early stage of lactation and to 9 udder halves infected with CNS at the end of lactation.

Table 2:
Number of sampled goats depending on parity and stage of lactation

DIM Median (Range) Sampling Day	2 (0 - 4) 0	57 (28 - 87) 1	263 (236 - 297) 2
Parity	Number of goats		
1	18	14	8
2	12	11	8
3	8	7	9
4	15	19	12

Table 3:
Number of goats and udder halves with and without CNS infection on different stages of lactation

DIM Median (Range)	Number of goats	CNS-infected animals	Number of udder halves	CNS-infected udder halves
2 (0 - 4)	64	21	128	29
57 (28 - 87)	60	11	120	13
263 (236 - 297)	47	7	94	9
Total	171	39	342	51

DIM = Days in milk

Among goats with IMI caused by CNS, 13 goats (62 %) had unilateral infections during the first days of lactation, the others had both half-udders infected. During early stage of lactation the rate with unilateral infections among infected animals increased to 81.8 % (9 goats) and

Table 4 :
Staphylococci isolated and prevalence of infection in left and right half udders on different sampling days during lactation

Species	DIM Median (Range)							
	2 (0 - 4)		57 (28 - 87)		263 (236 - 297)		Across lactation	
	Udder half		Udder half		Udder half		Udder half	
	Left (n = 64)	Right (n = 64)	Left (n = 60)	Right (n = 60)	Left (n = 47)	Right (n = 47)	Left (n = 171)	Right (n = 171)
Total CNS	14	15	6	7	5	4	25	26
S. epidermidis	9	6	6	4	5	1	20	11
S. xylosus	1	4	0	2	0	3	1	9
S. simulans	3	5	0	1	0	0	3	6
S. caprae	1	0	0	0	0	0	1	0

declined to 71.4 % (5 goats) at the end of lactation. The prevalence of CNS infections in left and right udder halves across lactation were similar on all tested days (Table 4) as well as described by Moroni et al. (2005b).

The IMI were caused by four *Staphyloccoccus* spp., *S. epidermidis*, *S. xylosus*, *S. simulans* and *S. caprae*. From the 51 udder halves diagnosed with CNS infection, 31 (60.8 %) were identified as *S. epidermidis*, 10 (19.6 %) as *S. xylosus*, 9 (17.6 %) as *S. simulans* and only one half udder as *S. caprae* (Table 4). *S. epidermidis* was the most prevalent bacteria over the entire lactation with 15 infected udder halves during the first days after kidding, 10 in the early stage of lactation and 6 at the end of lactation. The infection caused by *S. caprae* disappeared during lactation. The data indicating *S. epidermidis* as the most prevalent bacteria are in confirmation with findings from Moroni et al. (2005a, b) and Deinhofer und Pernthaner (1995), but in contrast to Bergonier et al. (2003) who described *S. caprae* as the most prevalent species in goats. Furthermore, previous studies suggested that a high prevalence of *S. epidermidis* might be explained when teat dipping was not practiced (Contreras et al., 1997; Poutrel, 1984). These explanations could not be used for the results of the presented study because in the investigated herd teat dipping was a common practice. Therefore other explanations should be proved like the different competition of species during udder colonization. Further studies are necessary to point it out.

Several studies have demonstrated the capacity of CNS to persist, also during the dry period (Poutrel, 1984; Contreras et al., 1997). Our data until now demonstrated that only a small proportion of pathogens were persistent over the entire lactation in the investigated herd. The persistence was detected in four udder halves, two infected with *S. epidermidis* and two with *S. xylosus*, respectively. The studies should be continued with involvement of further lactations for the evaluation of the pathogen's behaviour.

Infection status of the udder, traditionally described as the most important factor affecting milk SCC (Luengo et al., 2004) was investigated as a further question of the study.

According to the statistical analyses the parity had no significant effect on SCC in the presented investigation in contrast to results from Paape et al. (2007) and Sanchez et al. (1999). Our results agree with findings from Zeng and Escobar (1995) who also stated that parity did not affect the SCC. Therefore the factor parity was excluded from the model. Figure 1 illustrates the logarithm of SCC in connection with the infection statuses on the two sampling days, whereas day one represents the data in the early stage of lactation and day two at the end of lactation. SCC tended to increase with stage of lactation. This

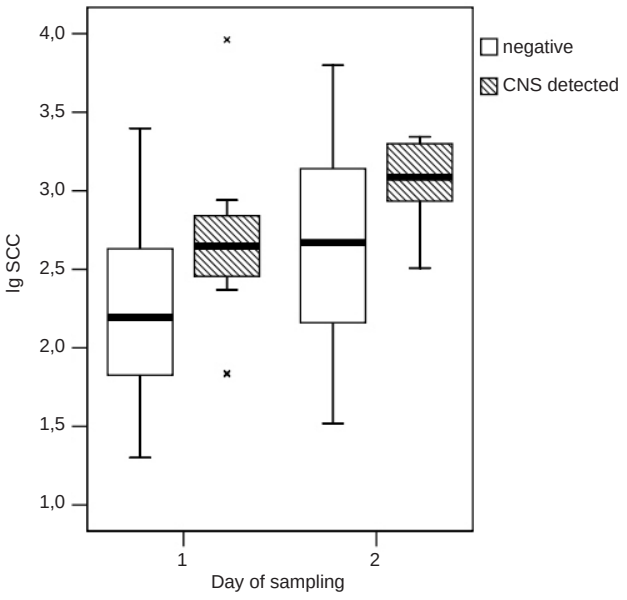

Figure 1:
The logarithm of SCC of early (1) and late (2) lactation milk samples in non-infected udder halves and halves infected with CNS

fact was also observed by other research groups (Luengo et al., 2004; Paape et al., 2007; Zeng und Escobar 1995; Min et al., 2007).

