Evaluation of a bovine cathelicidin ELISA for detecting mastitis in the dairy buffalo: Comparison with milk somatic cell count and bacteriological culture

Giulia Maria Grazia Puggioni, Vittorio Tedde, Sergio Uzzau, Jacopo Guccione, Paolo Ciaramella, Claudia Pollera, Paolo Moroni, Valerio Bronzo, Maria Filippa Addis

PII: S0034-5288(19)30768-4

DOI: https://doi.org/10.1016/j.rvsc.2019.11.009

Reference: YRVSC 3922

To appear in: *Research in Veterinary Science*

Received date: 2 August 2019

Revised date: 31 October 2019

Accepted date: 19 November 2019

Please cite this article as: G.M.G. Puggioni, V. Tedde, S. Uzzau, et al., Evaluation of a bovine cathelicidin ELISA for detecting mastitis in the dairy buffalo: Comparison with milk somatic cell count and bacteriological culture, *Research in Veterinary Science* (2019), https://doi.org/10.1016/j.rvsc.2019.11.009

Evaluation of a bovine cathelicidin ELISA for detecting mastitis in the dairy buffalo: comparison with milk somatic cell count and bacteriological culture

Giulia Maria Grazia Puggioni[a], Vittorio Tedde[a], Sergio Uzzau[a,b], Jacopo Guccione[c], Paolo Ciaramella[c], Claudia Pollera[d], Paolo Moroni[d,e], Valerio Bronzo[d], Maria Filippa Addis[a,d]*

[a]Porto Conte Ricerche, Alghero, Italy

[b]Dipartimento di Scienze Biomediche, Università degli Studi di Sassari, Sassari, Italy

[c]Dipartimento di Medicina Veterinaria e Produzioni Animali, Università degli Studi di Napoli, Napoli, Italy

[d]Dipartimento di Medicina Veterinaria, Università degli Studi di Milano, Milan, Italy

[e]Quality Milk Production Services, Animal Health Diagnostic Center, Cornell University, Ithaca, NY, USA

*Corresponding author at: Dipartimento di Medicina Veterinaria, Università degli Studi di Milano, Via Celoria 10, 20133 Milano, Italy

Abstract

A recently developed bovine cathelicidin (**CATH**) ELISA was evaluated in the dairy buffalo (*Bubalus bubalis*) by testing 618 quarter milk samples from a herd with subclinical mastitis cases. Somatic cell count (**SCC**) and bacteriological culture (**BC**) were carried out on the same samples for comparison. Out of 618 quarters, 258 (41.75%) were positive to CATH, 289 (46.76%) had SCC > 200,000 cells/mL, and 457 (73.95%) were positive to BC. The most prevalent microorganism was *Staphylococcus aureus* (**SAU**, 35.76% of all quarters), followed by non-aureus staphylococci (**NAS**, 22.17% of all quarters). Clinical mastitis quarters were only 7 (1.13%). CATH levels were significantly higher in clinical quarters and in high SCC, BC-positive quarters than in healthy, low SCC, BC-negative quarters. The highest median values were observed for SAU and the lowest for NAS. Differences among microorganism classes were generally more significant for SCC than for CATH. Test characteristics of the CATH ELISA, evaluated by considering as true positives all BC-positive quarters with SCC > 200,000 cells/mL (N=242), and as true negatives all sterile quarters with SCC < 200,000 cells/mL (N=44), were as follows: sensitivity 57.85%, specificity 84.09%, positive predictive value 95.24%, negative predictive value 26.62%, accuracy 61.82%. Therefore, the bovine CATH ELISA showed a fair sensitivity and a good specificity in detecting water buffalo mastitis.

Keywords: Subclinical mastitis; water buffalo milk; cathelicidin ELISA; somatic cell count; bacteriological culture.

1. Introduction

Mediterranean buffalo (**MB**) milk represents a relevant Italian economic resource in terms of sales volume and workforces employed (~ 320 million/year and ~ 15'000 people, respectively). Nevertheless, clinical management is often based on knowledge transferred from bovines, as limited specific information is available. One of the most prevalent diseases in dairy buffalo is mastitis with major economic, hygienic and welfare implications (Guccione and Ciaramella, 2017; Pisanu et al., 2019). Mastitis detection is typically based on the milk somatic cell count (**SCC**) with a threshold of 200×10^3 cells/mL as in bovine cows (Guccione and Ciaramella, 2017; Moroni et al., 2006; Piccinini et al., 2006). Under a clinical perspective, quarters with SCC < 200×10^3 cells/mL and negative to bacteriological culture (**BC**) are classified as healthy; quarters with milk SCC < 200×10^3 cells/mL and positive BC are classified as affected by intramammary infection (IMI); and quarters with milk SCC > 200×10^3 and positive BC are classified as affected by subclinical mastitis (**SCM**) or by clinical mastitis (**CM**) according to absence or presence of clinical signs, respectively (Guccione et al., 2017; Guccione and Ciaramella, 2017).

Several contagious (e.g. *Staphylococcus aureus, Streptococcus agalactiae*), environmental (e.g. *Streptococcus dysgalactiae, Escherichia coli*), and opportunistic microorganisms (e.g. Non-Aureus Staphylococci, *Streptococcus pluranimalium*) are recognized as mastitis-causing in MB (Fagiolo and Lai, 2007; Guccione et al., 2016; Moroni et al., 2006). Losses in milk production as well as changes in milk components, coagulation properties, and farm profitability are reported in MB herds positive for mastitis-causing bacteria (Guccione et al., 2014; Piccinini et al., 2006), but information regarding clinical effects or management strategies based on early diagnosis is still scarce (Guccione and Ciaramella, 2017).

More sensitive and specific SCM detection tools might improve hygienic and sensorial qualities of water buffalo milk and dairy products. Currently, the limited literature data available for water buffalo SCC when compared to bovine SCC creates some uncertainty on the best threshold to use for defining presence of inflammation (Fagiolo and Lai, 2007). Adding to this, an influence of days in milk and parity on SCC has been reported to occur in buffaloes (Cerón-Muñoz et al., 2002; Piccinini et al., 2006; Sahin et al., 2017, 2016;

Sahin and Ulutas, 2016). A better understanding of this parameter together with the implementation of alternative diagnostic strategies would greatly improve detection performance.

