Identifying the major bacteria causing intramammary infections in individual milk samples of sheep and goats using traditional bacteria culturing and real-time polymerase chain reaction

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ABSTRACT

Use of DNA-based methods, such as real-time PCR, has increased the sensitivity and shortened the time for bacterial identification, compared with traditional bacteriology; however, results should be interpreted carefully because a positive PCR result does not necessarily mean that an infection exists. One hundred eight lactating dairy ewes (56 Manchega and 52 Lacaune) and 24 Murciano-Granadina dairy goats were used for identifying the main bacteria causing intramammary infections (IMI) using traditional bacterial culturing and real-time PCR and their effects on milk performance. Udder-half milk samples were taken for bacterial culturing and somatic cell count (SCC) 3 times throughout lactation. Intramammary infections were assessed based on bacteria isolated in ≥ 2 samplings accompanied by increased SCC. Prevalence of subclinical IMI was 42.9% in Manchega and 50.0% in Lacaune ewes and 41.7% in goats, with the estimated milk yield loss being 13.1, 17.9, and 18.0%, respectively. According to bacteriology results, 87% of the identified single bacteria species (with more than 3 colonies/plate) or culture-negative growth were identical throughout samplings, which agreed 98.9% with the PCR results. Nevertheless, the study emphasized that 1 sampling may not be sufficient to determine IMI and, therefore, other inflammatory responses such as increased SCC should be monitored to identify true infections. Moreover, when PCR methodology is used, aseptic and precise milk sampling procedures are key for avoiding false-positive amplifications. In conclusion, both PCR and bacterial culture methods proved to have similar accuracy for identifying infective bacteria in sheep and goats. The final choice will depend on their response

time and cost analysis, according to the requirements and farm management strategy.

Key words: mastitis prevalence, small ruminant, subclinical mastitis, real-time polymerase chain reaction

INTRODUCTION

Milk provides the major source of income in dairy farms. Therefore, every disturbance in producing optimal milk quantity and milk quality will reduce farm profitability. Milk quantity and quality are related to genetics (i.e., breed) and environment (i.e., nutrition and management) as well as to animal health (i.e., udder health). Small ruminant production systems vary widely, from traditional hand milking to the most modern computerized milking parlors, and with different dairy breeds, herd sizes, and levels of milk yield. Despite these differences, all sheep and most goat milk is destined for manufacturing dairy products. Therefore, milk quality is key for high-quality dairy products and milk from intramammary infected glands alters its manufacturing ability (e.g., rennet coagulation and curd firmness) for dairy products.

Intramammary infection is one of the main causes of milk production losses (Gonzalo et al., 2002; Leitner et al., 2007, 2008) along with changes in its composition (Leitner et al., 2004a,b). In addition, IMI influences milk coagulation properties, depending on the species of bacteria (Leitner et al., 2006). Of the bacteria involved, some cause clinical infection [i.e., *Escherichia coli*, *Staphylococcus aureus*, and streptococci, whereas the majority cause only subclinical infection with no visible signs (i.e., *Staphylococcus* spp., including *Staph. aureus* and CNS)]. Consequently, to identify infected animals as well as the infecting bacteria, milk sampling and laboratory diagnoses are needed. However, these methods are expensive and the time of sampling is crucial. Conventional bacterial culturing often requires 48 to 72 h for incubation and additional confirmation tests that take time to be completed (Oliver et al., 2004).

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On the other hand, PCR assay kits available on the market for mastitis testing do not require a culture step and can be performed in 3 to 4 h total (Koskinen et al., 2009). Nevertheless, current prices are up to approximately \$30/sample. In clinical mastitis, such rapid results should shorten the total duration of treatment, improve the therapeutic outcome, and decrease unnecessary use of antimicrobials (Pyörälä, 2002; Barkema et al., 2006; van den Borne et al., 2010). For clinical cases of mastitis, early detection is critical to help animals to heal and to apply veterinary treatment if necessary. On the other hand, with regard to subclinical infection, the time of laboratory diagnostic procedure is not crucial for chronic forms; however, justification of sampling and testing are based on cost-benefit analysis aiming to improve milk yield and composition.

