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**Bovine mastitis, an evolving disease: Application of infrared  
thermography for the diagnosis of subclinical forms and presence of  
methicillin resistant *Staphylococcus aureus* in dairy farms**

**Direttore della Scuola:** Ch.mo Prof. Gianfranco Gabai

**Supervisore:** Ch.mo Prof. Massimo Morgante

**Dottorando:** Alessio Bortolami



*Dedicato a tutta la mia famiglia  
che mi ha sempre sostenuto ed aiutato,  
ai miei amici e ad Alice  
che mi ama piu' di quanto ritenessi possibile.*



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# 1. 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.

In the last years the fight against mastitis has seen the emergence of mastitis cases caused by multiresistant bacteria which poorly respond to antibiotic treatments. One of the most important pathogens because of this aspects of antimicrobial resistance and for its potential as zoonotic pathogen is the Methicillin Resistant *Staphylococcus aureus* (MRSA).

The aim of this thesis is to investigate two important aspects of bovine mastitis, in a first study we evaluated the potential of infrared thermography in the diagnosis of subclinical mastitis and in a second study it was investigated the presence and the prevalence of MRSA in the territory of the Veneto Region.

In the first 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. Thermographic images were 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.

In the second study we investigated the presence and prevalence of MRSA in the Veneto Region. In order to do that 70 farms holding at least 100 animals were selected. In each farm were collected composite milk samples and nasal swabs from lactating cows.

The samples collected were submitted for specific bacteriological culture for MRSA. The isolated strains were characterized to discover the presence of genes codifying for the resistance to several antimicrobial classes and to evaluate the epidemiological characteristics.

15 MRSA strains were isolated from 6 farms. 13 from milk samples and 2 from nasal swabs. The prevalence found was similar to what obtained in previous studies conducted in other European countries. The prevalence inside the farms was low and no farm was simultaneously positive for MRSA in milk and in nasal swabs. The low prevalence of colonization of the upper respiratory tract is somewhat surprising, considering the high prevalence of colonization in veal calves farms, in which are raised male calves coming from dairy farms.

The genetic characterization has determined the belonging of the isolated strains to well known clones (CC1, CC97, CC398) adapted to livestock (Livestock Associated MRSA) and different from the more dangerous strains associated to healthcare environment (Hospital Associated MRSA). The clones found have anyway already proven their zoonotic potential and represent not only a new challenge in the fight against bovine mastitis but also a risk for the exposed personnel.

## 2. RIASSUNTO

La mastite subclinica è causa di importanti perdite economiche per gli allevatori di vacche da latte. Il monitoraggio dello stato di salute della mammilla negli allevamenti è generalmente effettuato tramite la Conta delle Cellule Somatiche (SCC) ma a causa della scarsa sensibilità di questa metodica c'è la necessità di trovare nuove tecniche diagnostiche in grado di identificare rapidamente infezioni subcliniche della mammella. Ad aggiungersi alle difficoltà nella diagnosi di mastite negli ultimi anni sono comparse forme di mastite causate da batteri multiresistenti che causano un fallimento delle terapie antibiotiche. Uno dei batteri di primaria importanza sia per gli aspetti di antibioticoresistenza che per l'importanza quale agente zoonosico è lo *Staphylococcus aureus* meticillino-resistente (MRSA).

Lo scopo di questa tesi è stato quello di investigare due tematiche relative alla mastite bovina, in un primo studio è stata valutata l'applicazione di un nuovo strumento diagnostico per la diagnosi delle infezioni subcliniche, mentre nel secondo studio è stata valutata la presenza nel territorio Veneto di un patogeno emergente, L'MRSA.

Nel primo studio è stato valutato lo stato sanitario della mammella di 98 vacche di razza Frisona con elevata SCC, suddivise in 4 allevamenti della Regione Veneto. Da ogni vacca sono stati raccolti campioni di latte raccolti in maniera asettica su cui è stata eseguita la conta cellulare, l'esame batteriologico e la cultura per *Mycoplasma spp.*. Sono state inoltre raccolte immagini termografiche per la rilevazione delle temperature superficiali di tutti i quarti e i capezzoli funzionali. Al fine di valutare le differenti tecniche diagnostiche sono state effettuate Correlazioni di Pearson, Analisi della Varianza (ANOVA) e curve ROC.