A promising mastitis marker in this respect are milk cathelicidins (Addis et al., 2016c, 2016b; Smolenski et al., 2011). Cathelicidins are antimicrobial proteins released by milk neutrophils and epithelial cells in response to an IMI (Cubeddu et al., 2017; Zanetti, 2005). Being specifically produced by these cells, CATH may represent a more specific indicator of mastitis when compared to the total SCC that includes also other cell types. Our group developed an ELISA for detecting milk CATH by selecting and validating monoclonal antibodies against the bovine and ovine cathelicidin sequences. This pan-cathelicidin milk ELISA demonstrated remarkable diagnostic performances in both dairy species (Addis et al., 2016b, 2016a), and its successful application in MB would lead to a unique test for detecting milk CATH in different dairy ruminants with interesting perspectives for its implementation in other formats including pen-side tests. A recent shotgun proteomics study revealed that cathelicidins are the most increased protein family also in the milk of water buffalo with mastitis (Pisanu et al., 2019). Therefore, in this work we assessed the diagnostic performance of the bovine/ovine CATH ELISA in the quarter milk of a MB dairy herd with high incidence of subclinical mastitis, by carrying out a comparative evaluation with SCC and BC.

2. Materials and methods

2.1. Animals and milk samples

A total of 618 quarter milk samples were collected from a herd with 460 milking buffaloes in the context of a milk quality program in Campania (Italy) receiving an institutional approval by the Ethical Animal Care and Use Committee of the University of Naples "Federico II" (n. 2016/0052967). All procedures were carried out conforming to the relevant rules and regulations on animal welfare. Before sampling, all animals enrolled were submitted to clinical examination focused on the udder health status. Then, teats were carefully cleaned and disinfected with disposable towels embedded with chlorhexidine, stripped, and approximately 10 mL of milk was collected aseptically from each quarter into sterile vials. Samples were brought to the

laboratory and stored at 4°C for a maximum of 24 h until BC and SCC were performed. An aliquot was frozen and shipped in temperature-controlled containers to the Porto Conte Ricerche laboratories for ELISA testing.

2.2. Bacteriological culture (BC) and Somatic cell count (SCC)

BC was performed according to the National Mastitis Council standards (2017). Ten μl of each milk sample was spread onto blood agar plates (5% defibrinated sheep blood). Plates were incubated aerobically at 37°C and examined after 24 h. Colonies were classified based on Gram stain, morphology, and hemolysis patterns, and the number of each colony type was recorded. Representative colonies were then sub-cultured on blood agar plates and incubated again at 37°C for 24 h to obtain pure cultures. Gram-positive cocci were differentiated into staphylococci and streptococci by the catalase reaction. A coagulase test was performed on all isolated Staphylococci; those showing positive reaction were identified as *Staphylococcus aureus* (**SAU**), while strains showing negative reaction were classified as non-aureus species (**NAS**). Species identification was carried out with a gallery of biochemical tests. Only samples with more than five colonies with the same characteristics were considered positive, and non-sterile samples with at least one and less than five colonies with the same characteristics were classified as non-significant growth (**NSG**). If **SAU** was present, samples with less than five colonies were also considered positive. SCC was determined using an automated counter (Bentley Somacount 150; Bentley Instruments, Chaska, MN).

2.3. Pan-Cathelicidin ELISA

CATH abundance in buffalo milk was assessed with a pan-cathelicidin sandwich ELISA developed in-house at Porto Conte Ricerche for sheep and cow cathelicidins, as previously described (Addis et al., 2016b, 2016c). According to the UniProtKB/SwissProt Database (http://www.uniprot.org/) release 2019_02, the pan-cathelicidin peptide (**PCP**) sequence used to develop the pan-cathelicidin ELISA (Addis et al., 2016b) included a region having identities of 93.2% with Cathelicidin 1 (A0A0A7UX81), 80.0% with Cathelicidin 5 (V9LY96), 75.3% with Cathelicidin 7 (L0L830), 70.7% with Cathelicidin 6 (A0A0A7UXB6), 70.3% with

Cathelicidin 2 (A0A0A7V3V9) and 68.9% with Cathelicidin 4 (A0A0A7NSG7) protein sequences, respectively. The optical density value (**OD**) measured at 450 nm (**OD450**) was normalized against 6 culture-negative buffalo milk samples with <50,000 cells/mL, included in all ELISA plates as internal controls. Then, the average OD450 (+3 SD) of the internal controls was subtracted from all OD450 values to obtain the normalized OD450 value (**NOD450**). For easing logarithmic transformation and visualization, the adjusted OD450 value (**AOD450**) was calculated by adding a correction factor of 0.1 to all NOD450 values. Then, a threshold of > 0.114 AOD450 was applied for classification of samples as positive to CATH (Addis et al., 2016b).

2.4. Statistical analysis

According to the Shapiro-Wilk normality test, the data followed a non-normal distribution. Statistical significance of the differences among result distributions was therefore evaluated with the U-Mann-Whitney test (when comparing two parameters) or with the Kruskal-Wallis test and the Dunn's post-hoc paired comparisons (when comparing three or more parameters). Statistical analysis and descriptive statistics [median and interquartile range (IQR)] were carried out with GraphPad Prism version 5.03 for Windows (GraphPad Software, La Jolla, CA) and with MedCalc version 15.2.2 for Windows (MedCalc Software, Ostend, Belgium). Thresholds of 0.114 AOD450 and 200,000 cells/mL were used for cathelicidin (Addis et al., 2016a) and SCC (Moroni et al., 2006), respectively.

