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Evaluation of milk cathelicidin for detection of dairy sheep mastitis

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ABSTRACT

Mastitis due to intramammary infections is one of the most detrimental diseases in dairy sheep farming, representing a major cause of reduced milk productions and quality losses. In particular, subclinical mastitis presents significant detection and control problems, and the availability of tools enabling its timely, sensitive, and specific detection is therefore crucial. We have previously demonstrated that cathelicidins, small proteins implicated in the innate immune defense of the host, are specifically released in milk of mastitic animals by both epithelial cells and neutrophils. Here, we describe the development of an ELISA for milk cathelicidin and assess its value against somatic cell counts (SCC) and bacteriological culture for detection of ewe mastitis. Evaluation of the cathelicidin ELISA was carried out on 705 half-udder milk samples from 3 sheep flocks enrolled in a project for improvement of mammary health. Cathelicidin was detected in 35.3% of milk samples (249/705), and its amount increased with rising SCC values. The cathelicidin-negative (n = 456) and cathelicidin-positive (n = 249) sample groups showed a clear separation in relation to SCC, with median values of 149,500 and 3,300,000 cells/mL, respectively. Upon bacteriological culture, 20.6% (145/705) of the milk samples showed microbial growth, with coagulase-negative staphylococci being by far the most frequent finding. A significant proportion of all bacteriologically positive milk samples were positive for cathelicidin (110/145, 75.9%). Given the lack of a reliable gold standard for defining the true disease status, sensitivity (Se) and specificity (Sp) of the cathelicidin ELISA were assessed by latent class analysis against 2 SCC thresholds and against bacteriological culture results. At an SCC threshold of 500,000 cells/mL, Se and Sp were 92.3 and 92.3% for cathelicidin ELISA, 89.0 and 94.9% for SCC, and 39.4 and 93.6% for bacteriological culture, respectively. At an SCC threshold of 1,000,000 cells/mL, Se and Sp were 93.3 and 91.9% for cathelicidin ELISA, 80.0 and 97.1% for SCC, and 39.4 and 93.5% for bacteriology, respectively. In view of the results obtained in this study, the measurement of cathelicidin in milk by ELISA can provide added Se while maintaining a high Sp and may therefore improve detection of subclinical mastitis.
Key words: subclinical mastitis, ewe, small ruminant, ELISA

INTRODUCTION

Mastitis due to IMI is one of the major issues affecting dairy sheep worldwide and negatively affects milk production yields and quality (Bergonier et al., 2003). The difficulties in its detection and the high incidence of subclinical mastitis further exacerbate the problem. Therefore, the availability of tools enabling its timely, sensitive, and specific detection is key for ensuring productivity of the sheep farm. Adding to the clinical evaluation (Marogna et al., 2010), the most widespread approaches for monitoring and assessing flock health are milk SCC and bacteriological culture (Bergonier et al., 2003; Contreras et al., 2007). Nevertheless, both methods present drawbacks in terms of sensitivity (**Se**), specificity (**Sp**), costs, and trained personnel requirements and pose various practical or technical challenges (McDougall et al., 2001; Souza et al., 2012). The diagnostic value of SCC is based on the principle that the number of cells in milk increases when a bacterial infection occurs because of the alveolar influx of neutrophils that are recruited in the context of the inflammatory response. However, numerous noninfectious factors affect SCC, including age, breed, level of genetic selection, lactation stage, parity, milking technique, time of day, feeding, grazing style, udder shape, drought and other environmental stressors, and vaccinations or underlying viral infections (Bergonier et al., 2003; Souza et al., 2012). In addition, the type of causative agent can influence SCC in different ways, in terms of both intensity and duration. Accordingly, the single thresh-

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olds proposed in ewes range from 200,000 to 1.5 million cells/mL, but in most cases, values below 500,000 cells/mL are indicated (Bergonier et al., 2003; Contreras et al., 2007; Souza et al., 2012). Recently, it has been suggested that SCC of <500,000 or >1,000,000 cells/mL should reliably indicate the absence or presence of mastitis, respectively (Berthelot et al., 2005; Fragkou et al., 2014; Gelasakis et al., 2015), although a "gray area" remains between these threshold values with this approach. In addition, by using 500,000 cells/mL as the lower threshold, a higher Se assay may be required to ensure detection of all subclinical mastitis cases.

For a bacteriological culture, a positive result should provide direct evidence that an IMI is present, with the added advantage of identifying the causative microorganism. Nevertheless, milk culturing is known to suffer Se issues due to the intermittent shedding of microorganisms in milk, failure of the microorganism to grow in the culture medium, low multiplicities of infection, and the possible presence of antibiotics due to treatment as well as that of antimicrobial molecules produced by the host immune system itself, such as lysozyme, ferritin, lactoferrin, and antimicrobial peptides (Rainard and Riollet, 2006; Walker et al., 2011; Souza et al., 2012). In addition, some mastitis agents can be more difficult to isolate in culture. Specificity issues also exist due to the possible growth of environmental or commensal bacteria. In addition, possible species or genus misassignments following biochemical identification tests need to be taken into account (Plumed-Ferrer et al., 2013). However, and most importantly in the context of Se, Sp, and practical issues, the bacteriological examination of milk has the main aim of identifying the infectious agents so the correct measures can be implemented for controlling or eliminating the disease at the flock or herd level.