Il patogeno isolato con maggiore frequenza è lo *Staphylococcus aureus* seguito dagli Stafilococchi coagulasi negativi (CNS), *Streptococcus uberis*, *Streptococcus agalactiae* e altri isolati con minore frequenza. Il Somatic Cell Score (SCS), calcolato sulla base della conta delle cellule somatiche è stato

in grado di discriminare ( $p < 0.05$ ) i quarti positivi per un patogeno rispetto ai quarti negativi, con l'eccezione dei quarti infetti a causa di CNS. Le temperature rilevate con l'analisi delle immagini termografiche sono positivamente correlate al SCS ( $p < 0.05$ ) ma non sono in grado di discriminare tra quarti sani e quarti infetti. In conclusione la termografia sembra uno strumento promettente per una valutazione veloce ed economica dello stato infiammatorio della mammella e si è rivelata adatta all'applicazione in campo in quanto compatibile con la routine di mungitura ma i nostri risultati dimostrano uno scarso potere diagnostico.

Nel secondo studio è stata oggetto di indagine la presenza e la prevalenza nel territorio Veneto degli MRSA negli allevamenti di vacche da latte.

Sono stati raccolti campioni di latte individuale e tamponi nasali in 70 allevamenti del Veneto di almeno 100 capi. Sui campioni raccolti è stata effettuato un'esame batteriologico specifico per l'isolamento di MRSA. I ceppi isolati sono stati caratterizzati dal punto di vista genetico per valutare la presenza di geni codificanti la resistenza ad importanti classi antibiotiche e per valutarne le caratteristiche epidemiologiche.

Sono stati isolati 15 ceppi di MRSA da 6 allevamenti. 13 da campioni di latte e 2 da tamponi nasali. La prevalenza degli MRSA nel territorio oggetto di indagine si è rivelata simile a quella ottenuta in indagini condotte in diversi paesi europei. La prevalenza intraaziendale si è rivelata molto contenuta e non si sono registrati allevamenti positivi contemporaneamente nel latte e nelle cavità nasali. La bassa prevalenza a livello nasale è un dato sorprendente considerate l'altissima prevalenza intra e inter aziendale negli allevamenti di vitelli destinati a produrre carne bianca, dove una considerevole percentuale degli animali è costituita da vitelli di razza Frisona maschi provenienti da allevamenti di vacche da latte.

Le caratterizzazioni genetiche hanno determinato l'appartenenza dei ceppi isolati a cloni (CC1, CC97 e CC398) ben adattati agli animali di interesse zootecnico (Livestock Associated MRSA) e distinti da quelli circolanti nelle comunità ospedaliere (Hospital Associated MRSA). Si tratta comunque di

ceppi molto diffusi con capacità di causare zoonosi e che rappresentano quindi non solo una nuova sfida nella lotta alla mastite bovina ma anche un potenziale rischio per il personale esposto.



### 3. INTRODUCTION

Bovine mastitis is a result of inflammation of the mammary gland. It usually occurs as an immune response to bacterial invasion of the teat canal by variety of bacterial sources present on the farm, and can also occur as a result of chemical, mechanical, or thermal injury to the cow's udder.

Mastitis is a general term for inflammation of the mammary gland. Inflammation, in the classical sense, is seen as redness, heat, swelling, and pain. Thus, the clinical signs of mastitis are a hard, red tinged, warmer than normal, udder, which may elicit a painful reaction when touched. 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).

The presentation of mastitis can take many forms, namely the acute form, which is accompanied by systemic signs of fever and mild depression, and the more severe peracute form, which is characterized by the presence of fever, depression, shivering, loss of appetite, and loss of weight. There is also a gangrenous form where the affected quarter becomes markedly swollen and the teat becomes cold and cyanotic. A line of demarcation forms separating the living from the dead tissue. Although mastitis can exhibit many forms, greater than 95% of the cases of mastitis are subclinical. Subclinical mastitis has been described by Radostits et al. (2006) as an infection without visible changes in milk or the udder. Subclinical mastitis can cause substantial economic loss due to reduced milk production and dairy plant fines because of high BMSCC.