Bacterial identification by inoculating milk on agar plates is the gold standard of classical bacteriology (Oliver et al., 2004). In the last 20 yr, although DNA-based methods such as real-time PCR and other methods on the market have increased the sensitivity and shortened the time needed for bacterial identification, costs have remained relatively high. Moreover, PCR could also enable the differentiation between various genes; for instance, β -lactam positive and negative. Interpretation of the PCR results is questionable because they may either contain more than 1 bacterial species or give false-positive results for animals free of inflammation (Koskinen et al., 2010). Possible causes of PCR false-positive results could be little bacterial shedding (i.e., less than 3 cfu) or no growth of microorganisms in the culture media or in the contaminated milk (i.e., bacteria coming from other sources such as ovine and caprine). Hence, positive results in a sample showing more than a single bacterial specie from known clinically and subclinically infected glands, or from glands with no inflammation (no increase in SCC) or altered cell distribution, may be either true positive or derived from extramammary contamination of bacteria inhabiting the udder skin and teat canal (Taponen et al., 2009).

The objectives of the current study were to (1) compare the identification of the main bacteria causing clinical and subclinical IMI in individual milk samples taken from sheep and goat udder halves, using traditional bacterial culturing (**BAC**) and real-time PCR and (2) to study the influence of IMI on milk yield and SCC of dairy sheep and goats.

MATERIALS AND METHODS

Animals

The study was conducted at the Experimental Farm of the Servei de Granges i Camps Experimentals

(SGCE) of the Universitat Autònoma de Barcelona (UAB, Bellaterra, Spain). The Ethical Committee on Animal and Human Experimentation (CEEAH) of the UAB approved all the experimental and animal care procedures.

A total of 108 lactating dairy sheep (parity = 2.9 ± 0.2) of 2 breeds [Manchega (**MN**): $n = 56$; Lacaune (**LC**): $n = 52$] and 24 Murciano-Granadina dairy goats (parity = 4.0 ± 1.0) were studied after the weaning of the lambs and after parturition, respectively. Animals were kept under a semi-confinement system, allowed to graze for 6 h daily in an annual Italian ryegrass pasture, and supplemented indoors with alfalfa hay ad libitum (1.27 Mcal of NE_L /kg and 20.1% CP; DM basis) and concentrate at a flat rate of 0.8 kg/d (1.75 Mcal of NE_L /kg and 16.5% CP; DM basis) distributed during milking. Water and a commercial block of vitamins and minerals (Multi-Block; Agraria Comarcal del Vallès, Les Franqueses, Barcelona, Spain) were permanently available in the shelter.

Ewes were machine milked twice daily (0800 and 1700 h) in a double 12-stall parallel low-line milk pipeline milking parlor (WestfaliaSurge Ibérica SL, Granollers, Spain) equipped with recording jars and electronic pulsators at a vacuum of 42 kPa, 120 pulses/min, and 50% pulsation ratio. Goats were milked once per day (0900 h) in the same milking parlor using different electronic pulsators (90 pulses/min and 60% pulsation ratio). The milking routine included machine milking, without udder preparation, but with postmilking teat dipping in an iodine solution (P3-ioshield; Ecolab Hispano-Portuguesa SA, Barcelona, Spain) after cluster removal. Milk yield of individual ewes was recorded weekly (morning and evening) from the weaning of the lambs (35 DIM) to 120 and 135 DIM for MN and LC, respectively. For goats, individual milk yield was recorded weekly from 7 to 280 DIM.

Milk Sampling

Milk samples were taken aseptically by hand milking during the morning milking and submitted to the laboratory within 2 h. Before sampling, teats were disinfected by dipping in an iodine solution (P3-ioshield) and dried with disposable paper towels. The first 3 milk squirts were discarded. Following cleaning, the teats were disinfected with 70% ethanol and left for approximately 1 min to evaporate any remaining alcohol. Milk of the first squirts were again discarded and 3- to 4-mL samples were collected from each udder half into sterile tubes (Eurotubo Deltalab, code 429946; Deltalab SL, Rubí, Spain) for bacteriological testing done on the same day and California mastitis test (**CMT**) examination in situ (Drofilisa; Fatro Ibérica SL, Sant