Given the preceding considerations and limitations, a constant search is underway for other indicators of inflammation that would enable more efficient, sensitive, and specific detection of mastitis, to be used either as an alternative to SCC or as a supplement for assessing or improving SCC performance (Viguier et al., 2009; Gurjar et al., 2012). A feasible approach is to use the molecules that are specifically released in milk in response to a microbial infection as indicators or markers. Ideally, to maintain general mastitis screening capabilities, as with SCC, the marker should be a molecule, enzyme, or protein that is suitable for detection with enzymatic assays or immunoassay procedures and is released in milk as a result of inflammation within the mammary gland (Viguier et al., 2009). In keeping with this goal, dedicated biomarker discovery studies, carried out mainly in cows, have reported different proteins that are released in mastitic milk and might therefore have potential for IMI detection (Boehmer et al., 2010; Akerstedt et al., 2011; Ceciliani et al., 2012; Wheeler et al., 2012). Recent studies in sheep by our group have revealed that cathelicidins are among the most prominent and promising molecules for this purpose because they are released abundantly, specifically, and very early in milk following a microbial stimulus. In our studies, their significant and specific increase was seen in milk and in mammary tissues upon natural infection of sheep by *Mycoplasma agalactiae* as well as following experimental infection by *Streptococcus uberis* (Addis et al., 2011, 2013). Indications of cathelicidin release upon IMI have also been provided by other authors in cows (Murakami et al., 2005; Ibeagha-Awemu et al., 2010; Smolenski et al., 2011) and in goats (Brenaut et al., 2014).

Cathelicidins are a family of innate immune effectors that possess multiple functions, including direct antimicrobial activity and potent chemotactic and proinflammatory functions (Zanetti, 2004, 2005; Wiesner and Vilcinskas, 2010). Eight genes are known in sheep (Kościuczuk et al., 2012), and 4 have been demonstrated to be expressed in milk during an inflammatory response, including cathelicidin-1, -2, and -3 (Addis et al., 2013; Scumaci et al., 2015; Pisanu et al., 2015) and the cathelicidin-derived myeloid antimicrobial peptide (Addis et al., 2011). Milk leukocytes contain cathelicidin as the main component of the neutrophil secondary granules, accounting for about 4% of the total protein content (Zanetti et al., 1991). In these cells, the protein is stored preformed, and it is quickly and massively released on demand following a microbial stimulus, often even before the onset of clinical symptoms (Smolenski et al., 2011; Addis et al., 2013). In addition, cathelicidin is strongly associated with the neutrophil extracellular traps released in milk upon IMI (Lippolis et al., 2006; Reinhardt et al., 2013; Pisanu et al., 2015).

Notably, mammary epithelial cells also release cathelicidin and other antimicrobial proteins as one of the first events triggered by the entry of pathogens in the udder, in a rapid, sensitive, and specific manner (Addis et al., 2011, 2013). Cathelicidin release therefore occurs synergistically both in epithelial cells and in milk neutrophils, providing the first line of defense against the microbial invader by acting as a direct antimicrobial agent as well as a potent chemoattractant and proinflammatory mediator (Zanetti, 2004, 2005; Chromek et al., 2006; Nijnik and Hancock, 2009). Following this initial response, a massive influx of immune cells is recalled in the udder, with further degranulation and peaking of cathelicidin in milk (Addis et al., 2013). Therefore, the production of cathelicidin by epithelial cells as a sentinel act in response to microbial invasion makes these proteins even more relevant as early

and sensitive markers of an infectious event. Further advantages of cathelicidin compared with several other inflammation markers are that it is almost undetectable in the milk of healthy animals, unlike other antimicrobial proteins, and its levels increase quickly and significantly only upon exposure to an inflammatory stimulus. Moreover, if an infection is not established, cathelicidin levels rapidly return to baseline, and the same is thought to occur in late and resolving infections (Saad and Ostensson, 1990; Sladek et al., 2005; Smolenski et al., 2011). All these factors, combined with its proteinaceous nature and molecular dimensions, make cathelicidin a perfectly suitable target for the development of different immunoassay formats for diagnostic uses.

Based on these premises, the purpose of this study was to investigate the value of milk cathelicidin for improving Se and Sp of mastitis detection in ewes and to investigate its correlations with SCC and bacteriological culture. For these purposes, we implemented ELISA, one of the most standardized and widespread immunoassay platforms, for the detection of cathelicidin. In this article, we report the results obtained and present a critical discussion of them.

MATERIALS AND METHODS

Flocks

A total of 705 milk samples were collected in 2014 and 2015 from half-udders of Sarda ewes in full lactation belonging to 3 different semi-intensive dairy flocks (A, B, and C) located in northwestern Sardinia (Italy) and certified by the Istituto Zooprofilattico Sperimentale as being *Mycoplasma agalactiae*-free. Milk sampling occurred in the context of a project for improvement of sheep mammary health and reduction of CNS mastitis. Specifically, flock A was sampled once, for a total of 133 sheep. Flock B was sampled 3 times, once in 2014, for a total of 136 sheep, and twice in 2015, for a total of 299 sheep (113 and 186, respectively). Flock C was sampled twice in 2015, for a total of 137 sheep (54 and 83, respectively).

Milk Sampling

Before sampling, teat ends were carefully cleaned and disinfected with a commercial postmilking teat disinfectant with the following formulation: 0.5% chlorhexidine digluconate, 4.0% isopropyl alcohol, 4.0% glycerin, and 0.5% ethoxylated lanolin. The first streams of foremilk were discarded, and then approximately 40 mL of milk was collected aseptically from each teat into sterile 50-mL graduated test tubes. Milk samples were stored at 4 ± 2°C and analyzed for SCC and bacterial culture within 12 h at the National Reference Laboratory for Sheep and Goat Mastopathy.

Somatic Cell Content Determination

Somatic cell counts were determined by the fluoro-opto-electronic method with a Fossomatic FC instrument (Foss Electric, Hillerød, Denmark) according to ISO (2006).