Moreover, cows with subclinical mastitis should be considered as a risk for spread of mastitis pathogens within and between herds (Persson et al., 2011).

Mastitis usually occurs as an immune response to bacterial invasion of the teat canal by variety of bacterial sources present on the farm, and can also occur as a result of chemical, mechanical, or thermal injury to the cow's udder.

Milk-secreting tissues and various ducts throughout the udder can be damaged by bacterial toxins, and sometimes permanent damage to the udder occurs. Severe acute cases can be fatal, but even in cows that recover there may be consequences for the rest of the lactation and subsequent lactations. Practices such as close attention to milking hygiene, the culling of chronically-infected cows, good housing management and effective dairy cattle nutrition to promote good cow health are essential in helping to control herd mastitis levels.

Mastitis is most often transmitted by contact with the milking machine, and through contaminated hands or other materials, in housing, bedding and other equipment.

Whilst mastitis in cattle is well controlled in an historical context, there has been much debate about its apparent resurgence in recent years. In view of these recent changes, and the lack of a structured, coordinated approach to understanding and solving mastitis problems in dairy herds, it was felt that there was a need to investigate other technologies in order to provide to the dairy industry another instrument for disease diagnosis.

Moreover in the recent years we have seen in livestock the spreading of pathogens that have developed resistance to many antimicrobials of common veterinary use. One of the most important ones because of its zoonotic potential and the resistance to several classes of antibiotics is Methicillin Resistant *Staphylococcus aureus* (MRSA).

Since the first report of MRSA in livestock in clinical case of bovine mastitis in Belgium (Devriese et al., 1972), MRSA has been reported in livestock with increasing frequency, especially in pigs and veal calves.

Livestock-associated (LA)-MRSA colonization in food animals is an emerging problem that has put pressure on the scientific communities to define control strategies in order to reduce the spread in intensive livestock farming (Graveland et al., 2010). High prevalence of colonization in livestock may represent a work related health risk since people in direct contact with MRSA-positive animals have shown to have an increased risk of carrying the same MRSA strain as the animals (Catry et al.,



2010). Therefore, it is of importance to reduce LA-MRSA prevalence in food animals and consequently in exposed humans (Graveland et al., 2012).

### *3.1 Aetiological agents of mastitis*

Many bacteria, mycoplasmas, yeasts and algae may cause mastitis in dairy cows. Watts (1988) identified 137 different microorganisms as etiologic agent of mastitis but only a few of them are routinely isolated. Even several viruses may play a role in bovine mastitis aetiology (Wellenberg et al., 2002).

#### *3.1.1 Bacteria*

Bacterial mastitis pathogens have been divided in “contagious” and “environmental”.

##### *Contagious mastitis pathogens*

In essence, the contagious pathogens can be considered as organisms adapted to survive within the host, in particular within the mammary gland. They are capable of establishing sub-clinical infections, which are typically manifest as an elevation in the somatic cell count (leukocytes, predominantly neutrophils, and epithelial cells of milk from the affected quarter); they are typically spread from cow to cow at or around the time of milking (Radostits et al., 2006).

In the “contagious” group *Staphylococcus aureus*, *Streptococcus agalactiae* and *Mycoplasma bovis* are considered the major pathogens.

Often *S. aureus* mastitis is associated with a mild form of inflammation as noted by a Virginia Tech study where it was reported that approximately 40% of all cows with *S. aureus* mastitis infections had milk somatic cell counts of less than 400,000 cells/ml.<sup>5</sup> It has been noted that less than 100 colony forming units (cfu) of *S. aureus*/ml of milk can be isolated from infected quarters. The lack of somatic cell response, associated inflammatory mediators, and the low population of *S. aureus* within the gland are suggestive of mild cases of this disease. In chronic, either clinical or subclinical disease, there is loss of parenchymal and ductal tissue (Fox et al., 2002). Gudding et al. (1984) reported that *S. aureus* attaches to epithelium and appears to induce eroded and ulcerative tissue. It was also noted

that neutrophil extravasation is associated with the damaged epithelium. Presumably the neutrophil influx is a primary or secondary cause of the loss of milk production associated with increased milk somatic cell counts.

