Cathelicidin production and release by mammary epithelial cells during infectious mastitis

Tiziana Cubeddu[a], Carla Cacciotto[b], Salvatore Pisanu[b], Vittorio Tedde[b], Alberto Alberti[a], Marco Pittau[a], Simone Dore[c], Agnese Cannas[c], Sergio Uzzau[b,d], Stefano Rocca[a], Maria Filippa Addis[b,e,1]

[a] Università degli Studi di Sassari, Dipartimento di Medicina Veterinaria, Via Vienna 2, 07100 Sassari, Italy

[b] Porto Conte Ricerche, SP 55 Porto Conte/Capo Caccia, Km 8.400, Loc. Tramariglio, 07041 Alghero, Italy

[c] C.Re.N.M.O.C., Centro di Referenza Nazionale per le Mastopatie degli Ovini e dei Caprini – Istituto Zooprofilattico Sperimentale della Sardegna, Via Duca degli Abruzzi 8, 07100 Sassari, Italy

[d] Università degli Studi di Sassari, Dipartimento di Scienze Biomediche, Viale S. Pietro 43/B, 07100 Sassari, Italy

[e] Università degli Studi di Milano, Dipartimento di Medicina Veterinaria, Via Celoria 10, 20133 Milano, Italy

[1] Corresponding author at: Università degli Studi di Milano, Dipartimento di Medicina Veterinaria, Via Celoria 10, 20133 Milano, Italy. E-mail address: filippa.addis@unimi.it (M.F. Addis).

Abstract

Cathelicidins are well-characterized antimicrobial peptides (AMPs) that are present in significant amounts in mastitic milk. Neutrophils are believed to be the main producers of these AMPs, while the role of mammary epithelial cells (MECs) in their production and release is still unclear. In this work, cathelicidin production patterns were investigated in mammary tissues of ewes infected by Staphylococcus aureus, Streptococcus uberis, or Mycoplasma agalactiae, with a combined approach including immunohistochemistry, immune-colocalization, and fluorescent in situ hybridization. Our results confirm that MECs produce and release cathelicidins in response to different mastitis pathogens. As opposed to neutrophils, however, MECs do not seem to store the preformed protein precursor in their cytoplasm but appear to synthesize and release it only upon exposure to the microorganisms. Cathelicidin production by MECs appears to occur before leukocyte influx in the milk, suggesting a role for these cells in the initial response of the mammary epithelium to microbial infection. Once in the milk, infiltrating neutrophils release massive amounts of cathelicidin by degranulation and production of neutrophil extracellular traps, acting as the main contributor for cathelicidin abundance in mastitic milk. Taken together, our results support the active contribution of MECs to cathelicidin production and release and reinforce the value of cathelicidins as sensitive and pathogen-independent mastitis markers.

Keywords: Cathelicidin, Mammary epithelial cells, Mastitis, Milk, Immunomicroscopy, Antimicrobial peptides

1. Introduction

Mastitis is an inflammatory disease of the mammary gland mainly caused by an intramammary infection (IMI). In ewes, the most common etiological agents are gram-positive bacteria, including different environmental staphylococci and streptococci and contagious pathogens such as Staphylococcus aureus (S. aureus) and Mycoplasma agalactiae (M. agalactiae) (Dore et al., 2016). In the mammary gland, recognition of pathogen-associated molecular patterns (PAMPs) by pattern-recognition receptors (PRRs) triggers the release of effectors including antimicrobial peptides (AMPs) such as cathelicidins. The cathelicidin family of AMPs is quite widespread in nature, and it was initially identified in the secondary granules of leukocytes where it is stored as a precursor. Upon stimulation, cathelicidins are released outside the cell and cleaved by proteases to produce the C-terminal antimicrobial peptide (Zanetti et al., 1995).

The role of mammary epithelial cells (MECs) in the release of AMPs is not clearly defined, and conflicting data have been published in the latest years. It was demonstrated that murine lactating lobular units produce cathelicidins (Murakami et al., 2005). Tomasinsig and coworkers also demonstrated the constitutive expression of cathelicidin mRNA in the mammary gland of healthy cows, but they did not detect up-regulation of gene transcription during mastitis (Tomasinsig et al., 2010). Recently, a higher level of cathelicidin mRNA was detected in healthy tissues when compared to Staphylococci infected tissues (Kościuczuk et al., 2014). Accordingly, Smolensky and co-workers suggested that cathelicidins found in mastitic milk are derived by degranulating neutrophils recruited in the inflamed mammary alveolus (Smolensky et al., 2011). In previous studies conducted by our group, several AMPs and immunity-related proteins were identified among the
proteins that increase in milk upon infection (Addis et al., 2011, 2013). In preliminary IHC and immune co-localization experiments, cathelicidin positivity was observed in MECs of ewes experimentally infected with S. uberis (Addis et al., 2013), indicating the ability of these cells to produce and release cathelicidins.

In this study, cathelicidin production and release patterns were further investigated in mammary gland tissues of ewes naturally infected by S. aureus, S. uberis, and M. agalactiae. To this aim, different antibody-based techniques, including IHC, immune co-localization by confocal immunomicroscopy, and bacterial localization by fluorescent in-situ hybridization were applied. Results demonstrated that MECs produce and release cathelicidin in response to bacterial infections.

