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Original article

Evaluation of the udder health status in subclinical mastitis affected dairy cows through bacteriological culture, somatic cell count and thermographic imaging

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Abstract

Subclinical mastitis in dairy cows is a big economic loss for farmers. The monitoring of subclinical mastitis is usually performed through Somatic Cell Count (SCC) in farm but there is the need of new diagnostic systems able to quickly identify cows affected by subclinical infections of the udder. The aim of this study was to evaluate the potential application of thermographic imaging compared to SCC and bacteriological culture for infection detection in cow affected by subclinical mastitis and possibly to discriminate between different pathogens. In this study we evaluated the udder health status of 98 Holstein Friesian dairy cows with high SCC in 4 farms. From each cow a sample of milk was collected from all the functional quarters and submitted to bacteriological culture, SCC and *Mycoplasma spp*. culture. A thermographic image was taken from each functional udder quarter and nipple. Pearson's correlations and Analysis of Variance were performed in order to evaluate the different diagnostic techniques. The most frequent pathogen isolated was *Staphylococcus aureus* followed by Coagulase Negative Staphylococci (CNS), *Streptococcus uberis*, *Streptococcus agalactiae* and others. The Somatic Cell Score (SCS) was able to discriminate ($p<0.05$) cows positive for a pathogen from cows negative at the bacteriological culture except for cows with infection caused by CNS. Infrared thermography was correlated to SCS ($p<0.05$) but was not able to discriminate between positive and negative cows. Thermographic imaging seems to be promising in evaluating the inflammation status of cows affected by subclinical mastitis but seems to have a poor diagnostic value.

Key words: dairy cows, subclinical mastitis, bacteriological culture, somatic cell score, thermography

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Introduction

Bovine mastitis is a result of inflammation of the mammary gland. Depending on the severity of the inflammation, mastitis can be classified as sub-clinical, clinical or chronic. The degree of inflammation is dependent on the nature of the causative agent and on age, breed, immunological health and lactation state of the animal (Viguier et al. 2009). Many bacteria, mycoplasmas, yeasts and algae may cause mastitis in dairy cows. Watts et al. (1988) identified 137 different microorganisms as etiological agent of mastitis but only a few of them are routinely isolated.

Mastitis pathogens have been divided in „contagious" and „environmental". In the „contagious" group *Staphylococcus aureus*, *Streptococcus agalactiae* and *Mycoplasma bovis* are considered the major pathogens. The major environmental pathogens are Enterobacteriacee (as *Escherichia coli*), *Streptococcus dysgalactiae* and *Streptococcus uberis* (Blowey and Edmondson 2010). Coagulase Negative Staphylococci (CNS) are common environmental microorganisms but, in the last years, have been isolated more and more frequently from clinical milk samples and can be considered as emerging pathogens (Pyorala and Taponen 2009). Radostis et al. (2007) defines subclinical mastitis as an infection without visible changes in milk or the udder.

Subclinical mastitis is a big economic loss for farmers. Ott and Novak (2001) estimated a milk loss of 4.6% for Medium Bulk Tank Milk SCC (BTMSCC) (200,000 to 399,999 cells/ml) and 11.9% for High BTMSCC (> 400,000 cells/ml) compared to Low BTMSCC (< 200,000). Moreover, other money loss comes from the lower financial incentives for high quality milk and increased costs for veterinary and drug expenses. For bovine raw milk, European Union has set a limit for the sale of 400,000 BTMSCC (Reg. CE 853/2004). In many cases, the productivity of the cow will be permanently compromised (Halasa et al. 2007). Because of its financial impact, much attention has to be dedicated to the diagnosis and detection of subclinical mastitis problems in modern dairy farms. Diagnostic methods have been developed to detect of mammary gland inflammation and diagnosis of the infection and its causative pathogens. The gold standard is considered the bacteriology but has several limitations in identifying uninfected cows (Madouasse et al. 2012). Here are some possible reasons for that: no bacteria will grow when the bacteria have been terminated, the media used does not allow to grow the bacteria causing the infection, there are no bacteria in the milk sample collected although an Intra-Mammary Infection (IMI) is present as in the case of intermittent shedding (Sears et al. 1990). Because of these limitations, the costs and the time required for the response bacteriological sampling is not feasible as a routine test (Pyörälä and Taponen, 2009). Tests for indicators of inflammation are therefore necessary as screening tests to identify the quarters with IMI (Ruegg and Reinemann, 2002).