Resistance of *S. aureus* to antimicrobial agents can complicate treatment of its infections (Lowy, 2003). For treatment of mastitis, methicillin resistance, which is caused by the expression of the *mecA* gene, is of particular interest. Indeed, this mechanism confers resistance to almost all types of  $\beta$ -lactam antibiotics active against *S. aureus*, and these antibiotics are still frequently used in mastitis treatment (Sawant et al., 2005). However, methicillin-resistant *Staphylococcus aureus* (MRSA) has never been important in mastitis.

In 2005 a high prevalence of LA-MRSA was detected in pigs at Dutch slaughterhouses (De Neeling et al., 2007). Various reports confirmed this results also in Denmark (Guardabassi et al., 2007), Germany (Meemken et al., 2008) and Belgium (Denis et al., 2009). The predominant *spa* types found were t108, t034 and t011 all close relatives within CC398.

Graveland et al. (2010) in their survey involving 102 veal calves farms and farmers in Netherlands found positive the 88% of the farms and the 28% of the samples; most of the isolates belonged to the ST398 with 9 different *spa* types; in the same work were sampled also the farmers and their family and a prevalence of respectively of 33% and 8% was found, much higher than the estimated prevalence in humans in Holland which is around 1%.

MRSA has been described in mastitis only occasionally (Lee, 2003; Kwon et al., 2005; Moon et al., 2007; Hendriksen et al., 2008). From such studies, it seems that the prevalence of MRSA in mastitis is generally low. Yet, data on MRSA in mastitis need to be assessed carefully, as there are often ambiguities in the presence of *mecA*, level of investigation and origin of the detected MRSA strains. *Streptococcus agalactiae* is a highly contagious obligate parasite of the bovine mammary gland and was a major cause of mastitis in the pre-antibiotic era. It remains a significant cause of chronic mastitis in many herds, even though it can be readily eliminated. It generally causes a low-grade persistent type of infection and does not have a high self-cure rate. Unidentified infected cattle function as

reservoirs of infection, because they are not selected for treatment, segregation, or culling. For an obligate intramammary pathogen like *S. agalactiae*, the bovine udder is recognized as the only reasonable source of the organism in the milk.

A number of factors have been found to be associated with the prevalence of *S. agalactiae* in a herd. Foremost among these has been the failure to use post milking teat disinfectant and the selective or not use dry cow therapy. The use of a common wash rag or sponge has also been found to be a risk factor, as has the cleanliness of the cows, cleanliness of the exercise area, and the herd size.

Inadequate treatment of clinical cases of mastitis was observed more frequently in herds that were infected (Keefe, 1997).

The *Mycoplasma sp.* that cause mastitis are simple, cell wall-less, bacteria that can colonize and cause diseases in other extramammary sites in the bovine. Prevalence of mycoplasma mastitis appears to be increasing in many locations throughout the world (Fox et al., 2005).

Of the *Mycoplasma* species discovered, eight have been isolated from the bovine udder. By far the most frequent isolate recovered is *M. bovis*, but others such as *M. canadense*, *M. bovigentalium*, and *M. californicum* have also been cultured (Brandes and Kersting, 1999). The general consensus is that mycoplasma mastitis is a contagious mastitis pathogen that can be best controlled by strict milking time hygiene that includes post-milking teat asepsis, and identification and culling of infected animals (González and Wilson, 2003).

#### *Environmental mastitis pathogens*

The environmental pathogens are best described as opportunistic invaders of the mammary gland, not adapted to survival within the host; typically they 'invade', multiply, engender a host immune response and are rapidly eliminated (Bradley, 2002).

The major environmental pathogens are Enterobacteriaceae (as *Escherichia coli*, *Klebsiella pneumoniae* and *Pseudomonas aeruginosa*), *Streptococcus dysgalactiae*, *Streptococcus uberis* and *Corynebacterium bovis* (Blowey and Edmondson, 2010).

Despite the numerous studies carried out on mastitis caused by *Escherichia coli*, many open questions remain concerning the pathogenesis and clinical course of the disease.

The intensity of clinical signs reflects host response at the time of observation, and varies during the course of mastitis. The final outcome, as rapid elimination of bacteria, prolonged infection or death of the cow due to endotoxin shock, describes more the ability of the cow to limit deleterious inflammatory reactions and to clear the infection (Burvenich et al., 1994).