2. Materials and methods

2.1. Animal selection and tissue collection

In the context of a screening program for improvement of mammary health in sheep, several animals from different farms with mastitis problems due to S. aureus, S. uberis, or M. agalactiae were identified by physical examination, bacteriological tests, pan-cathelicidin ELISA, or western immunoblotting. When animals were selected for culling, tissues were retrieved at the slaughterhouse and brought to the laboratory, where milk and tissue samples were collected. Tissues were fixed in 10% neutral-buffered formalin, processed through graded concentrations of alcohol and xylene, and embedded in paraffin wax with a HISTO-PRO 200 vacuum tissue processor (Histo-Line, Milan) for histopathological and immunofluorescence examination, as previously described (Cacciotto et al., 2016).

2.2. Microbial culture

Milk samples collection and bacteriological examination of milk were carried out following standard procedures, according to the standards of the National Mastitis Council (1999), as previously described (Watts, 1990; Addis et al., 2016a,b). Briefly, 10 μL of milk were streaked onto 5% sheep blood agar plates and aerobically incubated at 37 °C. After 24 h and 48 h plates were examined and colony forming units (CFU) were counted. Isolated bacteria were classified for colony morphology, hemolytic activity, and Gram staining (Gram stain kit, Becton-Dickinson Co., Franklin Lakes, NJ). Gram-positive cocci were screened for catalase activity. BBL Coagulase Plasma Rabbit (with EDTA, BD), Staphylase Test kit (Oxoid, Thermo Scientific), and API Staph (bioMérieux) were used to identify suspected staphylococci. All commercial tests were performed according to the manufacturers' instructions. For M. agalactiae isolation, milk was plated onto blood agar base supplemented with 20% heat-inactivated horse serum and 500 μg/ mL ampicillin and plates were controlled up to 14 days, as previously described (Cacciotto et al., 2010, 2013). Mycoplasma species was confirmed by means of specific FS1/FS2 PCR, as previously described (Tola et al., 1996).

2.3. Immunoassays

The presence of cathelicidin in milk samples was assessed with an in-house pan-cathelicidin sandwich ELISA developed in our laboratories, as previously described (Addis et al., 2016a,b). For immunohistochemical analysis, 3 μm sections of mammary tissues were used. Prior to incubation with antibodies, tissue sections were dewaxed in xylene, rehydrated through graded alcohol series, and incubated with 0.3% hydrogen peroxide in PBS for 30 min to block endogenous peroxidases. After treatments, sections were incubated with a rabbit anti-CAMP (anti-cathelicidin, Sigma-Aldrich), diluted 1:1500. Reaction was revealed with the ImmPRESS™ Universal Antibody (anti-mouse IgG/anti-rabbit IgG, Peroxidase) (Vector) and ImmPACT™ DAB Peroxidase (Vector) following the manufacturer instructions. Nuclei was lightly counterstained with hematoxylin. IHC images were acquired with a Nikon Eclipse 80i microscope equipped with a Nikon DS-Fi1 camera (Nikon Instruments Inc.).

2.4. Co-localization assays

2.4.1. Streptococcus uberis, Staphylococcus aureus and cathelicidin

An integrated approach of immunofluorescence (IF) and fluorescent in situ hybridization (FISH) was used to co-localize cathelicidin with S. uberis and S. aureus. Three μm sections of mammary tissues were mounted on Superfrost™ slides (Thermo Scientific), dewaxed in xylene, rehydrated through graded alcohol series. Upon digestion with pepsin for 30 min (0.8% in 0.2 N HCl, 37 °C in PBS), sections were postfixed with an alcohol ascending scale (from 50% to 98%) and air dried. Sections were incubated for 2 h at 55 °C with 200 μL of prehybridization solution (50% of Hybridization Solution II [43% deionized formamide, 7% nuclease-free water]; Fluka). Then, slides were incubated with 200 μL of hybridization solution containing alternatively 1 nM biotynilated probes for S. uberis (5′-BtnGCGAAGTGGGACATAAAGTTA-3′) or S. aureus (5′-Btn-AGGTA TGCAATTTGATCGTGGTTATCAATCACCGTATATGGTTACTGATTC-3′), denatured for 8 min at 98 °C, and incubated for 16 h at 55 °C in a ThermoBrite StatSpin. Tissue sections were washed at room temperature for 5 min with 2X, 1X, 0.5X, and 0.1X SSPE (1X SSPE is 0.18 M NaCl, 10 mM NaH_2PO_4, and 1 mM EDTA [pH7.7]), and with PBS-T. Signals were revealed by incubating slides with streptavidin-Alexa Fluor 555 conjugate (Invitrogen) for 45 min at room temperature in the dark. Slides were then washed in PBS-T and processed as follows to detect cathelicidin. Non-specific sites were blocked with 2% BSA in PBS-T for 1 h at 37 °C and incubated overnight at 4 °C with anti-

CAMP, rabbit polyclonal antibody (Sigma Aldrich HPA029874), diluted 1:1500. After, the tissue sections were washed with PBS-T, incubated with a broad spectrum biotynilated antibody (Invitrogen) for 15 min, and the signal were revealed by incubating with streptavidin-Alexa Fluor 488 conjugate (Invitrogen), for 45 min at room temperature in the dark. Finally, after washing with Milli-Q water and nuclei counterstaining with Hoechst, slides were washed and mounted with Fluoromount™ Aqueous Mounting Medium (Sigma Aldrich).