3. Results

3.1. General statistics

Most animals were clinically healthy and had no udder, teat, or milk abnormalities. Only 7 quarters out of the 618 included in the study (1.13%) showed signs of clinical mastitis. Of all quarters, 457 (73.95%) were positive to BC, 289 quarters (46.76%) had SCC > 200,000 cells/mL, and 258 quarters (41.75%) were positive

to the CATH ELISA. All clinical mastitis quarters were positive to BC, had SCC > 200,000 cells/mL (median 4.091.000 cells/mL) and were positive to the CATH ELISA (median 0.31 AOD450). Results are summarized in **Table 1**. Detailed sample results are reported in **Supplementary File**.

3.2. Relationship between CATH ELISA and SCC

Quarter milk samples were grouped according to CATH results (CATH negative and CATH positive) and the distribution of the SCC value in the two groups was assessed, respectively. Result distribution is illustrated as boxplots in **Fig. 1**, and the respective median and interquartile ranges are reported in **Table 2**. SCC values were significantly lower in CATH-negative than in CATH-positive quarters ($P \leq 0.001$), indicating an association between increase in SCC and presence of CATH. In fact, the median SCC was 98,500 cells/mL in CATH-negative quarters, well below the 200,000 cells/mL threshold, and 713,000 cells/mL in CATH-positive quarters, well above it. However, there was an overlap of SCC values involving the upper quartiles of the CATH-negative group distribution and the lower quartiles of the CATH-positive group distribution.

3.3. Relationship of CATH ELISA with BC and clinical status

Quarters were then classified into negative, SCM, and CM, by considering all sterile quarters with SCC <200,000 cells/mL as true negatives, all BC-positive quarters with SCC >200,000 cells/mL and absence of clinical signs as SCM, and all quarters showing clinical signs of mastitis as CM, respectively (Guccione and Ciaramella, 2017). The distribution of CATH (AOD450) values among the groups is illustrated as boxplots in **Fig. 2**, and the respective median and interquartile ranges are reported in **Table 3**. As a result, all classes differed significantly from one another, indicating an association of CATH with mammary gland inflammation combined with presence of bacteria or clinical signs. The median CATH value of the negative group was below the 0.114 AOD450 threshold and the median CATH value of the SCM group was above it, although there was an overlap of CATH values in the upper and lower values of the two distributions, respectively. In the CM group, CATH values were considerably higher; in this case, the median CATH value

was over 0.3 AOD450, and the central quartiles of the distribution did not overlap with those of negative quarters.

3.4. Relationship of SCC and CATH results with the isolated microorganism

BC results for all quarters are detailed in **Table 4** and summarized in **Fig. 3**. SAU was the most prevalent microorganism (221 quarters, 35.76%), followed by NAS (137 quarters, 22.17%).

Median and IQR values measured for SCC and CATH according to the pathogen are detailed in **Table 4** and plotted in **Fig. 4**. Among all BC-positive quarters, the highest SCC and CATH median values were measured in SAU-positive ones. SAU-positive and NAS-positive quarters differed significantly in SCC and CATH levels; the SCC of SAU-positive quarters was also significantly different from sterile and other/NSG quarters.

Further, 38.46% quarters with SAU were positive to both SCC and CATH, *vs* 19.70% of quarters with NAS. Sterile quarters positive to both SCC and CATH were 17.46%. When SAU was isolated, SCC was > 200,000 cells/mL in 65.61% of cases, and CATH was above threshold in 48.87% of cases. On the other hand, when NAS were isolated, SCC was > 200,000 cells/mL only in 33.57% of cases, and CATH was above threshold only in 31.39% of cases.

3.5. Test characteristics of CATH ELISA in detecting culture-positive quarters with high SCC

Since neither BC nor SCC are perfect tests (Addis et al., 2016c; Dohoo and Leslie, 1991; Fox, 2013; Ruegg and Pantoja, 2013; Schukken et al., 2003; Walker et al., 2011), the diagnostic performance of the CATH ELISA was assessed by considering as true positives all BC-positive quarters with SCC > 200,000 cells/mL (N=242), and as true negatives all sterile quarters with SCC < 200,000 cells/mL (N=44), based on the standards set by previous studies (Guccione and Ciaramella, 2017). Test characteristics were as follows: sensitivity (Se) 57.85%, specificity (Sp) 84.09%, positive predictive value (PPV) 95.24%, negative predictive value (NPV) 26.62%, accuracy (A) 61.89%. The respective 95% confidence intervals (95% CI) are detailed in **Table 5**.

4. Discussion

Cathelicidin has emerged as an interesting marker for integrating and improving mastitis detection (Addis et al., 2013; Smolenski et al., 2011; Whelehan et al., 2014) also in the water buffalo (Pisanu et al., 2019). Its diagnostic performance, as evaluated in cows and ewes with a CATH ELISA, was demonstrated to be of interest (Addis et al., 2016b) and its applicability in goats was also recently described (Tedde et al., 2019). The ability to successfully measure the same marker with one diagnostic format in different dairy ruminants, including the water buffalo, would be of interest for its implementation in the diagnostic routine and for the development of other formats including pen-side tests.

A sensitive and specific mastitis test based on protein markers linked to innate immunity presents several advantages in comparison to somatic cells (Viguier et al., 2009), as discussed in our recent articles describing the cathelicidin ELISA (Addis et al., 2017, 2016b, 2016c). Adding to the closer correlation of protein markers than somatic cells with the presence of inflammation, an ELISA can be carried out on frozen samples in batch, even retrospectively, and does not require dedicated expensive instrumentation or specific technical skills, being a widespread and cheap laboratory assay technique. The development of a dedicated pen-side test might also enable its implementation in the field, providing the same logistic advantages of CMT with better diagnostic performance characteristics and ease of interpretation, although it will be a significant challenge to compete with the low cost, widespread diffusion and straightforward execution of the CMT.

With these premises, this study assessed the same CATH ELISA format in another valuable dairy species, the MB, revealing a good specificity and a fair sensitivity in discriminating bacteriologically positive, SCM quarters from sterile, healthy quarters. SCC values were significantly higher in CATH-positive than in CATH-negative quarters, indicating an association between mastitis and presence of CATH in milk. All clinical mastitis quarters showed high values for both SCC (median 4.091.000 cells/mL) and CATH (median 0.31), reinforcing the positive correlation of both parameters during mammary gland inflammation in agreement with the observations made in cows and ewes (Addis et al., 2016b, 2016c).