Different methods have been introduced for classifying the clinical course of *E. coli* mastitis. Cows can be divided into severe or mild responders based on intensity of general and local clinical signs, changes in appearance of milk and loss of milk production during mastitis (Lehtolainen, 2004).

Environmental streptococci are frequently isolated from bovine intramammary infections. Environmental streptococci include species of streptococci other than *Streptococcus agalactiae* and species of enterococci. *Streptococcus uberis* has been the most common species of environmental streptococci associated with bovine mastitis (Todhunter et al., 1995). Although *Streptococcus uberis* is a common cause of bovine mastitis, the epidemiology still is not fully understood and increasing rates of treatment failures are reported. *S. uberis* still must be mainly regarded as an environmental pathogen, but if conditions or cofactors promote infections, *S. uberis* seems to be able to spread like a contagious mastitis pathogen (Berger et al., 2012).

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 (Pyörälä and Taponen, 2009). *Trueperella pyogenes* (formerly known as *Arcanobacterium pyogenes*) the causative agents of the summer mastitis is routinely isolated from milk samples more frequently during the summer when flies can spread the pathogen or in farms with teat lesions problems (Unnerstad et al., 2009).

Environmental mastitis affects all dairy farms and generally is the major mastitis problem on modern, well managed dairy farms. Control measures effective against contagious pathogens are of little value in controlling of environmental pathogens. Control of environmental mastitis is achieved by reducing

exposure of teat ends to environmental pathogens and by maximizing the resistance of the cow to intramammary infection. Significant sources of environmental pathogens are organic bedding materials, manure covered alleyways, and wet or damp areas in barns, exercise lots, or pastures. Milking time hygiene can influence teat-end exposure. In general, exposure is minimized when all areas of the environment are clean, cool, and dry. Resistance is maximized by providing a stress-free environment that minimizes teat-end injury, and by feeding balanced diets sufficient in vitamin E and selenium. Antibiotic therapy during lactation or the dry period is of little value in the control of environmental mastitis in dairy herds, with the exception of preventing environmental streptococcal infection during the early dry period (Smith and Hogan, 1993).

### *3.1.2 Viruses*

Bovine herpesvirus 1, bovine herpesvirus 4, foot-and-mouth disease virus, and parainfluenza 3 virus have been isolated from milk from cows with clinical mastitis. Intramammary inoculations of bovine herpesvirus 1 or parainfluenza 3 virus-induced clinical mastitis, while an intramammary inoculation of foot-and-mouth disease virus resulted in necrosis of the mammary gland. Subclinical mastitis has been induced after a simultaneous intramammary and intranasal inoculation of lactating cows with bovine herpesvirus 4 (Wellenberg et al., 2002). Bovine herpesvirus 2, vaccinia, cowpox, pseudocowpox, vesicular stomatitis, foot-and-mouth disease viruses, and bovine papillomaviruses can play an indirect role in the aetiology of bovine mastitis (Burrows et al., 1971). These viruses can induce teat lesions, for instance in the ductus papillaris, which result in a reduction of the natural defense mechanisms of the udder and indirectly in bovine mastitis due to bacterial pathogens.

Bovine herpesvirus 1, bovine viral diarrhoea virus, bovine immunodeficiency virus, and bovine leukaemia virus infections may play an indirect role in bovine mastitis, due to their immunosuppressive properties (Yoshikawa et al., 1997).

### *3.1.3 Algae and yeasts*

A one-year examination of mammary secretions (n = 2,896) from Danish cattle with clinical or subclinical mastitis revealed 45 strains of fungi and algae. The strains originated from 44 mammary

secretions of 42 cows in 40 herds. The following species of fungi were identified: *Candida catenulata* (n = 2), *Candida kefyr* (n = 6), *Candida krusei* (n=17), *Candida rugosa* (n = 6), *Candida tropicalis* (n = 3), *Candida valida* (n=1), *Geotrichum capitatum* (n = 5). The algal species *Prototheca zopfii* was demonstrated in five samples (Aalbæk et al., 1994).

Since the first description by Lerche (1952) mastitis caused by *P. zopfii* has been reported worldwide, from temperate and tropical climatic zones as well.