2.4.2. Mycoplasma agalactiae and cathelicidin

To co-localize cathelicidin with M. agalactiae, a double immunofluorescence stain was performed. Three μm tissue sections were mounted on Superfrost™ slides (Thermo Scientific). Dewaxing, rehydration, and antigen retrieval were conducted in Dewax and HIER (heat-induced epitope retrieval) buffer L pH 6.2 (Thermo Scientific) at 98 °C for 20 min by means of an Electric Vegetable Steamer. Slides were then chilled in milli-Q water for 20 min and washed three times in PBST. Tissues were blocked with Normal Horse Serum (NHS) (VECTOR) for 30 min and then incubated overnight at 4 °C with a rabbit hyperimmune serum raised against M. agalactiae rP48 (Rosati et al., 2000; Cacciotto et al., 2016). On the following day, the tissue sections were washed with PBS-T, incubated with a broad spectrum biotinylated antibody (Invitrogen) for 15 min, and the signal were revealed by incubating with streptavidin-Alexa Fluor 555 conjugate (Invitrogen), for 45 min at room temperature in the dark. After washing 3 times with PBS, the sections were subjected to a new non-specific blocking step with NHS and incubated overnight at 4 °C with an anti-CAMP, rabbit polyclonal antibody (Sigma Aldrich HPA029874), diluted 1:1500. The signal was revealed as described above using streptavidin- Alexa Fluor 488 conjugate (Invitrogen).

2.4.3. Cathelicidin and cytokeratin

To co-localize cathelicidin and cytokeratin, tissue sections were subjected to a double immunofluorescence, as described above, with slight modifications. The anti-CAMP, rabbit polyclonal antibody (Sigma Aldrich HPA029874) was used in the first incubation and it was revealed with streptavidin-Alexa Fluor 555 conjugate (Invitrogen). In the second round, tissues were incubated with α-cytokeratin (peptide 18) FITC conjugated (Sigma Aldrich F4772) diluted 1:100. Nuclei were counterstained with Hoechst, washed with Milli-Q water, and slides were mounted with Fluoromount™ Aqueous Mounting Medium (Sigma Aldrich).

2.4.4. Confocal microscopy

Images were acquired with a Leica TCS SP5 confocal microscope (Leica Microsystems, Germany) and processed with the LAS AF Lite (Leica Microsystems) software.

3. Results and discussion

A detailed knowledge of the events taking place in mammary gland tissues during microbial infection might contribute to the development of alternative and more efficient strategies for detecting and contrasting IMI and mastitis in dairy ruminants. The description of novel innate immune defense mechanisms of the mammary gland such as the release of neutrophil extracellular traps (NETs) and related bactericidal factors brought AMPs into the spotlight (Brinkmann et al., 2004; Cacciotto et al., 2016). Among AMPs, cathelicidins have been the subject of several studies, and their release in milk is well established (Smolenski et al., 2011; Pisanu et al., 2015; Mudaliar et al., 2016). Milk cathelicidin was recently proposed as a tool for mastitis detection and monitoring, and a dedicated ELISA with high sensitivity and specificity for both sheep and cow mastitis was developed (Addis et al., 2016a; Addis et al., 2016b). It is well known that cathelicidins are released by neutrophils both through degranulation and NETosis, but the role of MECs in their production and secretion was unclear. In this study on ewes with IMI by three different mastitis agents (S. aureus, S. uberis, and M. agalactiae) we demonstrate that MECs produce and release cathelicidin in vivo during a natural infection.

The localization of cathelicidin in the mammary gland epithelium was first investigated by IHC (Fig. 1). Microscopic analysis revealed the presence of advanced acute inflammatory lesions in all mammary tissues of animals with IMI. Histopathological signs of mastitis included neutrophil infiltration, local degeneration, and desquamation of the secreting epithelial cells (Fig. 1). In infected tissues, cathelicidin reactivity was clearly visible in the epithelium lining the ducts, following a granular pattern. As expected, in IMI by S. aureus the epithelial lining was severely compromised, but anatomically distinct alveolar structures devoid of infiltrating leukocytes were still present (Fig. 1, column A, first row). There, cathelicidin was located in the epithelial cells surrounding the ducts, whereas the lumen was clear. In portions with severe inflammatory lesions (Fig. 1, column B, first row), the epithelium was still positive for cathelicidin, although with a less intense and more diffuse signal; concurrently, cathelicidin was abundantly detected in the alveolar lumen, indicating its release in the milk. As expected, infiltrating neutrophils were strongly positive for cathelicidin. The anatomical structure of S. uberis infected tissues was generally more conserved (Fig. 1, second row). In areas without leukocyte infiltration, epithelial cells lining the ducts were strongly positive for cathelicidin (Fig. 1, column A, second row). On the other hand, infiltration of inflammatory cells could be seen in fields with more severe lesions, with neutrophils being the most abundant cell type. Here, cathelicidin accumulated in the alveolar lumen, both in extracellular aggregates and within

neutrophil granules (Fig. 1, column B, second row). Finally, IMI by M. agalactiae was more detrimental for the mammary tissue, causing parenchyma thickening and fibrosis (Fig. 1, third row). Despite a general disruption of tissue architecture, some anatomically conserved areas could still be identified. There, cathelicidin was located in the epithelial cells surrounding the alveolar lumen (Fig. 1, column A, third row). In sections with more evident inflammatory damages, cathelicidin was mainly located in alveoli, both in neutrophil granules and in scattered areas of reactivity within the lumen (Fig. 1, column B, third row). Notably, residual MECs were still positive for cathelicidin. Macroscopically and microscopically normal tissues were always negative (Fig. 1, bottom row), although occasional traces of reactivity could be seen.

