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Relationship between milk cathelicidin abundance and microbiologic culture in clinical mastitis

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ABSTRACT

The availability of reliable tools to enable the sensitive and specific detection of mastitis in dairy cows can assist in developing control strategies and promote the more rational use of antibiotics. We have developed a milk cathelicidin ELISA that shows high sensitivity and specificity for dairy cow mastitis, based on latent class analysis. In this study, we investigated the effect of microbial agents on cathelicidin abundance in the milk of cows with clinical mastitis. We subjected 535 quarter milk samples (435 from quarters showing signs of clinical mastitis and 100 from healthy quarters as a control) to milk cathelicidin ELISA, somatic cell count (SCC), and microbiologic culture. Of the 435 clinical mastitis samples, 431 (99.08%) were positive for cathelicidin, 424 (97.47%) had SCC >200,000 cells/mL, and 376 (86.44%) were culture-positive. Of the 59 culture-negative samples, 58 (98.30%) were positive for cathelicidin and 55 (93.22%) had SCC >200,000 cells/mL. The abundance of cathelicidin and the extent of SCC increase depended on the causative agent: *Streptococcus agalactiae* and coagulase-negative staphylococci showed the highest and lowest changes, respectively. We also observed differences in behavior between the 2 markers depending on the pathogen: *Streptococcus agalactiae* induced the highest cathelicidin abundance, and *Serratia* spp. induced the highest SCC. Nevertheless, the different ability of microorganisms to induce cathelicidin release in milk did not compromise its value as a mastitis marker, given its higher sensitivity compared to SCC or microbiologic culture. All 100 negative control samples (collected from healthy quarters with SCC <100,000 cells/mL and culture-negative) were also negative for cathelicidin, corresponding to 100% specificity in the evaluated sample cohort. This study confirmed the value of the milk cathelicidin ELISA for detecting bovine mastitis, and highlighted the influence of mastitis-causing microorganisms on cathelicidin abundance. This influence did not compromise diagnostic performance; instead, it may have better reflected disease severity and evolution than SCC.
Key words: clinical mastitis, cathelicidin, dairy cow, ELISA, intramammary infection

INTRODUCTION

Sensitive and specific tools for the detection of mastitis in dairy cows are needed to reduce its economic impact. Effective diagnostic methods can lead to faster and more efficient control of mastitis in dairy ruminants and promote the more responsible use of antibiotic therapy. At present, mastitis monitoring is based mainly on SCC, but the immunologic detection of inflammation markers may be a valuable alternative, providing practical benefits and enabling the improvement of diagnostic performance (Viguier et al., 2009; Ceciliani et al., 2012). One of the most promising inflammation markers is cathelicidin, a class of proteins with antimicrobial activity and potent proinflammatory and chemotactic functions (Zanetti, 2004, 2005). Cathelicidin is released in milk, first by epithelial cells upon contact with an invading pathogen, and then by activated PMNL retrieved into milk by degranulation and the formation of neutrophil extracellular traps (Addis et al., 2013; Pisanu et al., 2015).

We have recently developed a high-performing milk pan-cathelicidin ELISA that shows elevated sensitivity and specificity for dairy cow mastitis relative to

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SCC and microbial culture (Addis et al., 2016b). We estimated the assay's performance using latent class analysis in a Bayesian framework (that is, without referring to a gold standard for true disease status). Other authors have observed the release of cathelicidin in milk in natural and experimental infections (Smolenski et al., 2011; Wheeler et al., 2012; Reinhardt et al., 2013). However, in their pioneering study, Smolenski et al. (2011) reported a lack of cathelicidin reactivity in about 25% of mastitis milk samples using immunoblotting analysis. They hypothesized, among other causes, that different microbial infections could induce different levels of cathelicidin release, compromising its diagnostic sensitivity, but the analytical technique and antibodies they used may also have influenced the sensitivity and specificity of pan-cathelicidin detection.