Currently, assays often used include measurement of SCCs, enzymatic analysis and CMT (Viguier et al. 2009). The presence of a pathogen in the mammary gland causes an increase in the number of immune cells, mostly neutrophils, to fight the infection. Therefore, an elevated cell concentration can be used as an indicator of infection (Madouasse et al. 2012).

Thermographic imaging has been recently applied in veterinary medicine and in animal production as a potential diagnostic and preventive tool, thus avoiding undue stress reactions.

In the bovine species, infrared thermography (IRT) has been used as a diagnostic tool for disease detection (Stelletta et al. 2012). In dairy cows, IRT has the potential to be a rapid, non-invasive, real-time method of detecting mastitis since a symptom of mastitis is the increase in local temperature from inflammatory reactions (Scott et al. 2000, Colak et al. 2008). Hovinen et al. (2008) infused *E. coli* lipopolysaccharides (LPS) into the left forequarter of the udder and showed that the treated quarter exhibited an increase in surface temperature of 1 – 1.5°C compared to the untreated right quarter. In a similar experiment, Pezeshki et al. (2011) compared several potential biomarkers of mastitis via induction with *E. coli*. Peak udder surface temperature was noted to increase 2 – 3°C but this increase was slower than the one in rectal temperature. In a study that included 62 Brown Swiss dairy cows, IRT ability to detect mastitis was compared to Somatic Cell Count (SCC) and California Mastitis Test (CMT); the study showed that sensitivity and specificity of IRT (95.6 and 93.6%, respectively) did not differ from those for CMT (88.9 and 98.9%, respectively) (Polat et al. 2010).

The aim of this study was to evaluate the potential application of thermographic imaging compared to SCC and bacteriological culture for infection detection in cow;s quarters affected by subclinical mastitis and possibly to discriminate between different pathogens.

Materials and Methods

Selection of animals and herd characteristics

98 Holstein Friesian cows with high SCC (>200.000 cells/ml) at the last test-day record performed by the Regional breeder association were se-

lected in 4 medium sized farms (80-200 lactating cows) in Veneto Region, Italy. The cows were selected taking into account also the parity order (1 to 4 calvings) and the conformation of the udder, excessive laxity of the udder ligament or excess of dirt on the udder skin were cause of the exclusion.

The selected animals were in different stage of lactation comprised between 14 and 270 days in milk. Cows with clinical sign of mastitis, as described by Radostis et al. (2007), were excluded. The sampling had been conducted from October to December 2014. No ventilators or water dropping was active at the time of sampling.

All the farms were free ranged and feed the cows with TMR (Total Mixed Ration) distributed twice a day. In all the farms cow's bedding was straw, changed from 2 to 3 times per week. Cows were milked twice a day with 12 hours interval between milking.

Collection of thermographic images

Milking-room temperature (14-22ºC) and humidity (72-94%) were recorded before the sampling and set on the camera. Thermographic images of the udder quarters and teat ends were taken using an infrared camera P25, Flir SystemsTM, as described by Berry et al. (2003). In brief, according to the type of milking facility, the camera was held at udder level, 1.0 m behind/on the site of the standing cow, with the tail held away. To obtain clear images of all functional quarters and teat ends 2-4 images were needed. The software used for image analysis was ThermaCam Researcher Basic 2.08, Flir SystemTM. Ten random spots of each quarter and the entire teat end area were analyzed to obtain the temperatures of the zones interested in this survey. Minimum, maximum and average temperature measures were collected.

