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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

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intensity and duration. Accordingly, the single thresholds 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 inva-