Bacteriological Culture

Milk samples were cultured following standard procedures (Watts, 1990). Ten microliters of milk was streaked onto the surface of 5% sheep blood agar plates. Plates were incubated at 37°C under aerobic conditions and examined for growth after 24 and 48 h. The isolation of 3 or more types of colony was considered to result from contamination. Preliminary characterization of isolates included colony morphology, hemolytic activity, and Gram staining (Gram stain kit, Becton-Dickinson Co., Franklin Lakes, NJ). Gram-positive cocci were screened for catalase activity. Suspected staphylococci were then identified by means of coagulase test (Coagulase Plasma Rabbit with EDTA, BD), production of clumping factor (Staphylase Test, Oxoid, Basingstoke, UK), and biochemical strips (API Staph, bioMérieux, Marcy l'Etoile, France). Gram-positive cocci with no or weak catalase production were identified by means of API 20 Strep (bioMérieux). Gram-negative bacteria were tested for oxidase production (Bactident Oxidase, Merck, Darmstadt, Germany) and then identified by API 20 E or API 20 NE (bioMérieux). All commercial tests were performed according to the manufacturers' instructions.

Pan-Cathelicidin ELISA

The pan-cathelicidin peptide (**PCP**) sequence used to develop the pan-cathelicidin ELISA included a region having identities of 100% with CTHL1_SHEEP, 72% with CHTL2_SHEEP, 70% with CTHL3_SHEEP, 81% with SC51_SHEEP, and 80% with SC52_SHEEP, respectively. All the sequences were retrieved from the nonredundant UniProtKB/SwissProt Database (http://www.uniprot.org/). The PCP was synthesized according to the solid-phase procedure of Merrifield (1963) using a microwave single-mode Discover SPS reactor insert in a peptide synthesizer Liberty (CEM Corporation, Matthews, NC) by fluorenylmethyloxycarbonyl chloride (Fmoc)-based solid-phase peptide synthesis. All Fmoc amino acids were obtained from Novabiochem (Laufelfingen, Switzerland). The crude peptide was purified by reverse-phase HPLC on an HP

1200 (Agilent Technologies, Santa Clara, CA) instrument connected to a UV-Vis detector, as described previously (Mura et al., 2011). The purified peptide mixture was analyzed and characterized on a quadrupole time-of-flight hybrid mass spectrometer equipped with a nano Z-spray source (Waters, Manchester, UK), as described previously (Maddau et al., 2009), after direct injection of the peptide solution into the ion source.

Anti-PCP monoclonal antibodies were obtained from Abbiotec (San Diego, CA) by using PCP as the antigen. The 2 best performing monoclonal antibodies were chosen among the 11 available clones by Western immunoblotting against PCP and against a panel of mastitic milk samples, according to standard procedures (Addis et al., 2011). Milk ELISA for pan-cathelicidin detection was developed in house according to standard procedures (Danowski et al., 2013; Miglio et al., 2013; Trend et al., 2015) in a sandwich format. The detection antibody was conjugated with horseradish peroxidase using a commercial kit (Lightning-Link, Horseradish Peroxidase, Innova Biosciences Ltd., Cambridge, UK). Six bacteriologically negative milk samples with less than 150,000 cells/mL were included in all ELISA plates and used for absorbance normalization. Normalization was done by subtracting from each optical density (**OD**) value measured in the plate, the average OD_{450} + 3 SD of the 6 samples measured in the same plate.

Statistics

According to the Shapiro-Wilk normality test, the data followed a nonnormal distribution. Therefore, a nonparametric Mann-Whitney U-test was applied. Statistical differences for categorical variables were evaluated using the chi-squared test. The best-fit line was obtained by calculating the nonlinear regression (quadratic function) by plotting the SCC versus the normalized OD_{450} (added to a correction factor of 0.1). R^2 was calculated as a parameter of goodness of fit by the software GraphPad Prism version 5.03 for Windows (GraphPad Software, La Jolla, CA). The descriptive statistical analysis (medians, interquartile ranges [IQR], and frequencies) was also carried out using GraphPad Prism. MedCalc Statistical Software version 15.2.2 (MedCalc Software bvba, Ostend, Belgium) was employed to generate receiver operating characteristic (**ROC**) curves and to calculate related values of area under the curve (**AUC**), Se, Sp, confidence intervals (CI), and the optimal cutoff values.

Latent class analysis (**LCA**) for calculation of Se, Sp, and 95% credible intervals (**CrI**) was carried out with BayesianLatentClassModels (BLCM) V. 1.13 (http://www.nandinidendukuri.com/blcm) (Dendukuri and Joseph, 2001; Dendukuri et al., 2009).

According to the latent class model, 3 assumptions need to be satisfied for method validation: the prevalence differs among the populations examined, Se and Sp are constant across subpopulations, and the tests are conditionally independent from each other. If the tests are not conditionally independent, the conditional dependence needs to be modeled.

The requirement for the difference in prevalence among the 3 examined populations was satisfied by using only the results from a sample subset, represented by flock A, flock B at the first sampling, and flock C at the second sampling, for the LCA calculations. The subset data were as follows: flock A, n = 133, prevalence 33.8%; flock B, n = 136, prevalence 14.0%; and flock C: n = 81, prevalence 63.0%. Prevalence was estimated by considering all samples having at least one positive result for ELISA with $OD_{450} > 0.014$, SCC > 500,000 cells/mL, or bacteriological culture positivity as being positive.

The requirement for constant Se and Sp across flocks was satisfied by consecutively excluding each of the populations from the model. An overlap of the CrI of Se and Sp for ELISA with normalized OD_{450} >0.014, SCC >500,000 and bacteriological culture positivity was observed in the 3 sample subsets used for LCA.