To confirm its MEC origin, cathelicidin was co-localized with an epithelial cytokeratin (Fig. 2). In tissues with IMI, cytokeratin and cathelicidin signals overlapped, with some differences depending on lesion severity. Specifically, in anatomically intact alveoli of S. aureus infected tissues, the two signals overlapped almost completely (Fig. 2A, first row). When local degeneration of the epithelial structure occurred, cathelicidin was located in neutrophils (Fig. 2B, first row). In areas showing acute inflammatory lesions, residual intact MECs still displayed positivity for cathelicidin. Comparable results were obtained in S. uberis infected mammary tissues, where epithelial cytokeratin and cathelicidin signals did also overlap in areas with conserved epithelial structures (Fig. 2A, second row). Moreover, droplet formations in the alveolar lumen were positive for both markers, indicating an epithelial origin with a possible apocrine derivation. In heavily inflamed areas, cathelicidin was mainly located in neutrophils, although cathelicidin was still detectable in residual epithelial cells (Fig. 2B, second row). A very similar scenario was seen in M. agalactiae infected tissues; despite severe alteration of the epithelium, cathelicidin was still detectable in intact MECs (Fig. 2A, third row). Moreover, cathelicidin was observed in the alveolar lumen within neutrophils and in aggregates of cellular debris positive to epithelial cytokeratin (Fig. 2B, third row). Negative control tissues were strongly positive for the epithelial marker and only faint cathelicidin signals were occasionally seen (Fig. 2A and B, bottom row).

Cathelicidin was then co-localized with the different microorganisms (Fig. 3). S. aureus was detected ubiquitously in infected tissues (Fig. 3A and B, first row). In areas devoid of neutrophil infiltration, epithelial cathelicidin co-localized with bacteria (Fig. 3A, first row). On the other hand, S. aureus was seldom detected in the desquamated epithelium of acutely inflamed areas, while cathelicidin was present in neutrophils (Fig. 3B, first row). On the contrary, S. uberis was always observed in correspondence with epithelial cells (Fig. 3A and 3B, second row). In areas with a normal tissue architecture, cathelicidin strongly overlapped with S. uberis. Moreover, the two signals co-

localized also in the alveoli, associated to structures resembling apocrine secretions (Fig. 3A, second row). Where tissue damage was more severe, bacteria and cathelicidin signals did not overlap, the latter being mostly located in the ducts and associated to neutrophils or aggregated in other structures (Fig. 3B, second row). M. agalactiae was mainly detected on the surface of MECs, as expected (Fig. 3A and B). Also in this case, cathelicidin and bacteria co-localized in morphologically normal areas. Moreover, as seen for S. uberis, cathelicidin and M. agalactiae were detected together in the lumen associated to apocrine secretion structures (Fig. 3A, third row). In more damaged tissue areas, mycoplasmas were less easily detected, and the cathelicidin signal was observed both in the epithelium and in the lumen. Here, cathelicidin was localized in neutrophils and in other extracellular formations (Fig. 3B, third row). Negative tissues did not show significant reactivity signals (Fig. 3A and B, bottom rows).

Collectively, results indicate common cathelicidin production and release patterns for all IMI agents. Healthy udder tissues are negative to cathelicidin, while the protein is clearly present in MECs of animals with IMI. In infected tissues, cathelicidin co-localizes with the epithelial cell marker, supporting the production of cathelicidin by MECs. In addition, cathelicidin signals colocalize with bacterial signals, suggesting that synthesis of cathelicidin in MECs is triggered by interaction with microorganisms. On the contrary, in areas with massive desquamation of the epithelium and leukocyte infiltration, cathelicidin is released in the alveolar lumen. Here, pathogens co-localize only partially with epithelial cathelicidin in the few residual MECs while, as expected, infiltrating neutrophils are strongly positive for cathelicidin.

The absence of cathelicidin in bacteria-free tissue areas in both ewes with and without IMI indicates that MECs do not constitutively produce and store preformed cathelicidin in their cytoplasm but produce and release the protein only upon stimulation. Then, in addition to direct antimicrobial activity, cathelicidin released by MECs can act as a chemoattractant, contributing to leukocyte influx in the infected area. Once in the alveolar lumen, recalled cells release massive amounts of cathelicidin and form NETs (Thammavongsa et al., 2013; Pisanu et al., 2015; Cacciotto et al., 2016).

The apparent contrast between our results and those reported by others may be explained when considering that a transcriptomics-based approach such as real-time RT-PCR might not be best suited for estimating cathelicidin expression. In fact, some authors reported similar or even higher levels of cathelicidin mRNA in mammary gland tissues before infection when compared to the same tissues after infection (Tomasinsig et al., 2010 Kościuczuk et al., 2014); nevertheless, we did not observe constitutive production of cathelicidin protein products in bacteriologically negative tissues. Notably, Chromek et al. (2006) observed that cathelicidin mRNA is pre-synthesized in human renal epithelial

cells. Only exposure to pathogens triggers its translation, leading to synthesis and secretion of cathelicidin with a concurrent time-related decrease in mRNA levels probably due to degradation. Further studies are clearly needed to test this hypothesis, but it can be speculated that, similarly to what observed by these authors, MECs synthesize and store cathelicidin mRNA but protein synthesis takes place only upon microbial stimulation. According to what observed in the human kidney, the decrease in cathelicidin mRNA seen in infected tissues might be due to its prompt degradation after protein synthesis (Chromek et al., 2006; Tomasinsig et al., 2010; Kościuczuk et al., 2014).