Collection of milk samples

Collection of mammary secretion was done aseptically according to National Mastitis Council guidelines (Hogan et al. 1999). In brief, teat ends were cleaned externally first with commercial pre-milking disinfectant solutions then dried with individual towel and after cleaned again with alcohol. The first few streams of foremilk were discarded and approximately 10 ml of milk for each quarter were collected into sterile tubes. Another milk sample (25 ml) was collected in sterile containers and preserved with sodium azide for assessing SCC with a Fossomatic cell counter (Foss Electric, Denmark). Milk samples were stored at 2-6ºC and cultured until 24 hours.

Bacteriological cultures and identification

Bacteriological cultures were carried out as described (Hogan et al. 1999). In summary, an approximate 10 fl aliquot from each milk sample was inoculated onto 5% defibrinated sheep blood (Allevamento Blood, Teramo, Italy) agar base (Biolife, Milano, Italy) plates containing 0.01% esculin (BD BBL, New Jersey, USA) using individual disposable sterile loop and incubated in aerobic conditions for 24 h and 48 h at $37^0 \pm 1$ºC.

A presumptive identification of microorganisms as *Staphylococcus* spp., *Streptococcus* spp., and coliform bacteria was made based on colony morphology, presence of pigmentation, haemolysis type, Gram stain appearance and catalase test. *Staphylococcus aureus* was differentiated from CNS on the basis of a positive coagulase reaction on rabbit plasma (Istituto Zooprofilattico Sperimentale delle Venezie, Legnaro, Italy). *Streptococcus agalactiae* was identified using colony morphology, absence of growth on Bile Esculin Agar (BEA) and a positive CAMP reaction. *Streptococcus uberis* was identified using colony morphology, absence of growth on BEA and negative CAMP reaction. A presumptive identification of *Corynebacterium spp*. was based on colony morphology after 48 h, a positive catalase reaction and Gram stain appearance.

A sample was considered contaminated when three or more dissimilar colony types were observed with no predomination of a single colony type (Hogan et al. 1999).

Mycoplasma culture and identification

2 ml of milk were centrifuged at 6000 rpm for 5 minutes, the supernatant was discarded and the pellet was inoculated into 2 ML of Mammal Mycoplasma Experience® (ME) broth medium (Reigate, UK). Approximately 200 µl of the inoculated broth were transferred in a second broth (2 Ml of ME) called „diluted", all inoculated broths were then incubated at 37± 1ºC under 5% CO2 conditions. Inoculated broths were checked daily for 7 days. If the broths changed color or turbidity, they were inoculated onto ME agar medium and checked daily for the presence of *Mycoplasma spp.* suspected colonies. If at the end of the 7th day no change was visible in the broths, an inoculation in a ME agar plate was performed. The samples were considered negative if at the 15th day no suspected colonies were reported. In order to confirm the identification of *Mycoplasma spp.* colonies, DNAs were extracted from 0.2 ml of suspect broths and a 16S-rDNA PCR and Denaturing Gradient Gel Electrophoresis

Table 1. Bacteriological culture results for farms A, B, C and D, number of cows and quarters sampled.

Farm	Quarters *(cows)*	Negative	*S. aureus*	*S. agalactiae*	*S. uberis*	CNS	*Mycoplasma bovis*	Others Pathogens
A	127 (32)	82	5	0	9	24	7	0
B	110 (30)	35	43	0	15	13	0	2 (Group D Streptococci) 1 (*E. coli*) 1 (*Corynebacterium spp.*)
C	80 (20)	21	36	8	0	14	0	1 (*Corynebacterium spp.*)
D	63 (16)	41	0	17	2	3	0	0
Total	380	179	84	25	26	54	7	7

CNS: Coagulase Negative Staphylococci.

Table 2. Means of SCC, SCS, SCC -1, and SCC -2 divided according to bacteriological results.