With regard to the requirement for conditional independence, because cathelicidin can be released by neutrophils as the main cell type, and neutrophils are also the main cell type found in mastitic milk (Smolenski et al., 2011; Addis et al., 2013; Pisanu et al., 2015), a conditional dependence of cathelicidin ELISA with SCC may exist. To account for this, we evaluated the dependence model between these 2 tests. According to the model diagnostics, the requirement for conditional independence was not satisfied. Therefore, the conditional dependence between cathelicidin and SCC was modeled. Uninformative priors, set as default parameters in the BLCM software, were used for cathelicidin ELISA and SCC because no reliable data were available in the current literature for these 2 parameters. For Se and Sp of bacteriological culture on a single milk sample, detailed and reliable estimates were available for cows (Dohoo et al., 2011). Therefore, the values of Se and Sp and their relative CI, reported by Dohoo et al. (2011) were used as priors, by considering condition E (defined in Dohoo et al., 2011), both for any organism and for CNS.

After definition of the parameters, Gibbs sampling was run. The first 500 iterations were discarded at the burn-in phase, and inferences were made based on the subsequent 10,000. Convergence of the Gibbs sampler

algorithm was verified according to the trace plots for all runs.

RESULTS

Bacteriological Culture Results

All 705 milk samples were subjected to standard bacteriological culture, and 20.6% of them (145/705) were positive. Coagulase-negative staphylococci were the most frequent finding (74.5%, 108/145), followed by *Enterococcus faecalis*, *Lactococcus lactis*, and *Mannheimia haemolytica* (4.1%, 6/145, for each). Among the CNS, *Staphylococcus epidermidis* was by far the most frequent (59.3%, 64/108).

ROC Curve Analysis of Cathelicidin ELISA Results

The presence of cathelicidin in ewe milk was evaluated by a pan-cathelicidin sandwich ELISA. The relationships of ELISA results with SCC and bacteriological culture were assessed by ROC curve analysis. As previously stated, values of SCC <500,000 or >1,000,000 cells/mL should reliably indicate the absence or presence of mastitis, respectively (Berthelot et al., 2005; Fragkou et al., 2014; Gelasakis et al., 2015). Therefore, we plotted separate ROC curves for these 2 SCC thresholds. The curves are reported in Figure 1A and 1B for SCC >500,000 and for SCC >1,000,000 cells/mL, respectively. Figure 1C reports the ROC curve plotted against bacteriological culture results. The evaluation of cathelicidin ELISA against SCC produced very high AUC values for both SCC thresholds (Table 1). In the comparison of cathelicidin ELISA against bacteriological culture, the AUC was also high, but it was lower than both AUCs seen for SCC. ROC curve analysis also enabled defining optimal ELISA cutoff values; that is, those providing the best Se/Sp trade-offs. At these optimal cutoff values, Se and Sp were very high for both SCC thresholds and bacteriological culture, although slightly lower in the latter case.

Of course, it should be highlighted that the Se/Sp trade-off can be balanced to the respective advantage of one or the other parameter when applying the test in practice by selecting different ELISA cutoff values. For evaluation of the test performances, in this work we used the optimal cutoff value of 0.014 as the threshold for defining cathelicidin ELISA-negative and cathelicidin-ELISA-positive samples.

The Inflammation Marker Cathelicidin and SCC

Out of 705 half-udder milk samples examined, 249 (35.3%) were positive for cathelicidin according to the