We identified the need to assess the influence of microbial agents on cathelicidin abundance as measured by our pan-cathelicidin ELISA, and to evaluate their effect on the diagnostic value of the ELISA compared to SCC. We investigated the relationships among cathelicidin, SCC, and culture results by considering the presence of clearly detectable clinical signs (clinical mastitis) as the gold standard for true disease status.

MATERIALS AND METHODS

Milk Sample Collection, Microbiologic Culture, and SCC

Milk samples were obtained from 16 dairy herds between March and May 2016. The farms were required to have trained technicians able to detect clinical mastitis; use an adequate milking routine that included forestripping to detect abnormal milk; and sample any case of clinical mastitis and freeze quarter milk immediately. Clinical mastitis was defined as the presence of udder or quarter swelling; heat, hardness, redness, or pain; or visible alterations in the milk, such as watery appearance, flakes, clots, or pus. Sample collection was carried out after careful cleaning and disinfection of teat ends with chlorhexidine-embedded disposable towels. For each sample, approximately 10 mL of milk was collected into a sterile vial after discharging the first stream. Samples were stored at −20°C and sent to the laboratory weekly for microbiological analysis and SCC determination. A total of 535 quarter milk samples were included in the sample cohort: 435 from cows with clinical mastitis, and 100 from clinically healthy cows with negative microbiologic culture and SCC <100,000 cells/mL.

At the laboratory of the Department of Veterinary Medicine, University of Milan, milk samples were thawed at room temperature, and 100 μL was spread onto blood agar plates (5% defibrinated sheep blood). Plates were incubated aerobically at 37°C and evaluated after 24 and 48 h. Microbiologic culture was carried out following guidelines from the National Mastitis Council (1999), with the exception of a 10× increase in inoculum volume. Provisional identification of colonies was based on Gram stain, morphology, and hemolysis patterns. Representative colonies were subcultured on blood agar plates and incubated again at 37°C for 24 h. Gram-positive, catalase-positive cocci were evaluated using the coagulase tube test to differentiate *Staphylococcus aureus* from other staphylococci. Gram-positive, catalase-negative cocci were identified as streptococci and differentiated by further biochemical tests (growth in 6.5% NaCl broth, esculin hydrolysis, fermentation of ribose, sorbitol, sucrose, and inulin) and by the Christie, Atkins, and Munch-Petersen test. Gram-negative bacteria were identified using Gram stain characteristics, oxidase reaction, and colony morphology on MacConkey's agar (Oxoid, Basingstoke, UK) and Eosin Methylene Blue agar (Laboratorios Conda, Madrid, Spain). Microorganisms other than bacteria were confirmed by microscopic appearance. When the growth of 2 different microorganisms was detected (25 samples of 376, 6.65%), the case was classified as a mixed infection. Samples with growth of 3 or more pathogens were considered contaminated and were not included in the study. We determined SCC using an automated somatic cell counter (Bentley Somacount 150; Bentley Instruments, Chaska, MN).

Pan-Cathelicidin ELISA

Cathelicidin abundance in milk was assessed at the Porto Conte Ricerche laboratories with a pan-cathelicidin sandwich ELISA based on 2 monoclonal antibodies developed against a pan-cathelicidin domain, as previously described (Addis et al., 2016a,b). At the end of the assay, the optical density at 450 nm (**OD450**) value of all samples was normalized against internal controls. To this aim, 6 culture-negative samples with <50,000 cells/mL were included in all ELISA plates, and their average OD450 (+3 SD) was subtracted from all OD450 values to obtain the normalized OD450 value (**NOD450**). To assess cathelicidin abundance, each milk sample was tested in duplicate aliquots of 10 and 1 μL. When the results of the 10-μL aliquot provided a NOD450 higher than 2.5, we considered the NOD of the 1-μL aliquot multiplied by 10. Finally, to enable comparison and logarithmic visualization, we added a correction factor of 0.1 to all NOD450 values to obtain the adjusted OD450 value (**AOD450**).