	N° of Quarters	SCC (cells/ml x 10^6)	SCS	SCC -1 (cells/ml x 10^3)	SCC -2 (cells/ml x 10^3)
Negative	179	1.01	4.1^{a}	1.49	0.96
S. aureus	84	1.99	5.8^{b}	1.25	0.89
S. agalactiae	25	4.66	$7.3^{b,c}$	1.04	1.86
S. uberis	26	4.24	$6.7^{b,c}$	2.50	1.55
CNS	54	1.97	$4.8^{a,d}$	1.91	0.87

^{a,b,c,d}: different letters indicates statistically different means.
SCC -1: Mean SCC at the last functional control (7-30 days before the sampling).
SCC -2: Mean SCC at the previous functional control (37-60 days before the sampling).
CNS: Coagulase Negative Staphylococci.

Table 3. Minimum, Maximum and Average temperatures ± Standard Deviation measured both at Nipple and udder Quarter level divided according to the bacteriological culture results.

	N° of Quarters	Min Nipple (°C)	Max Nipple (°C)	Average Nipple (°C)	Min Quarter (°C)	Max Quarter (°C)	Average Quarter (°C)
Negative	179	26.12 ± 0.42	33.34 ± 0.17	30.42 ± 0.26	30.82 ± 0.31	34.32 ± 0.14	33.08 ± 0.17
S. aureus	84	23.83 ± 0.58	32.49 ± 0.22	28.75 ± 0.33	28.70 ± 0.45	33.52 ± 0.20	31.95 ± 0.23
S. agalactiae	25	24.47 ± 0.93	32.58 ± 0.33	28.86 ± 0.51	30.67 ± 0.73	34.10 ± 0.31	32.93 ± 0.36
S. uberis	26	24.10 ± 0.83	32.23 ± 0.30	29.47 ± 0.35	29.29 ± 0.47	33.21 ± 0.20	31.85 ± 0.24
CNS	54	25.35 ± 0.61	33.04 ± 0.23	30.10 ± 0.45	30.56 ± 0.65	34.40 ± 0.28	32.90 ± 0.33

CNS: Coagulase Negative Staphylococci.

(DGGE) method were performed as described by McAuliffe et al. (2005).

Statistical analysis

SCS was calculated as described in literature: Score=log2(SCC/100.000)+3 (Reents et al. 1995). A Multivariate Analysis of Variance (ANOVA) was conducted in order to evaluate differences between SCC, SCS and Udder Surface Temperature (UST) according to the results of the bacteriological exam. To investigate the presence of an association between udder surface temperatures and SCS, Pearson's coefficients were calculated. Significance was defined at $p<0.05$. All the data were analyzed with SAS software (Littell 2006).

Results

Bacteriological and Mycoplasma culture

Bacteriological results are shown in Table 1. *S. aureus* was the most frequent pathogen isolated in subclinical mastitis cows in the farms involved in this study, accounting for the 41.8% of the positive samples and being present in 3 of 4 farms. *Streptococcus agalactiae* and *Streptococcus uberis* were present in the 12.4 and 11.9% of the positive samples respective-