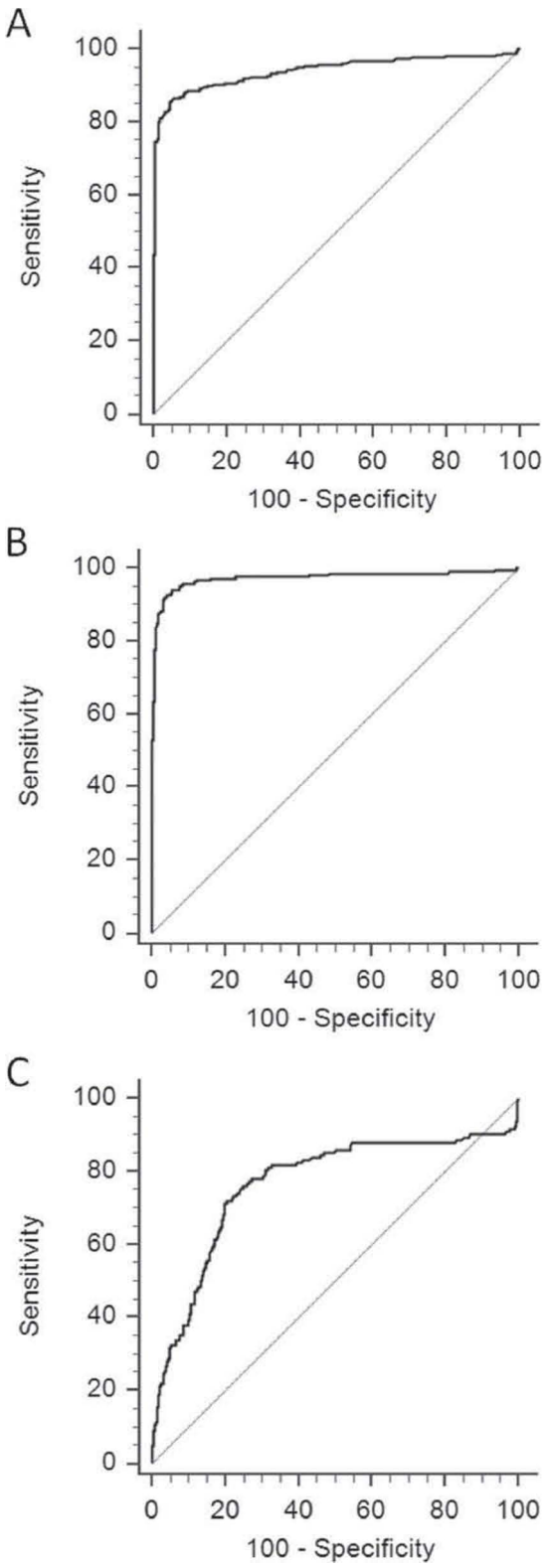

Figure 1. Receiver operating characteristic (ROC) curves illustrating the relationship of cathelicidin with SCC and bacteriological culture. (A) ROC curve plotted against SCC >500,000 cells/mL; (B) ROC curve plotted against SCC >1,000,000 cells/mL; (C) ROC curve plotted against bacteriological culture.

Table 1. Area under the curve (AUC), selectivity (Se), specificity (Sp), and optimal ELISA cutoff values obtained by receiver operating characteristic (ROC) curve analysis when setting SCC or bacteriological culture results as the reference

Item	AUC (95% CI)	Optimal ELISA cutoff value[1]	% Se (95% CI)	% Sp (95% CI)
SCC[2] >500,000	0.94 (0.92–0.95)	0.014	86.2 (81.4–90.1)	94.6 (92.1–96.5)
SCC >1,000,000	0.97 (0.96–0.98)	0.040	93.4 (89.9–96.8)	94.3 (91.8–96.2)
Bacteriological culture	0.76 (0.73–0.79)	0.039	71.1 (62.9–78.4)	80.1 (76.6–83.3)

[1]ELISA in normalized optical density $(OD)_{450}$.
[2]SCC in cells/mL.

optimal normalized OD_{450} cutoff value of 0.014. For assessing the relationship between the amount of cathelicidin present in milk and the SCC, we plotted the normalized OD_{450} cathelicidin ELISA values against the SCC measured for each sample (Figure 2). We subsequently observed an increasing trend of OD_{450} values at increasing SCC (Spearman ρ correlation 0.7335, $P < 0.0001$). Therefore, a direct quantitative relationship existed between the abundance of cathelicidin in milk and the number of immune cells recruited in the udder upon exposure to a microbial stimulus.

The ELISA-negative and ELISA-positive samples showed a statistically significant separation ($P < 0.0001$) according to SCC, as illustrated by the dot plot in Figure 3. SCC median (IQR) values of 149,500 (98,250–265,300) and 3,300,000 (1,225,000–13,831,000) cells/mL were observed, respectively.

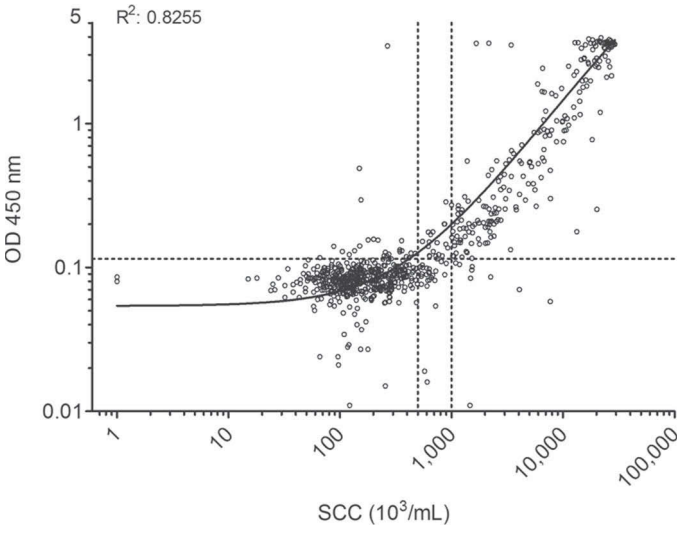

Figure 2. Distribution of cathelicidin positivity according to SCC values and normalized optical density $(OD)_{450}$. Dots indicate the value of all samples (n = 705) along the trend line (quadratic function) plotted by increasing SCC and normalized OD_{450}. A correction factor of 0.1 was applied to normalized OD_{450} values to enable an easier visualization in the plot. Horizontal dotted line = ELISA cutoff value (normalized $OD_{450} \geq 0.014$); vertical dotted lines = SCC thresholds of 500,000 and 1,000,000 cells/mL.

Correlations Among Cathelicidin, Bacteriological Culture, and SCC

To evaluate the distribution of cathelicidin and bacteriological culture results in relation to SCC, we grouped all samples into SCC classes according to Albenzio et al. (2012), as follows: class I, 1,000 to 300,000; class II, 301,000 to 500,000; class III, 501,000 to 1,000,000; class IV, 1,001,000 to 2,000,000; and class V, >2,000,000, to assess the absolute and relative frequency distribution (Figure 4A and 4B). As expected, cathelicidin positivity was mainly concentrated into classes with high SCC. However, cathelicidin positivity was also present in samples with low SCC, with 13.1% positivity in samples with <500,000 cells/mL and 42.3% positivity in samples between 501,000 and 1,000,000 cells/mL. For bacteriological culture, the number of positive samples increased according to increasing SCC and cathelicidin positivity.