However, the ELISA was less specific and sensitive in MB than in bovine cows and in ewes. In a recent shotgun proteomics study from our group (Pisanu et al., 2019), the most abundant CATH proteoform in buffalo milk with staphylococcal IMI was CATH 4. Unfortunately, this proteoform shares the lowest homology (68.9%) with the PCP sequence used for generating monoclonal antibodies (PCP, Addis et al., 2016a and 2016b). Optimization of antibody reactivity against CATH 4 might therefore improve diagnostic performances. Nevertheless, reactivity of the same antibodies by western immunoblotting in cathelicidin-positive buffalo quarter milk was very good (Pisanu et al., 2019) and we cannot rule out that CATH levels may actually be lower in buffalo milk than in the milk of other dairy species, reducing the diagnostic potential of this assay.

In addition to the diagnostic performance, other observations deserve some discussion. First, a very high number of quarters in the study herd were positive to bacteriological culture, although sampling procedures were carried out according to the NMC guidelines (Middleton et al., 2017) taking care not to contaminate the milk, and stripping was performed. This is in agreement with previous reports in MB (Moroni et al., 2006), but the number of SCC and CATH-positive quarters was not as high as expected with such a prevalence of BC-positive samples; indeed, with 73.95% of samples with positive BC, only 46.76% of samples had SCC > 200,000 cells/mL and only 41.75% were above threshold in the CATH ELISA. For comparison, in cows with 13.7% of samples with positive BC, 18.8% had SCC > 200,000 cells/mL, and 29.0% were above threshold in the CATH ELISA (Addis et al., 2016c). In ewes with 20.6% of samples with positive BC, 37.02% had SCC > 200,000 cells/mL, and 35.3% were above threshold in the CATH ELISA (Addis et al., 2016b). Second, SCC was also less performing in buffaloes than in bovines, even when considering the relationship of SCC with the etiologic agent (Addis et al., 2017). This might indicate a lesser increase of inflammation markers as a consequence of IMI in this dairy species, as it is known to occur in goats, another rustic dairy ruminant (Paape et al., 2007; Tedde et al., 2019). Accordingly, CATH levels may actually be lower in buffalo milk than in cow milk or in ewe milk.

In agreement with its higher pathogenicity and contagious nature, SAU was the most prevalent microorganism in clinical mastitis quarters and the strongest inducer of both SCC and CATH. Accordingly,

increases in SCC and CATH were seen in a lower number of quarters positive to microorganisms other than SAU. The behavior of CATH in SAU-positive quarters also suggests that a stronger inflammatory stimulus might be needed to elicit SCC and CATH increase in the water buffalo than in other dairy species such as cattle and sheep. On the other hand, presence of NAS was not related to SCC or CATH increase; CATH median levels were even lower in NAS-positive quarters than in sterile quarters, and about half of the quarters with SAU did not show any increase in SCC or CATH. Further investigations will be needed to better clarify the relationship of a positive milk microbial culture with IMI and mastitis in the water buffalo, also in light of the recent findings on the milk microbiome (Addis et al., 2016; Catozzi et al., 2017).

In conclusion, although test performances were not as good as in bovine and ovine milk, the CATH ELISA demonstrated a good specificity and a fair sensitivity in discriminating bacteriologically positive, SCM quarters from sterile, healthy quarters.

Acknowledgements

The authors wish to thank Dr. Antonio Casula for technical assistance with somatic cell counting. This work was partially supported by Sardegna Ricerche, Science and Technology Park of Sardinia, under grant program art.9 LR 20/2016 (2017) to Porto Conte Ricerche.

Conflict of Interest

The authors declare that they have no conflicts of interest.

References

Addis, M.F., Bronzo, V., Puggioni, G.M.G., Cacciotto, C., Tedde, V., Pagnozzi, D., Locatelli, C., Casula, A., Curone, G., Uzzau, S., Moroni, P., 2017. Relationship between milk cathelicidin abundance and microbiologic culture in clinical mastitis. J. Dairy Sci. 100, 2944–2953. https://doi.org/10.3168/jds.2016-12110

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. https://doi.org/10.1128/IAI.00291-13

Addis, M.F., Tanca, A., Uzzau, S., Oikonomou, G., Bicalho, R.C., Moroni, P., 2016a. The bovine milk microbiota: insights and perspectives from -omics studies. Mol. Biosyst. 12, 1–29.

https://doi.org/10.1039/C6MB00217J

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., 2016b. Evaluation of milk cathelicidin for detection of dairy sheep mastitis. J. Dairy Sci. 1–13.

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

Catozzi, C., Sanchez Bonastre, A., Francino, O., Lecchi, C., De Carlo, E., Vecchio, D., Martucciello, A., Fraulo, P., Bronzo, V., Cuscó, A., D'Andreano, S., Ceciliani, F., 2017. The microbiota of water buffalo milk during mastitis. PLoS One 12, e0184710. https://doi.org/10.1371/journal.pone.0184710

Cerón-Muñoz, M., Tonhati, H., Duarte, J., Oliveira, J., Muñoz-Berrocal, M., Jurado-Gámez, H., 2002. Factors Affecting Somatic Cell Counts and Their Relations with Milk and Milk Constituent Yield in Buffaloes. J. Dairy Sci. 85, 2885–2889. https://doi.org/10.3168/jds.S0022-0302(02)74376-2

Cubeddu, T., Cacciotto, C., Pisanu, S., Tedde, V., Alberti, A., Pittau, M., Dore, S., Cannas, A., Uzzau, S., Rocca, S., Addis, M.F., 2017. Cathelicidin production and release by mammary epithelial cells during infectious mastitis. Vet. Immunol. Immunopathol. 189, 66–70. https://doi.org/10.1016/j.vetimm.2017.06.002

Dohoo, I.R., Leslie, K.E., 1991. Evaluation of changes in somatic cell counts as indicators of new intramammary infections. Prev. Vet. Med. 10, 225–237. https://doi.org/10.1016/0167-5877(91)90006-N

Fagiolo, A., Lai, O., 2007. Mastitis in buffalo. Ital. J. Anim. Sci. 6, 200–206. https://doi.org/10.4081/ijas.2007.s2.200

Fox, L., 2013. Can somatic cells get too low? A question to be revisited. NMC Annu. Meet. Proc. 55–63.