In conclusion, this study highlights an active role of MECs during immune response to udder pathogens and reinforces the usefulness of cathelicidin as a sensitive and specific mastitis marker.

Acknowledgements

This work was supported by Sardegna Ricerche - Science and Technology Park of Sardinia, grant program “art. 26_2015” to Porto Conte Ricerche.

Figures

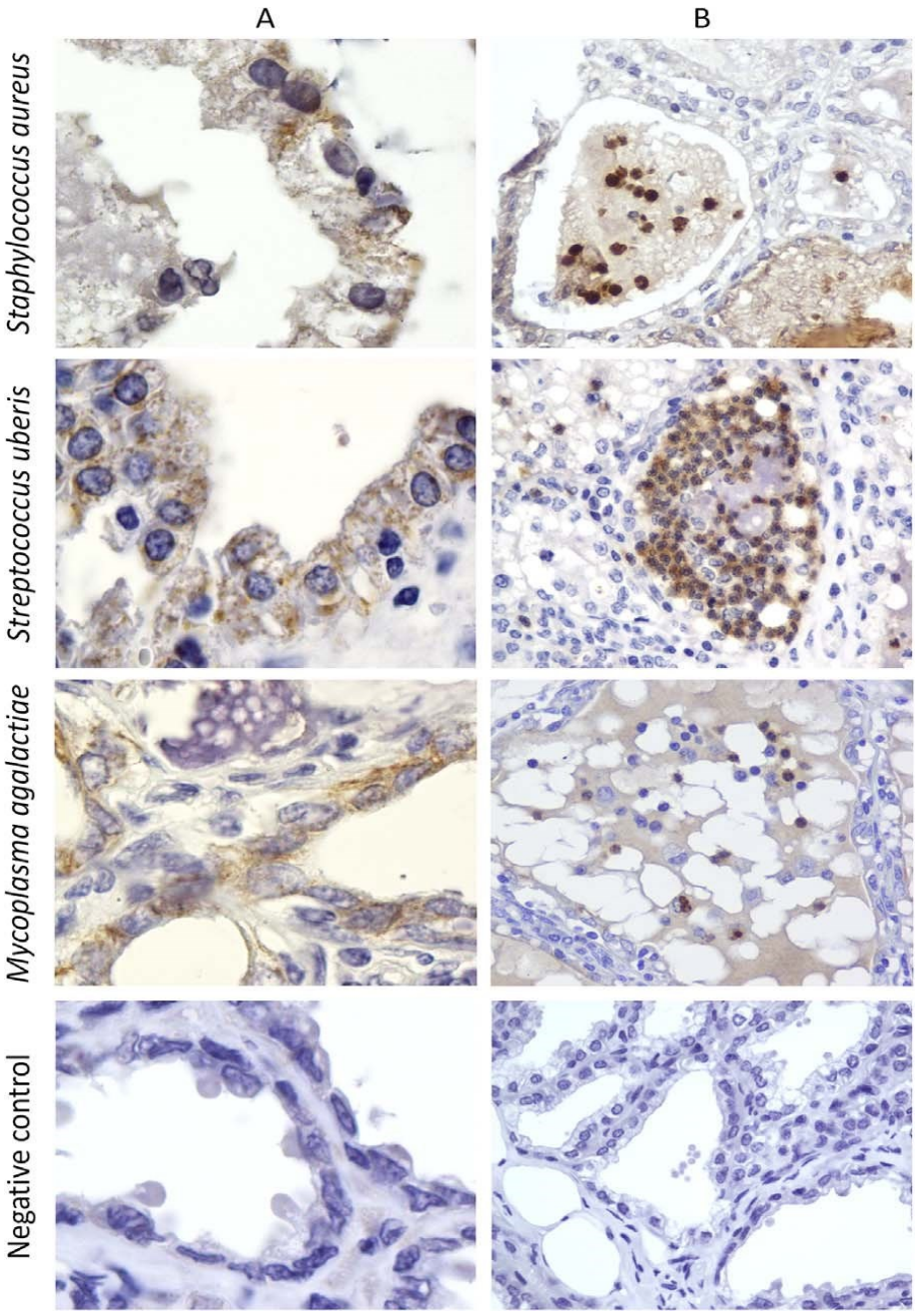

Fig. 1. Patterns of cathelicidin reactivity in sheep mammary gland tissues by immunohistochemistry. A: areas of tissue with visible epithelial lining. Magnification: 1000x. B: areas of tissue with acute inflammatory lesions. Magnification: 400x.