Table 2 reports the distribution of cathelicidin-negative and cathelicidin-positive samples according to the identified microorganism, within 3 SCC class groups;

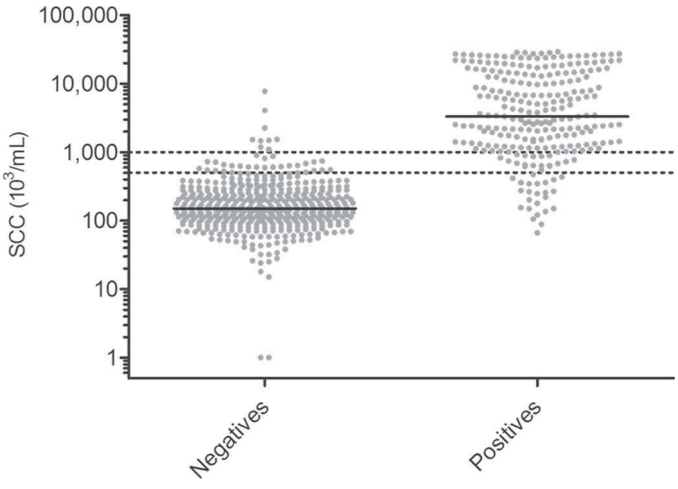

Figure 3. Dot plots indicating the distribution of SCC according to milk cathelicidin (negative or positive). The difference between negative and positive groups was statistically significant ($P < 0.0001$). Horizontal dotted lines indicate the SCC thresholds of 500,000 and 1,000,000 cells/mL.

namely, low, with SCC ≤500,000 cells/mL (classes I and II); intermediate, with SCC between 500,000 and 1,000,000 (class III); and high, with SCC >1,000,000 (classes IV and V). In the low SCC group, 6.0% (27/444) of samples were bacteriologically positive, and 18.5% (5/27) of these were positive for cathelicidin. In the intermediate sample group, 37.0% (17/46) of samples were bacteriologically positive, and 41.2% (7/17) of them were positive for cathelicidin. In the high SCC class, 47.0% (101/215) of samples were bacteriologically positive, and 97.0% (98/101) of them were positive for cathelicidin. Next, CNS were evaluated separately as a result of being the most frequent finding and one of the main etiological agents of subclinical mastitis in ewes (Contreras et al., 2007; Souza et al., 2012). With the focus on CNS species only, 4.3% (19/444) of samples had a CNS positivity in the low SCC group, and 26.3% (5/19) of them were positive for cathelicidin. In the intermediate sample group, 26.1% (12/46) of samples had a CNS positivity, and 41.7% (5/12) of them were positive for cathelicidin. In the high SCC group, 35.9% (77/215) of samples had a CNS positivity, and 98.7% (76/77) of them were positive for cathelicidin.

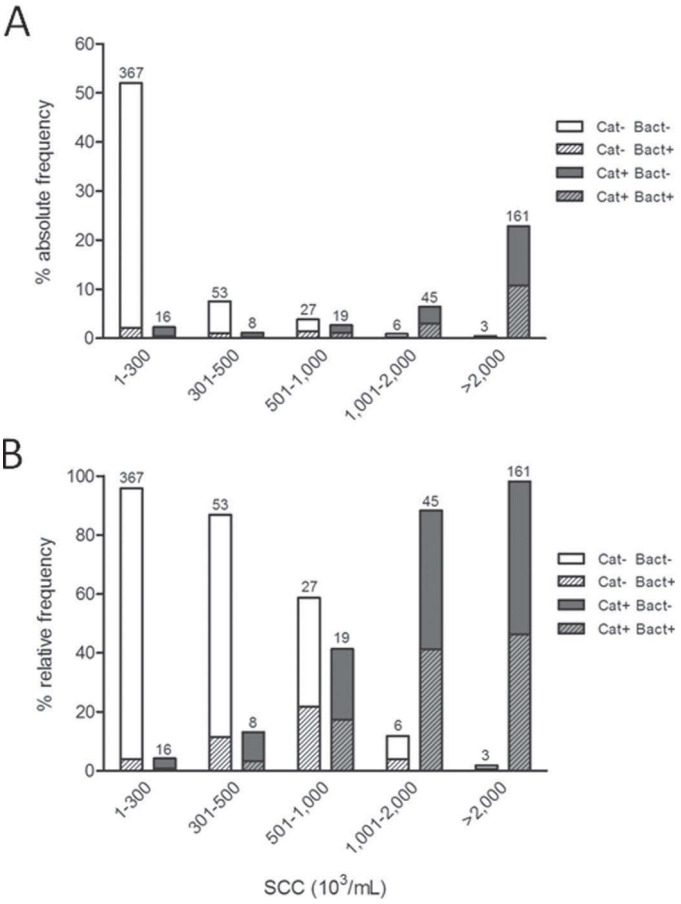

Figure 4. Frequency distribution of the cathelicidin (Cat) status according to SCC classes; n = 705; negative = 456; positive = 249. The number of samples within the class is reported above the bar. Shading indicates the relative proportion of bacteriologically (Bact+) positive samples within the class. (A) Absolute SCC class distribution and (B) relative SCC class distribution, according to Albenzio et al. (2012).

Statistical Evaluation of Test Characteristics

As previously stated, neither SCC nor bacteriological culture is a perfect mastitis test, so a serious bias might occur when estimating Se and Sp of cathelicidin with traditional approaches (Enøe et al., 2000). Therefore, LCA was carried out to evaluate the diagnostic performance of cathelicidin ELISA. In view of the close relationship of cathelicidin with SCC, conditional dependence between these 2 tests was modeled. As for the other test evaluations, LCA estimates were made by using 2 SCC thresholds, > 500,000 cells/mL and >1,000,000 cells/mL. LCA estimates of Se and Sp were also calculated by considering only CNS. Table 3 shows the Se and Sp values obtained for cathelicidin, bacteriological culture, and SCC at the 2 thresholds.