Just Desvern, Spain). Following milking, the milk yield of each gland was recorded individually and, after mixing the total amount collected, 40 mL of milk was sampled and preserved with bronopol (Broad Spectrum Microtabs II; D & F Control Systems Inc., San Ramon, CA) for SCC using a Fossomatic 5000 (Foss Electric A/S, Hillerød, Denmark) at the Interprofessional Milk Laboratory of Catalonia (**ALLIC**, Cabrils, Barcelona, Spain). These procedures were repeated 3 times during lactation (50 to 90 DIM, at approximately 15-d intervals). For comparing standard BAC and real-time PCR, additional milk samples (4 mL) were taken in sterile tubes from selected glands at the second and third sampling period and stored frozen at -20°C until analyzed at the ALLIC laboratory. Positive gland infection was based on bacteria isolation in at least 2 of the 3 samplings accompanied by an increase in somatic cells (CMT or SCC, or both).

Udder health was also visually evaluated before sampling according to a subjective classification based on gland morphology while animals were in the milking parlor: symmetrical (normal udder with no hardness at palpation), asymmetrical (abnormal udder with or without hardness in at least 1 gland), or involuted (<250 mL of milk/d). Animals were classified according to udder health as follows: (1) udders with no bacterial finding and CMT score ≤ 1 (**NBF**), (2) udders with bacterial finding and CMT score >1 in one half (**IMI-1**), or (3) udders with bacterial finding and CMT score >1 in both halves (**IMI-2**).

Bacteriological Examinations

Bacteriological analyses were performed separately at the UAB (**BAC1**), in fresh milk, and at the ALLIC (**BAC2**), in frozen samples, according to accepted standards (Oliver et al., 2004). At BAC1, 0.01 mL was streaked onto blood-agar plates (Bacto-Agar; Difco Laboratory, Le Pont-de-Claix, France) containing 5% washed sheep red blood cells. Plates were incubated at 37°C and examined for bacterial growth after 18 and 42 h. At BAC2, 0.02 mL of milk was streaked on esculin blood agar [tryptone soy agar (Biokar Diagnostics-Solabia, Pantin, France) and esculin (Merck Millipore, Darmstadt, Germany)] and incubated at 37°C for 48 h, with readings at 24 and 48 h. Cultures of colonies initially picked from the blood agar were tested by coagulase test in situ (Baird Parker RPF; Biokar Diagnostics) and Tryptone Bile X-Glucuronide BX selective agar (Chromocult; Merck Millipore). Samples were subjected to Gram stain, catalase, esculin hydrolysis (in situ), CAMP factor hemolysis, and agglutination tests for pathogen identification according to microbiology reference methods (Markey et al., 2013).

Real-Time PCR

Identification of bacterial DNA was done using a real-time PCR-based commercial reagent kit (PathoProof Mastitis Complete-12 kit PCR Assay; Thermo Fisher Scientific, Vantaa, Finland) for direct analysis of all milk samples at the ALLIC laboratory. The PathoProof kit for accurate identification of mastitis-causing bacteria from bovine milk can identify the following prevailing mastitis-causing bacterial species and groups: *Staphylococcus* spp. (including *Staph. aureus*) and all major CNS, *Streptococcus* spp. (*Streptococcus dysgalactiae*, *Streptococcus agalactiae*, and *Streptococcus uberis*), *E. coli*, *Enterococcus* spp. (including *Enterococcus faecalis* and *Enterococcus faecium*), *Klebsiella* spp. (including *Klebsiella oxytoca* and *Klebsiella pneumoniae*), *Serratia marcescens*, *Corynebacterium bovis*, *Arcanobacterium pyogenes*, and *Peptoniphilus* (*Peptostreptococcus*) *indolicus* as well as the staphylococcal β -lactamase penicillin resistance gene *blaZ*.

The assay was carried out following the manufacturer's procedure (PathoProof) and included all necessary reagents for DNA extraction and real-time PCR. The DNA extraction method used 350 μL of milk as the starting volume and involved an enzymatic lysis step, disrupting the cell walls of gram-positive and gram-negative bacteria, as well as spin column-based DNA purification and elution steps.