Mastitis caused by *P. zopfii* is most often recognized as a chronic, symptom-less process with very high somatic cell count (SCC) (over  $10^6$  cells/ml); however, acute, clinical mastitis may also occur (Anderson and Walker, 1988).

The most important aspect of algal mastitis is the lack of an effective therapy for veterinary use. In antimicrobial susceptibility tests performed in vitro, the majority of *P. zopfii* strains have proved to be moderately susceptible to a few antibiotics and some fungicides (polymyxin B, gentamicin, nystatin, amphotericin B). Because of its extremely high costs and because of veterinary public health considerations, this treatment procedure is not applicable in dairy cows and leaves the only option of culling the infected cows to control the spread of this disease (Jánosi et al., 2001).

### 3.2 Financial impact

Mastitis continues to be the most economically important disease of dairy cattle, accounting for 38% of the total direct costs of the common production diseases (Kossaibati and Esslemont, 1997). It is notoriously difficult to estimate the losses associated with clinical mastitis, which arise from the costs of treatment, culling, death and decreased milk production.

Clinical mastitis costs were evaluated by Heikkilä et al. (2012) and reported for Holstein Friesian cows costs from €458 to €946 for each episode, mainly caused by long-term production losses. In Italy Colombo et al. (2010) calculated a cost that can vary from €50 to €350/cow/year. Also subclinical mastitis has an important impact on the finances of farms, 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). In many cases, the productivity of the cow will be permanently compromised (Halasa et al., 2007). Other money losses for the farm can come 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).

The effect of udder health on the yield and quality of milk and, consequently, on cheese production and quality has been established. If the milk has a high SCC, the deterioration during syneresis with a longer coagulation time and a weak coagulum leads to an increased moisture content in the cheese and a lower dry matter yield (Auldust et al., 1996).

This effect has also been observed with Parmigiano Reggiano cheese by the reduced cheese yield is the consequence of a decrease of both milk casein and recovery of milk fat in cheese, as SCC increases. The data reported suggest that there was a significant decrease in 24 h actual cheese yield when the value of SCC was above 300 000 cells/ml, therefore before milk SCC reaches the legal limit of 400 000 cell/ml. Thus causing a decrease in profit of 46 euro for a cheese batches made with milk SCC within the range 300 000–400 000 cells/ml compared to batches made with milk SCC below 300 000 cells/ml.

### *3.3 Tests for diagnosis of mastitis*

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 method to demonstrate the presence of an infection is considered bacteriological culture due to its ability to detect bacterial, mycotic and algal infection (Madouasse et al., 2012).

Currently, assays often used include measurement of SCCs, enzymatic analysis and CMT (Viguier et al., 2009).

### *3.3.1 Bacteriological culture*

Bacteriological culture is the standard method for identifying IMI. However, logistic and financial considerations involved in sampling all quarters at the time of calving have precluded widespread adoption of this strategy in the dairy industry.

If an effective means to identify fresh cows at a high risk for IMI were available and validated, it might increase the adoption of bacteriological culture (Sargeant et al., 2001).

The gold standard, as previously stated, 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).

### *3.3.2 Somatic Cell Count*

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). Inflammation of the mammary gland that results from the introduction and multiplication of pathogenic microorganisms in the mammary gland is a complex series of events leading to reduced synthetic activity, compositional changes, and elevated SCC. The magnitude and temporal relationships of these responses vary with nutritional status, other animal factors, and the pathogen involved. Because the elevation of SCC is a response to an insult to the mammary gland and is modulated by inflammatory mediators, the major factor influencing SCC is infection status. The effects of stage of lactation, age, season, and various stresses on SCC are minor



if the gland is uninfected. Except for normal diurnal variation, few factors other than infection status have a significant impact on milk SCC (Harmon, 1994).

Rapid movement of leukocytes into the mammary gland is one of the most important natural defense mechanisms against mastitis. Leukocytes, also called somatic cells, are present at concentrations less than 200,000 cells/ml in milk from uninfected glands, and are the majority of cells comprising the somatic cell count (SCC). Leukocyte numbers increase markedly in response to invading pathogens, and may reach concentrations of millions/ml in acute mastitis cases.