DISCUSSION

Currently, a need exists for improving mastitis monitoring and detection in small ruminants because of the known limitations of SCC and the difficulties in the precise definition of the best diagnostic SCC threshold in ewes and in goats (Souza et al., 2012). This need is especially prominent with regard to the high prevalence of subclinical mastitis and of the significant problems encountered in its detection and control (Contreras et al., 2007; Souza et al., 2012). Therefore, novel methods providing an increase in Se without compromising Sp are to be sought. In addition, the identification of a reliable mastitis indicator, such as an inflammation marker, might also be useful for assessing, reinforcing, or integrating the SCC value in small ruminants. Cathelicidin emerged as a potentially reliable candidate in our previous studies carried out in ewes with natural and experimental infection with various mammary pathogens (Addis et al., 2011, 2013; Pisanu et al., 2015), and it is supported by studies from other authors in cows and in goats (Murakami et al., 2005; Ibeagha-Awemu et al., 2010; Tomasinsig et al., 2010; Smolenski et al., 2011; Brenaut et al., 2014). The value of cathelicidin resides in its well-known role in innate immunity of the mammary gland (Zanetti, 2004, 2005). In fact, it is released early, in a sensitive and specific manner, by epithelial cells and then by activated neutrophils recruited in the udder in response to a microbial stimulus, and it is therefore present in abundant amounts in mastitic milk (Addis et al., 2013; Pisanu et al., 2015). In the

Table 2. Distribution of cathelicidin-negative (Cat−) and cathelicidin-positive (Cat+) samples according to the identified microorganism and to the SCC class

			Low SCC[1]			Intermediate SCC[2]			High SCC[3]		
Group	Bacterial species	Total	Total	Cat−[4]	Cat+[5]	Total	Cat−	Cat+	Total	Cat−	Cat+
CNS	*S. epidermidis*	64	11	9	2	8	4	4	45	—	45
	S. chromogenes	12	2	2	—	1	1	—	9	1	8
	S. warneri	6	2	1	1	2	2	—	2	—	2
	S. haemolyticus	4	—	—	—	—	—	—	4	—	4
	S. auricularis	2	—	—	—	—	—	—	2	—	2
	S. hyicus	2	—	—	—	—	—	—	2	—	2
	S. xylosus	1	1	1	—	—	—	—	—	—	—
	Other CNS	17	3	1	2	1	—	1	13	—	13
	Total CNS	108	19	14	5	12	7	5	77	1	76
Other species	*Enterococcus faecalis*	6	—	—	—	1	—	1	5	—	5
	Lactococcus lactis	6	3	3	—	—	—	—	3	1	2
	Mannheimia haemolytica	6	—	—	—	1	—	1	5	—	5
	Streptococcus uberis	3	—	—	—	—	—	—	3	1	2
	Staphylococcus aureus	2	—	—	—	1	1	—	1	—	1
	Corynebacterium spp.	4	2	2	—	2	2	—	—	—	—
	Other gram-negative bacteria	4	1	1	—	—	—	—	3	—	3
	Multiple pathogens	3	1	1	—	—	—	—	2	—	2
	Mixed flora	3	1	1	—	—	—	—	2	—	2
	Total other species	37	8	8	—	5	3	2	24	2	22
Totals	Positives	145	27	22	5	17	10	7	101	3	98
	Negatives	560	417	398	19	29	17	12	114	6	108
	All samples	705	444	420	24	46	27	19	215	9	206

[1]SCC below 500,000 cells/mL.

[2]SCC between 500,000 and 1,000,000 cells/mL.

[3]SCC above 1,000,000 cells/mL.

[4]Cathelicidin ELISA with normalized optical density $(OD)_{450} < 0.014$.

[5]Cathelicidin ELISA with normalized $OD_{450} \geq 0.014$.

present study, we investigated the suitability of cathelicidin as a marker for improving detection of subclinical mastitis, and we compared results of these assessments with SCC and microbiological findings in half-udder milk samples. To this purpose, we implemented a pan-cathelicidin assay in an ELISA format for measurement of cathelicidin. The ELISA text has a wide range of advantages, being widespread and highly standardized, quantitative, and easily implemented in the laboratory workflow, even within low-budget infrastructures, without requiring dedicated or expensive instrumentation or advanced personnel training. In addition, it can be easily automated and carried out in multiplexing with extremely high throughputs.

We observed a very high correlation of cathelicidin with SCC, as demonstrated by (1) the results of the ROC curve analysis (Figure 1A and 1B), (2) the increase in milk cathelicidin abundance at increasing SCC (Figure 2), and (3) the clustering of cathelicidin-negative and cathelicidin-positive samples with low and high SCC, respectively (Figure 3). Nevertheless, some discrepancies were apparent, with several milk samples

Table 3. Mean and 95% credible intervals (CrI) for selectivity (Se) and specificity (Sp) of cathelicidin, SCC at 2 cells/mL thresholds, and bacteriological culture (BC), for the detection of mastitis according to the latent class analysis (LCA)

	SCC >500,000 cells/mL		SCC >1,000,000 cells/mL	
Item	% Se (95% CrI)	% Sp (95% CrI)	% Se (95% CrI)	% Sp (95% CrI)
All pathogens				
Cathelicidin[1]	92.3 (79.9–99.1)	92.3 (86.6–97.0)	93.3 (81.8–99.1)	91.9 (85.9–97.2)
SCC	89.0 (76.0–97.7)	94.9 (89.7–98.6)	80.0 (65.4–93.8)	97.1 (92.7–99.5)
BC	39.4 (36.0–42.8)	93.6 (91.9–95.0)	39.4 (36.0–42.8)	93.5 (91.9–95.0)
CNS				
Cathelicidin	91.1 (77.1–98.9)	92.1 (86.1–96.9)	91.2 (77.6–98.9)	91.8 (85.7–97.0)
SCC	86.3 (71.5–97.0)	94.4 (89.0–98.1)	77.8 (62.5–92.4)	96.9 (92.3–99.5)
BC	38.8 (34.9–42.7)	96.2 (94.9–97.3)	38.8 (35.0–42.8)	96.2 (94.9–97.3)