Statistical Analyses

Statistical analyses were carried out with JMP software (SAS Institute, 2000). The experimental unit was the individual animal. For sheep, the analyzed parameters were milk yield and logarithm of SCC by a 3-way ANOVA model:

$$Y_{ijkl} = \mu + \alpha_i + \beta_j + \delta_k + \alpha\beta_{ij} + \alpha\delta_{ik} + \beta\delta_{jk} + e_{ijkl},$$

where Y_{ijkl} = individual analysis parameter value, μ = overall mean, α_i = breed (MN or LC), β_j = IMI status (NBF, IMI-1, or IMI-2), δ_k = parity (1, 2, 3, or ≥ 4), $\alpha\beta_{ij}$ = breed $\times$ IMI status interaction, $\alpha\delta_{ik}$ = breed $\times$ parity interaction, $\beta\delta_{jk}$ = IMI status $\times$ parity interaction, and e_{ijkl} = residual error. The breed $\times$ IMI status $\times$ parity number interaction was not included in the model due to a missing combination (no MN sheep in the 1st lactation with IMI-2).

For goats, breed effect was excluded from the model. Multiple comparisons between means were done by the Tukey Honestly significant difference test and significance was declared at $P < 0.05$, unless otherwise indicated. Correlations between all the continuous variables and the analyzed parameters were determined for all data.

RESULTS

Milk Production and SCC Score

The MN ewes produced less milk than LC sheep ($P < 0.001$) as shown in Table 1. Breed had no effect on SCC levels ($P = 0.38$) but IMI status affected milk yield and SCC of udder halves ($P < 0.001$). The logarithm of SCC of uninfected milk samples ranged between 4.96 and 5.22, whereas it ranged between 6.56 and 6.80 for the ewes with IMI-2 (Table 1). Figure 1 illustrates the individual MN and LC logarithm of SCC values according to IMI status.

For each sheep breed, animals with IMI-2 showed significantly lower milk yield than NBF ($P < 0.001$) but no differences were found between IMI-2 and IMI-1 ($P > 0.05$; Table 1). No significant interactions existed except for the case of breed $\times$ SCC ($P = 0.032$) in which the LC SCC values were greater. Milk yield increased according to parity in both breeds ($P < 0.001$) but SCC did not vary ($P = 0.84$).

Infected goats tended to produce less milk ($P = 0.080$) and had slightly higher SCC than uninfected goats (Table 1; $P < 0.05$), as shown in Figure 1. No correlation was found between milk yield and SCC in both sheep and goats.

Bacterial Isolation

Throughout the study, only 3 (2.8%) ewes were diagnosed with clinical mastitis, 2 were infected with *E. coli*, and 1 was infected with *Pseudomonas* spp. Clinical mastitis was treated after sampling and those animals were excluded from the study thereafter. Comparing the BAC results, 87% of the udder halves were 3 times identical either for single bacteria specie colonies or NBF and 11% only repeated in 2 samplings. Moreover,

2% of samples showed contaminated milk in at least 1 of the samplings (more than 2 colony types per plate). According to BAC results, 17 MN ewes were classified as IMI-1 and 7 were IMI-2, resulting in 42.9% subclinically infected ewes (Table 1). On the other hand, 17 LC ewes were classified as IMI-1 and 9 were IMI-2, resulting in 50.0% of ewes that were subclinically infected. Two-thirds of the infected glands were asymmetrical. Regarding the uninfected sheep, 89.7% had symmetrical udders and 10.3% had asymmetrical ones (4 MN and 2 LC ewes).

Of the 24 Murciano-Granadina goats, 1 doe (4.2%) was diagnosed with clinical mastitis caused by *E. coli*. Eight were found uninfected (33.3%) with symmetrical udders, 10 were classified as IMI-1 (of which 2 had asymmetrical glands), and 3 were classified as IMI-2 with symmetrical glands (Table 1). Two more goats had high SCC in one of the glands with no bacterial finding.

Comparison of Bacterial Culture and Real-Time PCR of Individual Milk Samples

A total of 90 milk samples from both breeds of sheep and goats were selected from individual glands with known bacterial infection (IMI-1 and IMI-2) and from uninfected glands with low SCC (NBF with CMT = 0) or high SCC (NBF with CMT >1). Results of the BAC are summarized in Table 2. Of the 90 samples, including sheep of both breeds and goats, 30 (33.7%) were classified as NBF, of which 18 had low SCC and 12 had high SCC. Regardless of CMT, all 30 NBF samples were confirmed by BAC (BAC1 and BAC2) and 29 by real-time PCR as NBF. Only a single NBF sample was found positive by real-time PCR for *Staphylococcus* spp. (Table 2) and considered as BAC false negative.