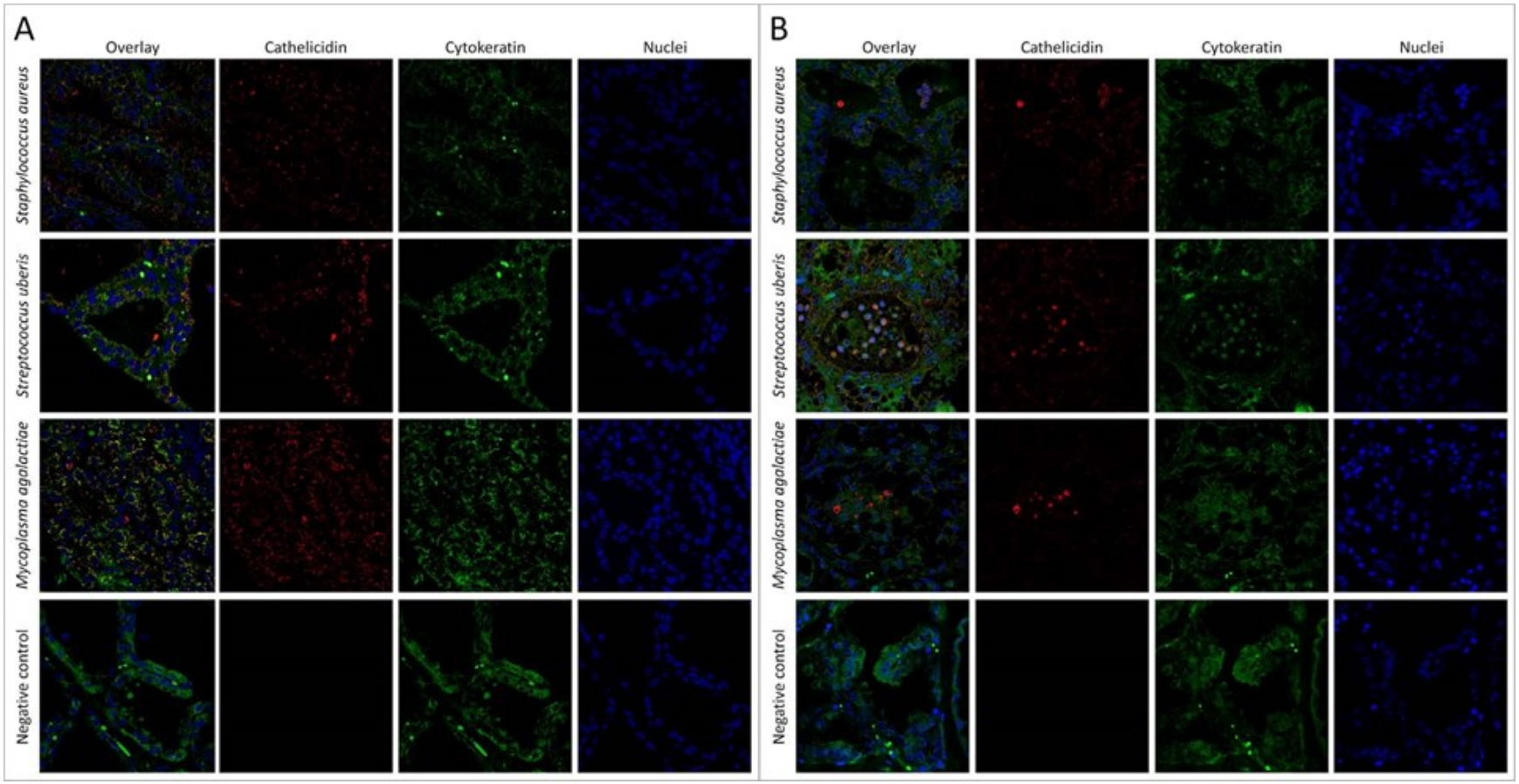

Fig. 2. Co-localization of cathelicidin reactivity (red) with epithelial cells (green), and nuclear DNA (blue) in mammary gland tissues. Panel A: areas of tissue with visible epithelial lining. Panel B: areas of tissue with acute inflammatory lesions. The bright green elliptical formations are due to red blood cell autofluorescence. The images show an overlay of the three signals (left columns) and the three channels acquired separately for each marker (cathelicidin, epithelial cytokeratin, and nuclei, from left to right, respectively). Magnification: 400x.