Guccione, J., Borriello, G., Ciaramella, P., Di Loria, A., 2017. Clinical evaluation of poor milking procedures effects on dairy Mediterranean buffaloes udder health. Large Anim. Rev. 23, 155–157.

Guccione, J., Ciaramella, P., 2017. Mastitis in Mediterranean Buffaloes. J. Dairy Vet. Sci. 2, 1–4. https://doi.org/10.19080/JDVS.2017.02.555590

Guccione, J., Cosandey, A., Pesce, A., Di Loria, A., Pascale, M., Piantedosi, D., Steiner, A., Graber, H.U., Ciaramella, P., 2014. Clinical outcomes and molecular genotyping of Staphylococcus aureus isolated from milk samples of dairy primiparous Mediterranean buffaloes (Bubalus bubalis). J. Dairy Sci. 97, 7606–7613. https://doi.org/10.3168/jds.2014-8455

Guccione, J., Perreten, V., Steiner, A., Thomann, A., Pesce, A., Ciaramella, P., Bodmer, M., 2016. Short communication: Role of Streptococcus pluranimalium in Mediterranean buffaloes (Bubalus bubalis) with different udder health statuses. J. Dairy Sci. 99, 2945–2949. https://doi.org/10.3168/jds.2015-10291

Middleton, J.R., Fox, L.K., Pighetti, G., 2017. Laboratory Handbook on Bovine Mastitis. National Mastitis Council, Madison, WI, New Prague, MN.

Moroni, P., Sgoifo Rossi, C., Pisoni, G., Bronzo, V., Castiglioni, B., Boettcher, P.J., 2006. Relationships Between Somatic Cell Count and Intramammary Infection in Buffaloes. J. Dairy Sci. 89, 998–1003. https://doi.org/10.3168/jds.S0022-0302(06)72165-8

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. https://doi.org/10.1039/C6MB00290K

Paape, M.J., Wiggans, G.R., Bannerman, D.D., Thomas, D.L., Sanders, A.H., Contreras, A., Moroni, P., Miller, R.H., 2007. Monitoring goat and sheep milk somatic cell counts. Small Rumin. Res. 68, 114–125. https://doi.org/10.1016/j.smallrumres.2006.09.014

Piccinini, R., Miarelli, M., Ferri, B., Tripaldi, C., Belotti, M., Daprà, V., Orlandini, S., Zecconi, A., 2006. Relationship between cellular and whey components in buffalo milk. J. Dairy Res. 73, 129. https://doi.org/10.1017/S0022029905001548

Pisanu, S., Cacciotto, C., Pagnozzi, D., Uzzau, S., Ciaramella, P., Guccione, J., Penati, M., Pollera, C., Moroni, P., Bronzo, V., Addis, M.F., 2019. Proteomic changes in the milk of water buffaloes (Bubalus bubalis)

with subclinical mastitis due to intramammary infection by Staphylococcus aureus and by non-aureus staphylococci. Sci. Rep. in press.
Ruegg, P.L., Pantoja, J.C.F., 2013. Understanding and using somatic cell counts to improve milk quality. Irish J. Agric. Food Res. Vol. 52, 101–117.
Sahin, A., Ulutas, Z., 2016. Changes in some physico-chemical content of anatolian buffalo milk according to some environmental factors. Buffalo Bull. 35.
Sahin, A., Yildirim, A., Ulutas, Z., Ugurlutepe, E., 2017. The effects of stage of lactation, parity and calving season on somatic cell counts in Anatolian water buffaloes. Indian J. Anim. Res. 51, 35–39. https://doi.org/10.18805/ijar.11461
Sahin, A., Yldrm, A., Ulutas, Z., 2016. Effect of various environmental factors and management practices on somatic cell count in the raw milk of Anatolian Buffaloes. Pak. J. Zool.
Schukken, Y.H., Wilson, D.J., Welcome, F., Garrison-Tikofsky, L., Gonzalez, R.N., 2003. Monitoring udder health and milk quality using somatic cell counts. Vet. Res. 34, 579–596. https://doi.org/10.1051/vetres:2003028
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. https://doi.org/10.1016/j.vetimm.2011.06.034
Tedde, V., Bronzo, V., Puggioni, G.M.G., Pollera, C., Casula, A., Curone, G., Moroni, P., Uzzau, S., Addis, M.F., 2019. Milk cathelicidin and somatic cell counts in dairy goats along the course of lactation. J. Dairy Res. 86, 217–221.
Viguier, C., Arora, S., Gilmartin, N., Welbeck, K., O'Kennedy, R., 2009. Mastitis detection: current trends and future perspectives. Trends Biotechnol. 27, 486–493. https://doi.org/10.1016/j.tibtech.2009.05.004
Walker, J.B., Rajala-Schultz, P.J., Walker, W.L., Mathews, J.L., Gebreyes, W.A., DeGraves, F.J., 2011. Variation in daily shedding patterns of Staphylococcus aureus in naturally occurring intramammary infections. J. Vet. Diagnostic Investig. 23, 1114–1122. https://doi.org/10.1177/1040638711425587
Whelehan, C.J., Barry-Reidy, A., Meade, K.G., Eckersall, P.D., Chapwanya, A., Narciandi, F., Lloyd, A.T., O'Farrelly, C., 2014. Characterisation and expression profile of the bovine cathelicidin gene repertoire in mammary tissue. BMC Genomics 15, 128. https://doi.org/10.1186/1471-2164-15-128
Zanetti, M., 2005. The role of cathelicidins in the innate host defenses of mammals. Curr. Issues Mol. Biol. 7, 179–196.