Table 1. Average daily milk throughout the experiment and SCC of dairy sheep and goats according to IMI status of the udder

IMI status according to breed group	No.	Milk, L/d		Log SCC	
		Mean $\pm$ SE	Range	Mean $\pm$ SE	Range
Manchega ewes					
Uninfected	32	0.91 $\pm$ 0.05 ^a	0.31–2.01	4.96 $\pm$ 0.04 ^c	4.73–5.56
1 gland infected	17	0.82 $\pm$ 0.06 ^{ab}	0.40–1.24	6.09 $\pm$ 0.11 ^b	5.13–6.86
2 glands infected	7	0.72 $\pm$ 0.08 ^b	0.35–1.24	6.80 $\pm$ 0.11 ^a	6.38–7.27
Lacaune ewes					
Uninfected	26	1.45 $\pm$ 0.07 ^a	0.44–2.24	5.22 $\pm$ 0.04 ^c	4.78–5.64
1 gland infected	17	1.26 $\pm$ 0.11 ^{ab}	0.50–2.28	6.11 $\pm$ 0.04 ^b	5.49–7.07
2 glands infected	9	1.06 $\pm$ 0.10 ^b	0.50–1.50	6.56 $\pm$ 0.14 ^a	5.61–7.05
Murciano-Granadina goats					
Uninfected	8	1.39 $\pm$ 0.15 ^x	1.14–1.65	5.55 $\pm$ 0.03 ^b	5.30–6.23
1 or 2 glands infected	15 ¹	1.14 $\pm$ 0.13 ^y	0.78–1.68	6.18 $\pm$ 0.06 ^a	5.67–7.01

^{a–c}Means within the same breed with different superscript letters differ at $P < 0.05$.

^{x,y}Means within the same breed with different superscript letters tend to differ at $P < 0.10$.

¹Including 3 goats with both udder halves infected.

Figure 1. Average logarithm of SCC in dairy goats (A) and dairy sheep (B) according to IMI status determined by the presence of bacterial infection and inflammation in 1 (○) or 2 (●) udder halves or with no bacterial finding (▲).

Of the 60 infected samples, 38 were identified as CNS species in BAC1 and BAC2 (Table 2). The real-time PCR results mainly agreed with BAC, 36 samples being classified as *Staphylococcus* spp. or β -lactamase-positive *Staphylococcus* spp., and the remaining 2 samples were identified as no CNS (*Staph. aureus* and *S. uberis*).

Eight more samples were identified as *Staph. aureus* in BAC1 and BAC2, of which 7 matched *S. aureus* and 1 *Staphylococcus* spp. by real-time PCR. Moreover, 5 isolates identified as *Streptococcus* spp. by BAC1 and BAC2 failed to be confirmed by real-time PCR despite being specific to *Strep. agalactiae*, *Strep. dysgalactiae* and *Strep. uberis*.

Table 2. Summary of the number of bacteria found in bacterial cultures [BAC1: Universitat Autònoma de Barcelona (UAB, Bellaterra, Spain) and BAC2: Interprofessional Milk Laboratory of Catalonia (ALLIC, Cabriels, Barcelona, Spain)] and real-time PCR (RT-PCR) using clinical and subclinical sheep and goats mastitic samples

Item	NBF ¹	CNS	<i>Staphylococcus aureus</i>	<i>Streptococcus</i> spp.	<i>Arcanobacterium pyogenes</i>	<i>Escherichia coli</i>	<i>Enterococcus</i> spp.	<i>Enterobacteriaceae</i>
BAC1	30	38	8	5	2	2	3	2
BAC2	30	38	8	5	2	2	3	2
RT-PCR								
<i>Staphylococcus</i> spp.	1	19	1	—	—	—	—	—
<i>Staphylococcus</i> spp. and β -lactam	—	17	—	—	—	—	—	—
<i>Staphylococcus</i> spp. and <i>Staph. aureus</i>	—	1	7	—	—	—	—	—
<i>Streptococcus agalactiae</i>	—	—	—	—	—	—	—	—
<i>Streptococcus dysgalactiae</i>	—	—	—	—	—	—	—	—
<i>Streptococcus uberis</i>	—	1	—	—	2	—	—	—
<i>Arcanobacterium pyogenes</i>	—	—	—	—	—	—	—	—
<i>Peptoniphilus indolicus</i>	—	—	—	—	—	—	3	—
<i>Enterococcus</i> spp.	—	—	—	—	—	2	—	—
<i>E. coli</i>	—	—	—	—	—	—	—	—

¹No bacterial finding.