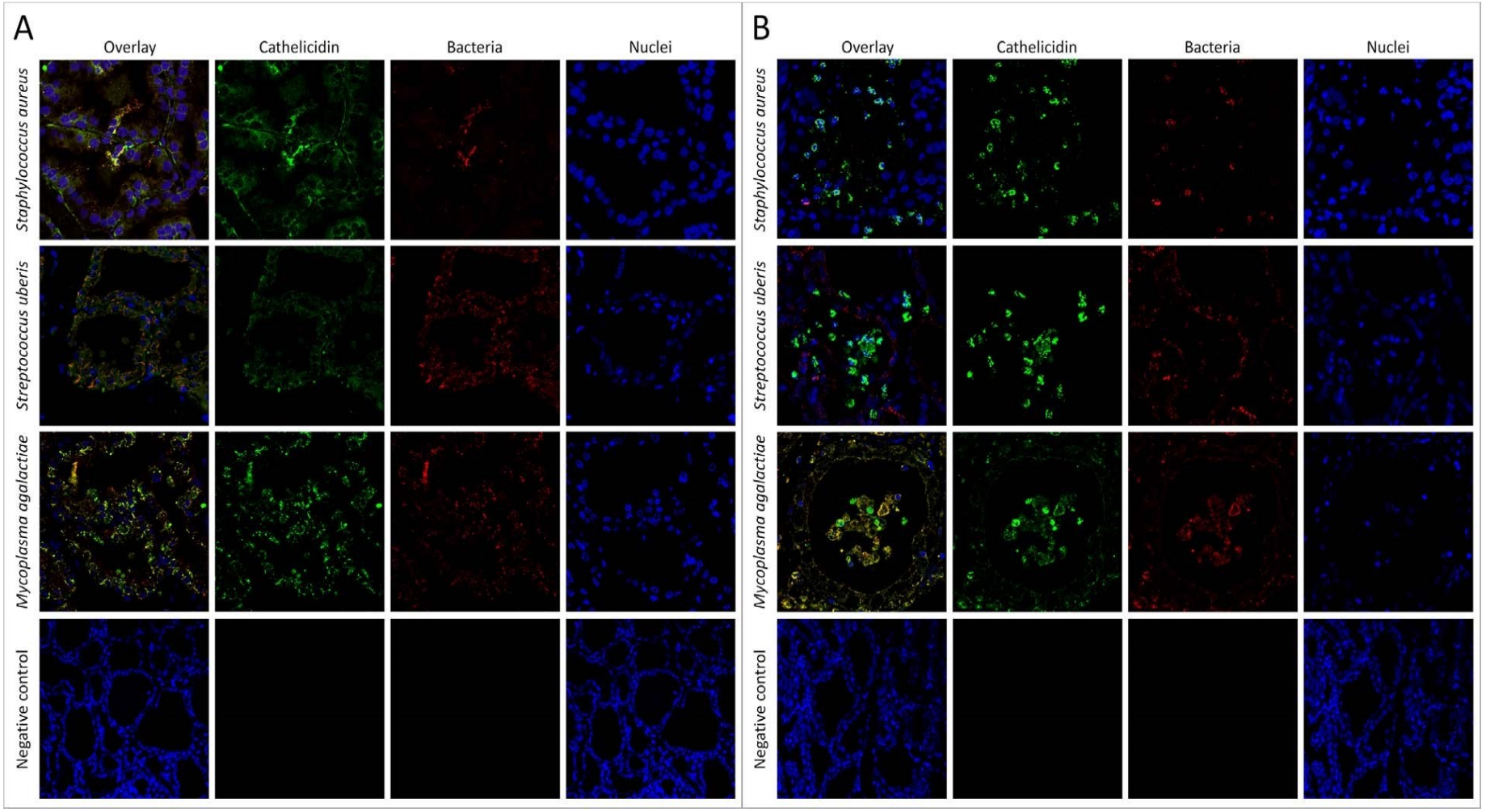

Fig. 3. Co-localization of cathelicidin (green) reactivity with bacteria (red), and nuclear DNA (blue) in sheep mammary gland tissues. Panel A: areas of tissue with visible epithelial lining. Panel B: areas of tissue with acute inflammatory lesions. The bright green elliptical formations are due to red blood cell autofluorescence. The images show an overlay of the three signals (left columns) and the three channels acquired separately for each marker (cathelicidin, bacteria, and nuclei, from left to right, respectively). Magnification: 400x.

References

Addis, M.F., Pisanu, S., Ghisaura, S., Pagnozzi, D., Marogna, G., Tanca, A., Biosa, G., Cacciotto, C., Alberti, A., Pittau, M., Roggio, T., Uzzau, S., 2011. Proteomics and pathway analyses of the milk fat globule in sheep naturally infected by Mycoplasma agalactiae provide indications of the in vivo response of the mammary epithelium to bacterial infection. Infect. Immun. 79, 3833–3845. http://dx.doi.org/10.1128/IAI. 00040-11.

Addis, M.F., Pisanu, S., Marogna, G., Cubeddu, T., Pagnozzi, D., Cacciotto, C., Campesi, F., Schianchi, G., Rocca, S., Uzzau, S., 2013. Production and release of antimicrobial and immune defense proteins by mammary epithelial cells following Streptococcus uberis infection of sheep. Infect. Immun. 81, 3182–3197. http://dx.doi.org/10.1128/ IAI.00291-13.

Addis, M.F., Tedde, V., Dore, S., Pisanu, S., Puggioni, G.M.G., Roggio, A.M., Pagnozzi, D., Lollai, S., Cannas, E.A., Uzzau, S., 2016a. Evaluation of milk cathelicidin for detection of dairy sheep mastitis. J. Dairy Sci. 99, 6446–6456. http://dx.doi.org/10.3168/jds. 2015-10293.

Addis, M.F., Tedde, V., Puggioni, G.M.G., Pisanu, S., Casula, A., Rota, N., Bronzo, V., Moroni, P., Uzzau, S., 2016b. Evaluation of milk cathelicidin for detection of bovine mastitis. J. Dairy Sci. 99, 8250–8258. http://dx.doi.org/10.3168/jds.2016-11407.

Brinkmann, V., Reichard, U., Goosmann, C., Fauler, B., Uhlemann, Y., Weiss, D.S., Weinrauch, Y., Zychlinsky, A., 2004. Neutrophil extracellular traps kill bacteria.
Science 303, 1532–1535. http://dx.doi.org/10.1126/science.1092385.

Cacciotto, C., Addis, M.F., Pagnozzi, D., Chessa, B., Coradduzza, E., Carcangiu, L., Uzzau, S., Alberti, A., Pittau, M., 2010. The liposoluble proteome of Mycoplasma agalactiae: an insight into the minimal protein complement of a bacterial membrane. BMC Microbiol. 10, 225.