The vast majority of leukocytes in milk during inflammation are the polymorphonuclear neutrophilic leukocytes (PMN) which enter the mammary gland from the blood. The PMN are attracted to the quarter by the presence of infecting microorganisms. The PMN recognize bacterial cells coated with antibodies, engulf these microorganisms, and kill them, a process known as phagocytosis. A 12 to 24-hour lag after initial infection generally occurs before a high leukocyte concentration is found in milk, although some microorganisms such as *Escherichia coli* cause a more rapid response.

Other leukocyte types in the udder are lymphocytes and macrophages. Lymphocytes are the "conductors" of the immune system, and coordinate the activities of all cells in the immune system by releasing soluble mediators called cytokines. Cytokines are hormone-like proteins that are produced locally in the udder in very low levels. These proteins play an important role in stimulating the recruitment of cells into the udder following bacterial invasion. Cytokines also are responsible for initiating the development of antibody-producing plasma cells from activated lymphocytes.

Like the PMN, macrophages can phagocytize and kill bacteria. However, a major role of macrophages is their ability to stimulate the migration of PMN into milk by releasing cytokines and leukotrienes.

The cell concentration in uninfected quarters is still debated. In a review of 21 studies Djabri et al. (2002) estimated the geometric mean somatic cell count of uninfected quarters to be of 68,000 cells/ml. In Europe, elevated SCCs above 200,000 cells/ml are often used as an indicator of mastitis (Schukken et al., 2003).

### *3.3.3 California Mastitis Test*

In 1957 Schalm and Noorlander [54](54) reported the development of a rapid, simple test for mastitis which they called the California Mastitis Test (CMT). They indicated that the degree of precipitation and gel formed by a mixture of the reagent and milk reflected the cell count of the milk. They showed that when milk from normal gland was mixed with abnormal milk and tested, a gel or precipitate was produced. Jensen (1957) reported that the reaction was due solely to the formation of a gel of leucocyte proteins and found that the test was negative until the count exceeded 500,000 cells per ml. Since the first development CMT has been widely used worldwide as simple and economic cow side test. The sensitivity and specificity of CMT compared to bacteriological culture have been extensively studied in literature with different results (Barnum and Newbould, 1961).

CMT had a sensitivity and specificity of 0.47 and 0.61 for detecting IMI in cattle that had recently calved, and another study 11 found that sensitivity and specificity of the CMT on day 4 of lactation were 0.82 and 0.80 for detecting IMI caused by major mastitis pathogens.

At the present day is widely used by farmers of North-East of Italy as screening method since even if the specificity for mastitis is low it has a good predictive value for SCC, making it useful to individuate the animals that may affect to a greater extent the Bulk Tank Milk Somatic Cell Count (BTMSCC).

### *3.3.4 Electric conductivity*

The only cow-side test which can be (has been) applied on each quarter automatically (in-line measurement) is the measurement of Electric Conductivity (EC). This has been investigated in many studies worldwide, but with often contradictory results.

The electrical conductivity values in milk of healthy quarters may show a high variability due to influences such as stage of lactation, breed, milking interval, or due to factors related to the general cow status (for example oestrus). Therefore, the distribution of electrical conductivity values in healthy quarters could overlap with the distribution of electrical conductivity values in milk of mastitic quarters. Even if some data suggest that electrical conductivity could be applied to the

diagnosis of clinical mastitis, the application of electrical conductivity as a parameter to indicate mastitis by measurements of composite or foremilk samples may not be sufficient in comparison with other mastitis diagnostic parameters.

Depending on the thresholds used, it was found that for subclinical mastitis in the case of low thresholds the frequency of false-positives was high (low specificity), while with high thresholds the frequency of false-negatives was high (low sensitivity) (Hamann and Zecconi, 1998).

In another study, traits reflecting the level rather than variation of EC, and in particular the Inter Quarter Ratio (IQR), performed best to classify cows correctly. By using this trait, 80.6% of clinical and 45.0% of subclinical cases were classified correctly. Of the cows classified as healthy, 74.8% were classified correctly (Norberg et al., 2004).

For the reasons explained even if in some of the farms electric conductivity is measured by the milking equipment, the data obtained are hard to interpretate.