FIGURE LEGENDS

Fig. 1. Distribution of the SCC value (cells x 10^3/mL) in CATH-negative and CATH-positive quarters. Boxes indicate values within the 25th and 75th percentiles, and central lines indicate the median value. Whiskers indicate values within the 10th and 90th percentiles, and individual dots represent values outside the whiskers. Median and IQR values are reported in **Table 2**. The dashed line indicates the SCC threshold at 200,000 cells/mL. The difference between classes was statistically significant (***P ≤ 0.001) according to the Mann-Whitney test.

Fig. 2. Boxplots illustrating cathelicidin (CATH) ELISA AOD450 values in negative quarters, subclinical mastitis quarters (SCM), and clinical mastitis quarters (CM), as defined in the text. Boxes indicate values within the 25th and 75th percentiles, and central lines indicate the median value. Whiskers indicate values within the 10th and 90th percentiles, and individual dots represent values outside the whiskers. Median and IQR values are reported in **Table 3**. The dashed line indicates the CATH ELISA threshold at AOD 0.114. Statistical significance according to the Mann-Whitney test with Dunn's post-test correction is indicated for each comparison (***P ≤ 0.001; *P ≤ 0.05).

Fig. 3. Bacteriological culture results (n=618). The bars indicate the number of quarters positive to the different microorganisms. The legend reports the detail on other bacterial species (shades of blue) and *Streptococcus* and *Enterococcus* spp. (shades of orange). Numbers are detailed in Table 4.

Fig. 4. Boxplots illustrating the distribution of somatic cell count (SCC) and cathelicidin (CATH) AOD450 values according to the bacteriological culture (BC) result. Boxes indicate values within the 25th and 75th percentiles, and central lines indicates the median value. Whiskers indicate values within the 10th and 90th percentiles, and individual dots represent values outside the whiskers. Median and IQR values are detailed in **Table 4**. Dashed lines indicate the SCC threshold at 200,000 cells/mL and the CATH threshold at AOD

0.114, respectively. Statistical significance according to the Mann-Whitney test with Dunn's post-test correction is indicated in the plots for each comparison (***P ≤ 0.001; *P ≤ 0.05). "Other" refers to other microorganisms and non-SAU quarters with less than 500 colony-forming units/mL (non-significant growth).

Table 1. Median, interquartile range, mean and SD values observed for somatic cell count (SCC) and cathelicidin (CATH) in all quarters, in quarters with a positive bacteriological culture (BC) result, and in quarters with clinical mastitis.

Description	Sample number	Median (IQR) SCC (cells x 10^3)	Median (IQR) CATH (AOD450)
All quarters	618 (100%)	169.5 (54.75-740.5)	0.108 (0.092-0.129)
Positive BC	457 (73.95%)	222 (64-985)	0.109 (0.092-0.132)
Clinical mastitis	7 (1.13%)	4091 (2789-5173)	0.306 (0.133-0.485)

Table 2. Median and interquartile ranges (25^{th} and 75^{th} percentile, respectively) of the somatic cell count (SCC) in cathelicidin (CATH) ELISA-negative and in CATH ELISA-positive quarters, respectively.

CATH ELISA result	Median SCC (IQR) in cells x 10^3/mL
Negative	98.5 (49.25-283)
Positive	713 (79-2097)

Table 3. Median and interquartile ranges (25th and 75th percentile, respectively) observed for cathelicidin (CATH) ELISA in negative quarters, in bacteriologically positive quarters with subclinical mastitis (SCM), and in clinical mastitis quarters (CM).

Description	Sample number (%)	Median (IQR) of CATH AOD450
Negative	44 (7.12%)	0.100 (0.088-0.110)
SCM	235 (30.03%)	0.120 (0.100-0.189)
CM	7 (1.13%)	0.303 (0.133-0.485)

Table 4. Summary of bacteriological culture (BC) findings with the respective somatic cell count (SCC) and cathelicidin (CATH) ELISA values measured in that class. Details and distribution of BC results are reported in Figure 3.

Description	Sample number (%)	Median (IQR) SCC (cells x 10^3)	Median (IQR) of CATH AOD450
Staphylococcus aureus	221 (35.76%)	324.0 (103.5-1556)	0.112 (0.094-0.138)
Non-aureus staphylococci	137 (22.17%)	105.0 (46.0-473.5)	0.102 (0.092-0.122)
Other/NSG*	126 (20.39%)	77.0 (37.2-348.0)	0.110 (0.090-0.131)
Sterile	63 (10.19%)	90 (45.0-382.0)	0.103 (0.09-0.118)
Strepto/Enterococcus spp.	49 (7.93%)	259.0 (59.5-721.0)	0.105 (0.080-0.1305)
Gram-negatives	22 (3.56%)	199.5 (62.5-667.8)	0.110 (0.090-0.131)

*Other microorganisms and non-SAU quarters with less than 1500 colony-forming units/mL (quarters with non-significant growth).

Table 5. Test characteristics of the cathelicidin (CATH) ELISA in the Mediterranean buffalo.