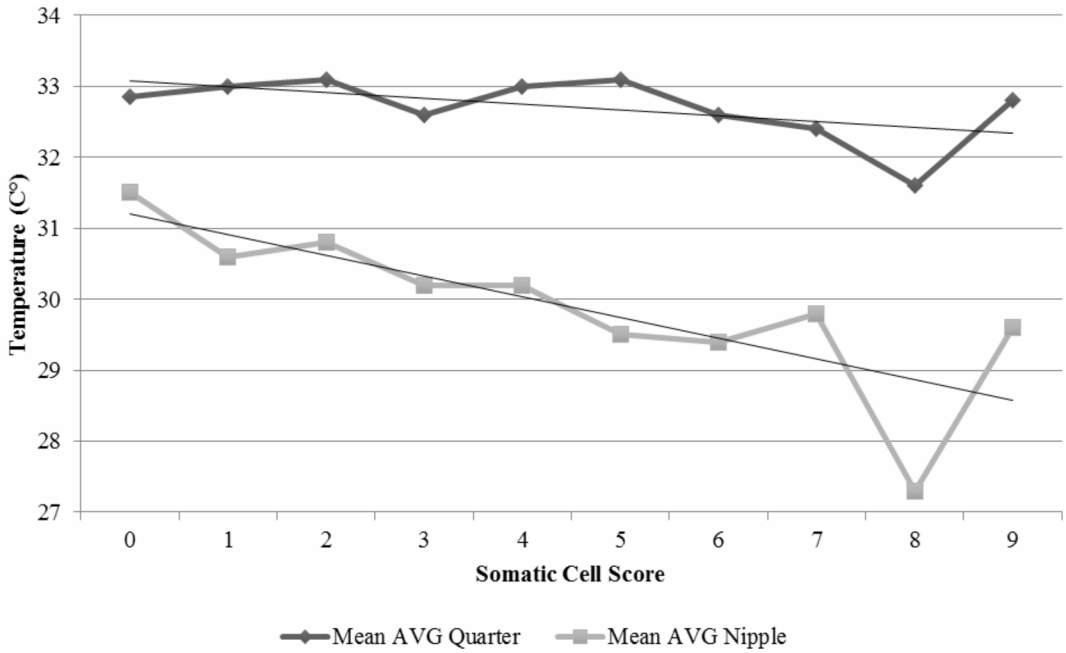

Fig. 1. Mean Average (AVG) temperatures (°C) at Quarter and Nipple level according to Somatic Cell Score.

ly. Other pathogens as Group D Streptococci, *E. coli* and *Corynebacterium spp*. were isolated from the farms involved in the study. In Farm A seven quarters resulted positive for *Mycoplasma bovis* and belonged to three different animals.

Somatic Cell Count

In Table 2 the results of SCC and SCS according to the bacteriological results and the farm records of SCCs of the cows enrolled in this study are summarized. Streptococci caused the highest raise in SCC (4.66 and 4.24x10^6 cells/ml for *S. agalactiae* and *S. uberis* respectively) while the lowest SCC was determined in the quarters negative for bacterial growth. *Staphylococcus aureus* and CNS infected quarters had similar means for SCC (1.99 and 1.97x10^6 cells/ml respectively) but *S. aureus* positive quarters had a higher mean SCS (5.8 ± 0.3 vs 4.8 ± 0.4). The SCS resulted more sensitive than SCC for the identification of quarters positive for bacterial growth. Only SCS of quarters positive for CNS did not differ from negative quarters. Comparing the positive quarters, SCS of quarters infected by Streptococci significantly ($p<0.05$) differed from quarters infected with CNS, but SCS was not able to differentiate between *S. aureus* and CNS.

Udder surface temperatures

The mean temperatures measured both at quarter and nipple levels at increasing SCS are shown in Fig. 1. The statistical analysis revealed a negative association between averages (AVG) temperatures at quarter (-0.12) ($p<0.05$) and nipple (-0.28) ($p<0.01$) level and SCS. The same significant ($p<0.05$) association was observed also in nipple minimum (-0.22) and maximum (-0.20) temperature and quarter maximum (-0.16) temperature. No associations were found between a positive bacteriological culture and temperature measured at both nipple and quarter level. UST resulted higher in negative quarters (30.42 ± 0.26°C at nipple level and 33.08 ± 0.17°C at quarter level) than in positive quarters.

Table 3 presents the average, minimum and maximum temperatures at both nipple and quarter level associated to the pathogen isolated from bacterial culture.

Discussion

The high prevalence of *S. aureus* in our study support the findings of Bertocchi et al. (2012) who investigated the prevalence of different mastitis pathogens in the Northern part of Italy, and confirmed the important role of this pathogen in subclinical mastitis issues. Coagulase Negative Staphylococci were present in 26.9% of the positive samples and these results confirm the recent literature that describes CNS as an

emerging cause of subclinical mastitis (Pyörälä and Taponen 2009). Udder infection caused by *Mycoplasma bovis* is well described in the literature (Jasper 1977) but the prevalence of this contagious microorganism as a cause of clinical and subclinical mastitis is still unknown in Italy. Since many diagnostic laboratories in Italy do not routinely include *Mycoplasma spp.* culture in the diagnostic investigation of milk samples, the role of this pathogen as a mastitis and subclinical mastitis causative agent could be underestimated.