[1]ELISA cutoff: normalized optical density $(OD)_{450} \geq 0.014$.

being positive for cathelicidin without evident increases in the SCC. Specifically, the dot plots (Figure 3) clearly show that several cathelicidin-positive samples had very low SCC, and the distribution of cathelicidin positivity within SCC classes (Figure 4) highlights this finding. However, although most of the negative samples fall in class I (<300,000 cells/mL) and most of the positive samples fall in classes above IV (>1,000,000 cells/mL), a slight superimposition exists in class II (from 301,000 to 500,000 cells/mL), and an almost equivalent distribution is present in class III (from 501,000 to 1,000,000 cells/mL). In milk samples falling in SCC classes II and III, therefore, the measurement of cathelicidin might provide significant benefits in terms of increased subclinical mastitis detection by revealing the presence of otherwise undetected inflammatory events. This possibility suggests that, in cases in which the individual half-udder milk sample has a SCC between 300,000 and 1,000,000 cells/mL and cathelicidin is positive, a treatment or intervention decision may be undertaken or bacteriology done to define the etiology of the inflammation. If cathelicidin is negative, then a SCC false positive would be likely, and an unnecessary bacteriological culture, treatment, or culling would be avoided. In fact, several non-IMI causes are known to cause transient SCC increases in sheep without a concurrent release of inflammation markers (Berthelot et al., 2006; Souza et al., 2012), and residual, spent somatic cells may still be found in milk after an inflammation stimulus has ceased. However, further investigations would be advised to better dissect the modes and kinetics of cathelicidin release in milk as well as its relationships with the specific IMI agent. In fact, we cannot rule out that samples with high SCC that are negative for cathelicidin might reflect a short half-life of cathelicidin or some bias in the measurement assay.

All these observations also highlight that, as already known, neither SCC nor bacteriological culture represents a reliable gold standard for evaluating the diagnostic performance of a novel diagnostic test such as the cathelicidin ELISA because their respective biases could lead to significant errors in estimating the Se and Sp of the new test (Enøe et al., 2000). An option that enables circumventing this issue is represented by the LCA approach (Hui and Walter, 1980), which is based on a probabilistic cross-classification of results and provides a better estimate of Se and Sp of a novel diagnostic test in the absence of a true gold standard (Georgiadis et al., 2003). Latent class analysis is increasingly being implemented in the evaluation of methods for detecting mastitis and IMI (Dohoo et al., 2011; Koop et al., 2011; Fosgate et al., 2013; Vissio et al., 2014) For the latent class model to be applicable, 3 assumptions need to be satisfied: the prevalence differs among the populations examined, Se and Sp are constant across subpopulations, and the tests are conditionally independent from each other. Conditional independence among tests implies that when the disease status of a test subject is known, the probability of a test result is unaffected by the outcome of another test (Dawid, 1979; Toft et al., 2005). If the tests are not conditionally independent, conditional dependence needs to be modeled. In this work, the results clearly indicated that cathelicidin and SCC are strongly related. Therefore, LCA was applied by taking into account the conditional dependence of cathelicidin and SCC.

As a result, cathelicidin always showed a superior Se compared with SCC. In addition, Sp of cathelicidin was also high, especially in the comparison with SCC >500,000 cells/mL (Table 3). This finding is especially relevant because it may provide significant advantages in the detection of subclinical mastitis. As expected, Se of bacteriological culture was always the lowest. Notably, Sp of cathelicidin and SCC was very close to Sp of bacteriological culture, and both cathelicidin and SCC outperformed bacteriological culture when 1,000,000 cells/mL was the diagnostic SCC threshold.

Although LCA compensates for the absence of a gold standard test, cathelicidin Sp might still be slightly underestimated. In fact, because cathelicidin has a higher Se than SCC and bacteriological culture, several samples will produce a positive result for cathelicidin and negative results for both SCC and bacteriological culture. These results will be classified as false positives, but they may actually be false negatives of the other 2, less sensitive, tests. Further studies may be needed to better define the ELISA cathelicidin Sp, which might be even higher than the one observed upon LCA against SCC and bacteriological culture.

The performance of cathelicidin as a mastitis marker, its proteinaceous nature, and its strict correlation with inflammation (Frohm et al., 1997; Zanetti, 2004, 2005; Smolenski et al., 2007; Nijnik and Hancock, 2009; Addis et al., 2013) support the implementation of other immunoassay platforms for its detection. In most cases, the availability of quality antibodies is a limiting factor in developing sensitive, specific, and reproducible laboratory or field immunoassay tests (Baker, 2015; Bradbury and Plückthun, 2015). Here, the availability of specific monoclonal antibodies against a pan-cathelicidin domain opens the way to its exploitation in a wide range of immunoassay formats enabling pen-side and on-line measurement of the marker.

As a final consideration, in view of the nature of the cathelicidin marker and its involvement in innate immunity processes, the possible implementation in other dairy ruminants, such as cows, goats, or water buffaloes, seems to represent a tangible perspective. Particularly

in the case of goats, for which the SCC problem is well known and has an even greater impact than in sheep, the possible applicability of this marker for mastitis detection deserves further investigation.

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