Of the remaining isolates, identified by BAC1 and BAC2 as 2 *A. pyogenes*, 2 *E. coli*, and 3 *Enterococcus* spp., all were confirmed with certainty by real-time PCR. Finally, PCR failed to identify 2 BAC1 and BAC2 as *Enterobacteriaceae*.

DISCUSSION

Despite the low incidence of clinical IMI in this study, the subclinical IMI prevalence indicated that almost 4 of 10 ewes or goats showed signs of udder inflammation, as measured by SCC, CMT, and BAC. Prevalence of clinical mastitis in dairy sheep is generally <5% (Berger et al., 2004), as observed in our data, but prevalence of subclinical mastitis infections were in 21% of glands and 34% of ewes according to Las Heras et al. (1999). Cuccuru et al. (2011) reported in sheep an increase of subclinical IMI prevalence with parity, varying from 35 to 65% for second to fifth, respectively. Prevalence of subclinical infections in dairy goats ranged from 7 to 34% of the glands and 17 to 44% of the goats (Contreiras et al., 1995). It is generally accepted that 20 to 30% of milk samples from IMI glands result in no growth by BAC in dairy cows (Bradley et al., 2007; Koskinen et al., 2010).

Many animals that were found subclinically infected in our study had normal udders without signs of IMI (no detectable changes in the milk, the udder, or the animal) but with increased SCC levels. Asymmetrical udders in uninfected sheep and goats also have high SCC, probably as a result of previous infections. These results indicate that (1) by adequate and careful aseptic milk sampling, it is possible to detect a few bacteria types in BAC or real-time PCR, or both in most samples; (2) a positive bacteriological result alone is not a sufficient indication of IMI and should always be coupled with an inflammatory response test (e.g., SCC or CMT); and (3) not all inflammation responses as measured by SCC or CMT are indications of the presence of bacteria (i.e., IMI). Therefore, adequate milk sampling for standard bacteriology, combined with an inflammatory response test, is necessary to identify true mammary infections. Moreover, in the case of disagreement between the 2 tests, second and third samplings are needed.

A real-time PCR assay was performed on milk samples without the need for BAC. Strong agreement between the BAC, performed independently by 2 laboratories (BAC1 and BAC2) and the real-time PCR was found in the present study. Moreover, udders presumed to be uninfected by BAC and with low milk SCC were detected as NBF by PCR.

These results were confirmed by those obtained from independent BAC tests, with 98.9% agreement between them and, whenever the real-time PCR was positive, a

single bacterium was detected. Moreover, 1 of 30 (3%) of samples identified as NBF by BAC were positive as *Staphylococcus* spp. by real-time PCR. The advantage of the real-time PCR was its ability to differentiate between β -lactam-positive and -negative CNS species. This information is of extreme importance, as approximately half of the CNS were gene encoding for β -lactamase (i.e., resistant to penicillin). However, it does not mean that the all β -lactam-negative species are sensitive to it, because more than 1 gene is involved in the drug resistance and, consequently, in the efficacy of antibiotics. The β -lactam-negative *Staph. aureus* found in this study do not guarantee drug sensitivity and therefore can be considered resistant. The real-time PCR failed to identify the 5 *Streptococcus* spp., probably because they are different from the 3 *Streptococcus* spp. detectable by the kit used (i.e., *Strep. agalactiae*, *Strep. dysgalactiae*, and *Strep. uberis*). All in all, the real-time PCR commercial kit results were almost identical to those of BAC, and produced an outcome in a few hours compared with days needed for conventional BAC. However, the real-time PCR disadvantages are bacterial species limitations for which the diagnostic kit was designed (bovine specific) and the high cost of the test, which could currently limit its use.