Cacciotto, C., Addis, M.F., Coradduzza, E., Carcangiu, L., Nuvoli, A.M., Tore, G., Dore, G.M., Pagnozzi, D., Uzzau, S., Chessa, B., Pittau, M., Alberti, A., 2013. Mycoplasma agalactiae MAG_5040 is a Mg^{2+}-dependent, sugar-nonspecific SNase recognised by the host humoral response during natural infection. PLoS One 8, e57775. http://dx. doi.org/10.1371/journal.pone.0057775.

Cacciotto, C., Cubeddu, T., Addis, M.F., Anfossi, A.G., Tedde, V., Tore, G., Carta, T., Rocca, S., Chessa, B., Pittau, M., Alberti, A., 2016. Mycoplasma lipoproteins are major determinants of neutrophil extracellular trap formation. Cell. Microbiol. 18, 1751–1762. http://dx.doi.org/10.1111/cmi.12613.

Chromek, M., Slamová, Z., Bergman, P., Kovács, L., Podracká, L., Ehrén, I., Hökfelt, T., Gudmundsson, G.H., Gallo, R.L., Agerberth, B., Brauner, A., 2006. The antimicrobial peptide cathelicidin protects the urinary tract against invasive bacterial infection.
Nat. Med. 12, 636–641. http://dx.doi.org/10.1038/nm1407.

Dore, S., Liciardi, M., Amatiste, S., Bergagna, S., Bolzoni, G., Caligiuri, V., Cerrone, A., Farina, G., Montagna, C.O., Saletti, M.A., Scatassa, M.L., Sotgiu, G., Cannas, E.A., 2016. Survey on small ruminant bacterial mastitis in Italy, 2013–2014. Small Rumin. Res. 141, 91–93.

Kościuczuk, E.M., Lisowski, P., Jarczak, J., Krzyżewski, J., Zwierzchowski, L., Bagnicka, E., 2014. Expression patterns of β-defensin and cathelicidin genes in parenchyma of bovine mammary gland infected with coagulase-positive or coagulase-negative Staphylococci. BMC Vet. Res. 10, 1–14. http://dx.doi.org/10.1186/s12917-0140246-z.

Mudaliar, M., Tassi, R., Thomas, F.C., McNeilly, T.N., Weidt, S.K., McLaughlin, M., Wilson, D., Burchmore, R., Herzyk, P., Eckersall, P.D., Zadoks, R.N., 2016.
Mastitomics, the integrated omics of bovine milk in an experimental model of Streptococcus uberis mastitis: 2. Label-free relative quantitative proteomics. Mol.
BioSyst. 12, 2748–2761. http://dx.doi.org/10.1039/C6MB00290K.

Murakami, M., Dorschner, R.A., Stern, L.J., Lin, K.H., Gallo, R.L., 2005. Expression and secretion of cathelicidin antimicrobial peptides in murine mammary glands and human milk. Pediatr. Res. 57, 10–15.

Pisanu, S., Cubeddu, T., Pagnozzi, D., Rocca, S., Cacciotto, C., Alberti, A., Marogna, G., Uzzau, S., Addis, M.F., 2015. Neutrophil extracellular traps in sheep mastitis. Vet.
Res. 46, 59. http://dx.doi.org/10.1186/s13567-015-0196-x.

Rosati, S., Robino, P., Fadda, M., Pozzi, S., Mannelli, A., Pittau, M., 2000. Expression and antigenic characterization of recombinant Mycoplasma agalactiae P48 major surface protein. Vet. Microbiol. 71, 201–210. http://dx.doi.org/10.1016/S0378-1135(99) 00164-9.

Smolenski, G.A., Wieliczko, R.J., Pryor, S.M., Broadhurst, M.K., Wheeler, T.T., Haigh, B.J., 2011. The abundance of milk cathelicidin proteins during bovine mastitis. Vet. Immunol. Immunopathol. 143, 125–130.

Thammavongsa, V., Missiakas, D.M., Schneewind, O., 2013. Staphylococcus aureus degrades neutrophil extracellular traps to promote immune cell death. Science 342, 863–866. http://dx.doi.org/10.1126/science.1242255.

Tola, S., Idini, G., Manunta, D., Galleri, G., Angioi, A., Rocchigiani, A.M., Leori, G., 1996. Rapid and specific detection of Mycoplasma agalactiae by polymerase chain reaction. Vet. Microbiol. 51, 77–84. http://dx.doi.org/10.1016/0378-1135(96)00023-5.

Tomasinsig, L., De Conti, G., Skerlavaj, B., Piccinini, R., Mazzilli, M., D'Este, F., Tossi, A., Zanetti, M., 2010. Broad-spectrum activity against bacterial mastitis pathogens and activation of mammary epithelial cells support a protective role of neutrophil cathelicidins in bovine mastitis. Infect. Immunol. 78, 1781–1788. http://dx.doi.org/10. 1128/IAI.01090-09.

Watts, J.L., 1990. Bovine mastitis. In: Carter, G.R., Cole, J.R.J. (Eds.), Diagnostic Procedures in Veterinary Bacteriology and Mycology, 5th ed. Academic Press, San Diego, pp. 469–478.

Zanetti, M., Gennaro, R., Romeo, D., 1995. Cathelicidins: a novel protein family with a common proregion and a variable C-terminal antimicrobial domain. FEBS Lett. 374, 1–5. http://dx.doi.org/10.1016/0014-5793(95)01050-O.