	Value	95% Confidence Interval
Sensitivity	57.85%	51.36% - 64.15%
Specificity	84.09%	69.93% - 93.36%
Positive Predictive Value	95.24%	90.95% - 97.55%
Negative Predictive Value	26.62%	22.97% - 30.61%
Accuracy	61.89%	55.98% - 67.54%

Highlights

- A bovine/ovine cathelicidin ELISA was evaluated for detecting mastitis in buffaloes
- The cathelicidin ELISA showed good specificity and fair sensitivity
- Optimization on species-specific sequences might further improve diagnostic performance

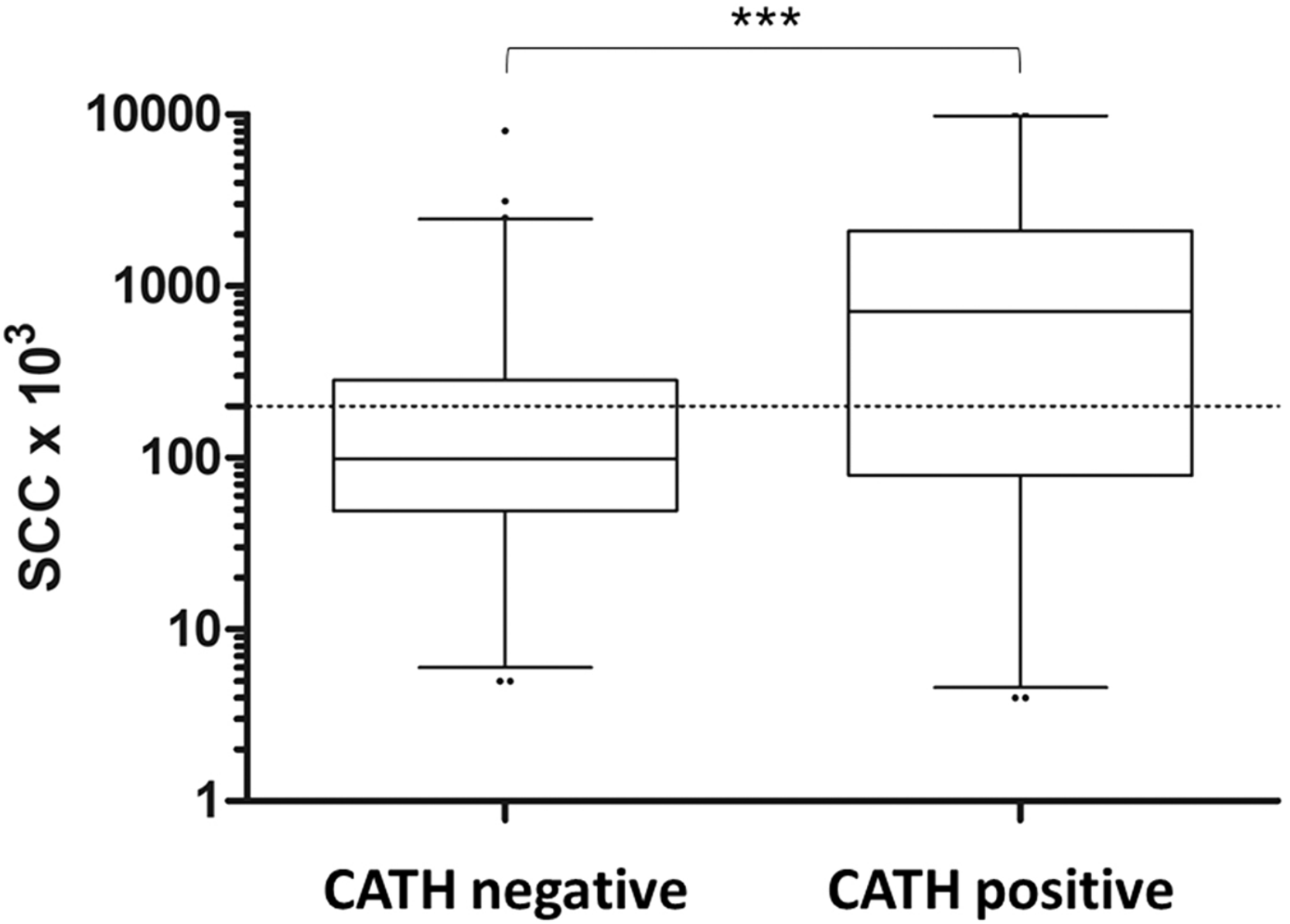

Figure 1

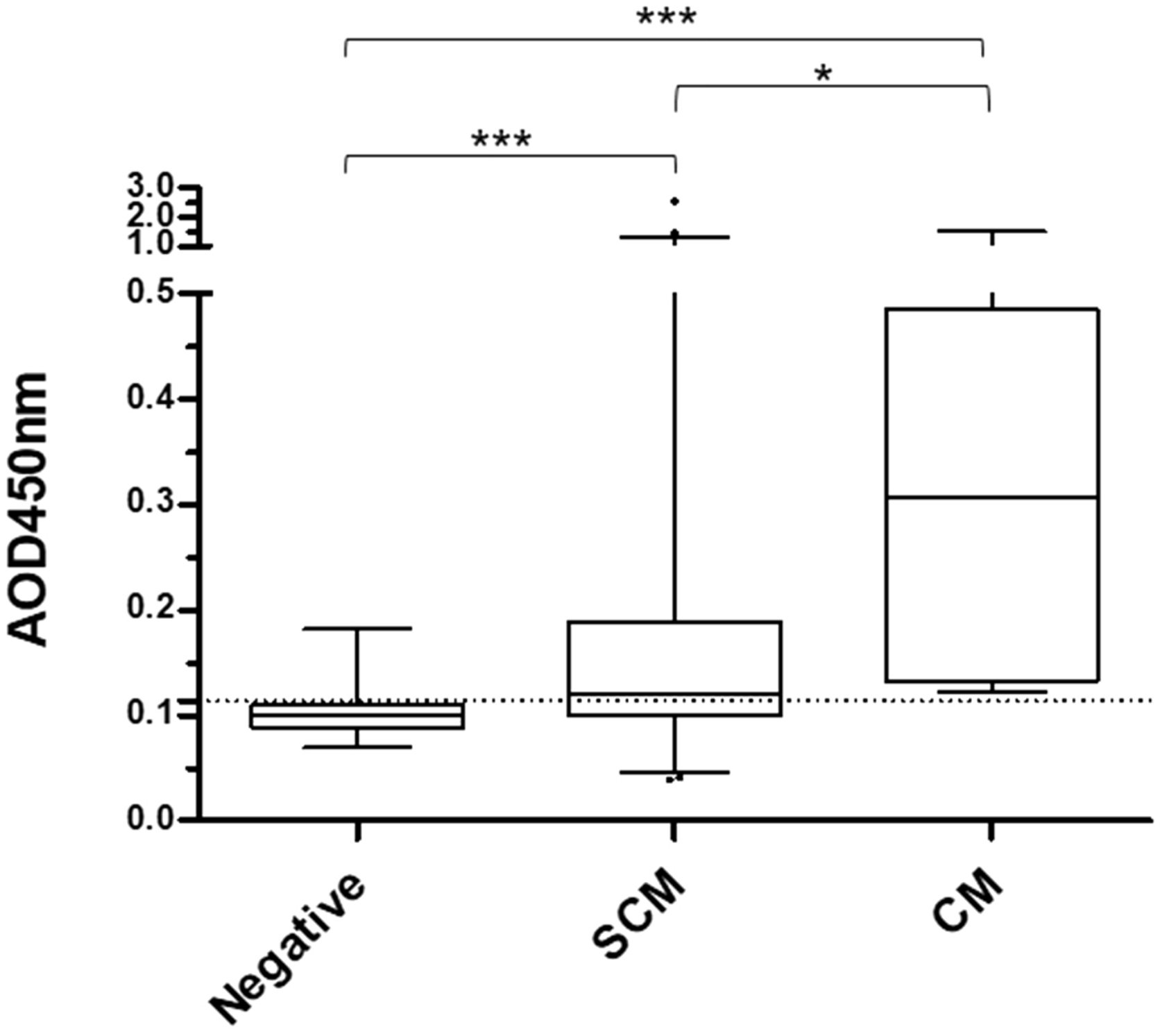

Figure 2

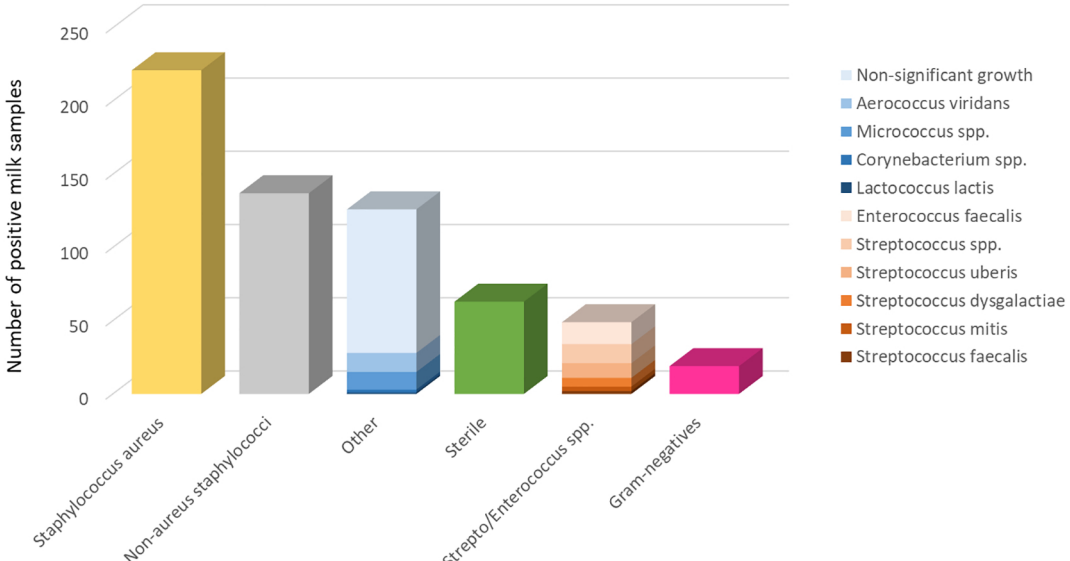