Our results disagree with previous studies using the same kit comparing bacterial cultures and real-time PCR in bovine milk samples. Real-time PCR provided bacteriological diagnosis for almost 50% of the negative results obtained from conventional culturing of mastitic milk samples (Taponen et al., 2009). Koskinen et al. (2010), using 1,000 cow samples, identified more than 15% of the clinical and subclinical quarters with 2 or more bacteria species using BAC, whereas the PCR identified around 50% of the same samples. Moreover, of the healthy quarters with low SCC, one-third of the samples were positive, mostly with 1 bacterial species tested by the PCR.

The sensitivity and reliability of the 2 methods used for bacterial detection mainly depend on a set of key factors such as (1) secretion of bacteria is not uniform through repeated milk sampling; therefore, a low bacteria count can show a negative result especially in BAC; (2) the teat end is colonized with different bacteria, including mastitis pathogens; thus, small amounts of bacterial DNA, detectable by PCR, may end in false-positive results; thus, one sampling may not be sufficient to determine an infected gland and, therefore, inflammation response, such as increase in SCC and change in the distribution of somatic cells, is essential; moreover, if DNA methodology is used, sampling is the key to avoid contamination; and (3) both methods are not feasible outside reference laboratories, but more of a limitation exists if a specific DNA kit is

used (i.e., aerobic bacteria in BAC or pathogens in the DNA-specific kit). This is a major issue when applying DNA methods, which could result in a positive finding, and in many instances with more than a single species, which suggests that many milk samples from healthy glands with low SCC are actually infected even with a highly pathogenic bacterium. The point of the present work is to draw attention to the fact that there is a high correlation between proper milk sampling and classic bacteriology. Therefore, in milk from animals with no identified bacteria and no increase in SCC, PCR findings were also negative.

Cost-benefit analyses of commercial clinical and subclinical diagnostic tests can rely on controlled studies. In clinical mastitis, the time it takes to obtain the test results is important and in most cases the number of the causative bacteria is high; therefore, DNA methods are preferred. Thus, the time required for sampling and testing (in hours) is important in the treatment decision. In subclinical mastitis, duration of the diagnostic procedure is not crucial so that the cost of diagnostics, the tools for coping with the infection, and the influence of the infection on productivity must to be considered in making the decision. Antibiotic treatment of animals that are not at risk (as in the case of clinical mastitis) needs to be justified with respect to the costs of medicine and milk losses (Barlow et al., 2009; van den Borne et al., 2010) as well as the occurrences of hazards to consumer health and safety. Therefore, diagnosis and treatment of subclinical mastitis is complicated and can vary among animal species and individual animals, and according to milk price, drug cost, and availability of medicine.

Based on the current study, infection reduced milk production by approximately 20% in the 2 sheep breeds and by 7% in the Murciano-Granadina goats. Moreover, the milk quality as measured by SCC decreased. These losses are greater than those estimated in other studies of sheep and goats (Leitner et al., 2004a,b, 2008) in which it ranged from 4 to 13% for Assaf sheep and from 1 to 2% for Saanen, Shami, and crossbred goats with 25 to 75% udder infection. Martí De Olives et al. (2013) reported 15 and 38% milk loss in subclinical IMI of Manchega dairy sheep in the short term in early lactation, using whole-udder or half-udder models. Additionally, estimated curd loss by IMI varied between 5 and 16% in sheep and 3 to 10% in goats (Leitner et al., 2008).

CONCLUSIONS

Subclinical infection caused mainly by *Staph. aureus* and various CNS species resulted in lower milk yield and decreased quality. Bacteria colonies or culture-

negative growth were repeatable throughout samplings, and BAC and real-time PCR had 98.9% agreement. It should be emphasized that one sampling may not be sufficient to determine IMI and, therefore, other inflammatory responses should be monitored. Moreover, when PCR methodology is used, aseptic and precise milk sampling procedures are the key for avoiding false-positive amplifications. However, sampling is problematic because in many cases no inflammation signs appear and diagnostics by BAC or DNA methods are expensive. Moreover, although identification of the bacteria is the beginning of the process, it is important only if treatment and prevention of new infections are feasible.

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