The mean SCC including infected and non-infected animals on day one was 2.28 (SD 0.52), increased to 2.70 (SD 0.61) on day two. The mean SCC in milk samples from udder halves that showed no infection with CNS on day one was 2.2 (SD 0.5), while the mean SCC from infected halves was 2.6 (SD 0.5). With increasing days in milk (mean = 263 days), the SCC in non-infected udder halves reached the level of infected udder halves on day 1 with 2.6 (SD 0.6). The SCC in CNS infected udder halves was 3.1 (SD 0.3) (Figure 1). It has been shown recently that SCC increased so strong with stage of lactation, that Moroni et al. (2005b) could not observe significant differences in SCC between healthy and infected animals at the end of lactation. Taking this fact and the own data into consideration, the independence of the udder halves' reactions should be proved. Flock and Zeidler (1968) investigated the degree of independence of udder quarters with respect to mastitis incidence in dairy cows and showed evidence that the quarters are affecting each other. Results of variance analysis are shown in Table 5. The least square mean (LSM) of SCC was 2.35 if both udder halves were non-infected with CNS. To consider the non-infected udder halves while the other half was infected with CNS, the LSM of SCC was 2.81 and tended to increase. LSM of SCC in infected udder halves whereas the other half was non-infected was 3.02 and significant different from non-infected udder halves ($P < 0.01$). If both udder halves were infected with CNS the LSM of SCC was 2.82 and not significant different from the other groups of tested halves. This fact should be proved in further investigations under consideration of more lactations and inclusion of more animals. Nevertheless, the data indicated the dependency of udder halves on each other with respect to the infection status.

Figure 2 illustrates the logarithm of SCC in early and late lactation milk samples of non-infected udder halves and of udder halves affected by different CNS species. The difference between infected and non-infected halves showed a tendency to increase for all identified bacteria, except of *S. simulans*. The database for *S. simulans* was marginal (n = 1) during the investigated lactation and should be disregarded. As expected, SCC was lowest for non-infected udder halves (mean of both sampling days: 2.41). SCC was elevated in udder halves infected with *S. epidermidis* and *S. xylosus*, with 2.85 and 2.86, respectively. The differences were not significant. Among CNS, infections with *S. epidermidis* are associated with the highest values of SCC (Contreras et al., 1999; Deinhofer und Pernthaner, 1995). In contrast, recently Moroni et al. (2005a) observed differences in SCC increase among bacteria species. The SCC for infected udder halves with *S. caprae* was greater than with *S. epidermidis* and other CNS. The number of the remaining infected udder halves in the presented study was insufficient, therefore conclusions were not necessary with regard to that topic.

Table 5:

Least square means (LSM) of SCC, standard error and confidence intervals in udder halves with different infection status

Status of udder halves	LSM	SE	Confidence interval (95 %)
Both non-infected	2.35 ^{A}	0.04	2.27 - 2.44
Non-infected halves, other infected	2.81 ^{A,B}	0.17	2.47 - 3.15
Infected halves, other non-infected	3.02 ^{B}	0.17	2.67 - 3.36
Both infected	2.82 ^{A,B}	0.23	2.37 - 3.28

^{A,B} Means with different superscripts differ significantly ($P < 0.01$)

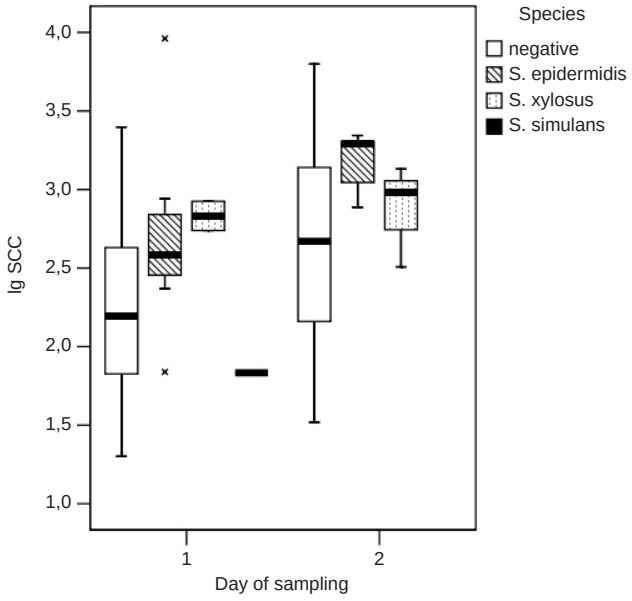

Figure 2:

The logarithm of SCC of early (1) and late (2) lactation milk samples in non-infected udder halves and in halves affected by different CNS species

The presented data showed the highest SCC values regarding infections with *S. epidermidis* and *S. xylosus* . As well as Figure 1, Figure 2 reveals the increase of SCC for all detected bacteria species with the stage of lactation. Nevertheless, further studies are necessary to prove the data.

5 Conclusions

The study showed that *S. epidermidis* was the most prevalent species in the investigated herd. The prevalence of CNS decreased during lactation.

The data indicated the dependency of the udder halves with regard to their infection status. This fact needs further investigations with a higher number of animals.

It could be shown that any infection with CNS caused an increase of SCC in goat`s milk as well as in cow´s milk.

SCC increase was observed with stage of lactation.

The different factors influencing the SCC of goat milk should be taken into account if diagnostic thresholds will be defined for evaluation of milk quality.

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