Number of positive milk samples
250
200
150
100
50
0
Staphylococcus aureus
Non-aureus staphylococci
Other
Sterile
Strepto/Enterococcus spp.
Gram-negatives
Non-significant growth
Aerococcus viridans
Micrococcus spp.
Corynebacterium spp.
Lactococcus lactis
Enterococcus faecalis
Streptococcus spp.
Streptococcus uberis
Streptococcus dysgalactiae
Streptococcus mitis
Streptococcus faecalis

Figure 3

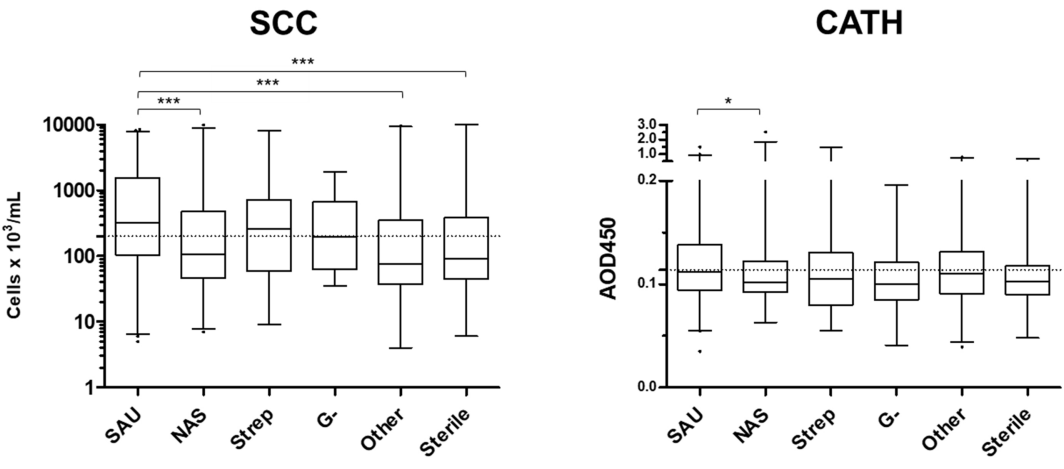
SCC
***
***
***
10000
1000
100
10
1
Cells x 10³/mL
SAU
NAS
Strep
G-
Other
Sterile
CATH
*
3.0
2.0
1.0
0.2
0.1
0.0
AOD450
SAU
NAS
Strep
G-
Other
Sterile

Figure 4