Other pathogens were isolated from the milk samples, as Group D Streptococci, *E. coli* and *Corynebacterium spp*., with lower frequency and together with *M. bovis* the number of samples were too low to be used for statistical purposes.

The SCS resulted more sensitive than SCC for the identification of positive quarters. Only the CNS positive quarters did not differ from the negatives which was probably caused by the moderate increase in SCC of cows infected with CNS as previous observations showed (Lam et al. 1997). The cell concentration in uninfected quarters is still debated. In Europe, elevated SCCs above 200 000 cells/ml are often considered as an indicator of mastitis (Schukken et al. 2003). The SCC of negative quarters found in this study is high but an extreme variability has been previously reported by Djabry et al. (2002) with a range of 7 000 to 1 849 000 cells/ml in herds characterized by high SCC (> 700 000 cells/ml). The presence of an increased risk for a positive bacteriological culture in cows with high SCC has been previously reported (Sgorbini et al. 2014) and the effect of pathogens is more evident if quarter SCC is used (Schwarz et al. 2010).

The decrease in temperature observed in cows with high SCC may be explained as a decrease in functionality and blood flow to the affected udder (McGavin et al. 2007). For *S. aureus* a severe effect on the udder tissues has been well described in the literature with lesions of the alveolar secretory epithelial cells of variable severity, shrinkage of alveoli, proliferations of conjunctive tissues (Sutra and Poutrel 1994).

For other bacteria, the mammary tissue damage during IMI has not been studied so extensively but is probably underestimated because of the lack of sensitive and non-invasive detection methods (Zhao and Lacasse 2008). These results are in contrast with previous findings (Polat et al. 2010), which reported a positive association between SCC and USTs. SCC is, however, an unspecific indicator of inflammatory process and a raise in SCC is not necessary caused by bacterial infections (Hillerton 1999). In our work, the decrease in temperature at increasing SCC may be related to inflammation processes limited to deeper portions of the udder quarter thus not causing an increase in UST or even, the deviation of the blood flow could cause a decrease in the UST. Invasion and localization into deeper tissue after the entrance into the udder quarter is typical of bacterial strains that cause persistent infections (Haveri et al. 2005). A similar decrease in temperature observed in cows with high SCS were observed in the animals with positive bacteriological culture. The higher effect, although not significant, has been observed in cows positive for *S. aureus* and *S. uberis*, two pathogens that frequently colonize the udder with IMI poorly responsive to antibiotic treatments and that often evolve in chronic forms of mastitis (Pedersen et al. 2003).

The cows resulted negative at the bacteriological exam seems to have higher temperatures at all levels. This result could be explained by a successful clearance of the infection with fewer tissue damages and a restoration of the normal blood flow (McGavin et al. 2007). The USTs of the healthy quarters found in this study are similar to the results obtained in previous works (Berry et al. 2003, Polat et al. 2010) where the temperature measured at udder level was respectively 33.42ºC and 33.45ºC. The temperature obtained in subclinical mastitis cows in this work, however, was lower than the temperatures reported by Polat et al. (2010) who investigated the use of thermal imaging in subclinical mastitis investigations, even if in a different breed (Holstein Friesian vs Brown Swiss) and classified only according to SCC without taking into account the aetiology of mastitis. These differences in findings may be caused by the differences in the stage of the infection, since a late stage of infection is characterized by having more repair process and less inflammatory response compared to earlier stage of infection, or by differences in the aetiological agent (Benites et al. 2002).

In conclusion, the SCS has been able to identify cows infected by different pathogens except from the animals infected with CNS. The lack of a significant association between aetiological agent and udder temperatures confirm the poor diagnostic value of termography in subclinical mastitis aetiology investigations.

However, the association found between SCS and temperatures suggests the use of thermographic imaging as a screening tool helpful in the evaluation of an inflammation status of the udder. At the moment, SCC and SCS remains the method of election to monitor the udder status of the herd and further studies are needed to confirm the discriminatory potential of SCS in subclinical mastitis investigations.

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