### *3.3.5 Other indicators of bovine mastitis*

#### Enzymatic activity

Many of the indigenous enzymes increase in milk during inflammation. The enzymes dealing with the synthesis of milk decrease and the enzymes related to inflammation increase. The enzymes originating from phagocytes increase exponentially, and they include N-acetyl-D-glucosaminidase (NAGase), beta-glucuronidase and catalase (Pyörälä, 2003). The activity of the enzymes originating from the blood also increase, for example plasminogen which then is locally activated to plasmin, a proteolytic enzyme that degrades fibrin and casein. NAGase activity has in several studies been found to reliably detect IMI with mastitis pathogens (Kitchen, 1981). Milk NAGase activity correlates very closely with SCC (Mattila et al., 1986). It accurately reflects the degree of inflammation so that in mastitis caused by major pathogens, milk NAGase level is significantly higher than in mastitis due to minor pathogens (Mansfeld et al., 2001). The capacity of milk NAGase activity to detect sub-clinical mastitis was tested considering SCC above 400 000 cells/mL as a positive reference for mastitis (Ball

and Greer, 1991). It was concluded that the test performed well since it gave an average of 17% false positive and 2% false negative diagnoses (Pyörälä, 2003).

#### Acute phase proteins

The acute phase response refers to a group of non-specific host responses to a wide variety of stimuli and it is characterised by changes in the concentrations of a number of hepatically synthesised plasma proteins - the so-called acute phase proteins. Serum amyloid A and haptoglobin are the two major acute phase proteins in cattle (Nielsen et al., 2004).

In an experiment performed by Eckersall et al. (2001) the concentrations of the two acute phase proteins, serum amyloid A and haptoglobin, in serum and milk were compared in 10 cows with clinical mastitis, 11 cows with extramammary inflammatory conditions and 10 clinically healthy control cows. The concentrations of both acute phase proteins were higher in the serum and milk of the cows with mastitis than in the cows in the other two groups. Four of the cows with extramammary inflammatory conditions had serum amyloid A concentrations in serum above 100 µg/ml, but negligible concentrations in milk, indicating that a pathogen must be present in the mammary gland for serum amyloid A to accumulate in milk.

In another research project which focused on assessing milk samples from cows with various forms of mastitis were undertaken with a view to identifying new biomarkers for bovine mastitis. In this study the acute phase protein concentrations in milk increased significantly with increasing somatic cell count, suggesting that they may be indicators of the severity of an infection. The applicability of acute phase proteins and/or their changing patterns as mastitis biomarkers would require their adaptation to rapid (on farm) and robust measurement formats (Thomas, 2015).

#### *3.4 Infrared thermography*

Infrared thermography is a technique that allows the remote measurement of the surface temperature of an object. A thermal imaging camera provides colour images, where each colour corresponds to a specified temperature.

This is possible because thermography detects the infrared (IR) radiation spontaneously emitted from all bodies with a temperature higher than  $-273^{\circ}\text{C}$  and which has a wavelength between  $0.75\ \mu\text{m}$  and  $1000\ \mu\text{m}$  (Sparrow and Cess, 1978). This technology typically measures radiated electromagnetic energy in the  $3\text{--}12\ \mu\text{m}$  wavelength range (Schaefer et al., 2004).

The intensity and frequency of the emitted radiation are closely related to the temperature of the source through well-known laws of physics. In the late nineteenth century, physicists Stefan and Boltzmann established first experimentally, and then theoretically, the relationship existing between the quantity of energy emitted by a black body and its temperature. The energy ( $E$ ) is proportional to the fourth power of the absolute temperature ( $T$ ) and this is called the law of black body emission:

$$E = \sigma T^4$$

Where  $\sigma = 5,67 \cdot 10^{-8}\ \text{W/m}^2\ \text{K}^4$  is the Boltzmann constant.

Furthermore, Wien's law describes the relationship between the peak wavelengths of emission of a black body and its temperature ( $T$ ):

$$\lambda_{\text{max}} T = 2,910\ \text{m K}$$

Using these laws, modern thermal imaging cameras provide real-time thermal images accurate to hundredths of a degree which allow the smallest details to be seen. Modern detectors are of extremely high sensitivity and coupled to the excellent quality of the optical systems employed ensure the sensitivity of current thermal imaging cameras is capable of producing high resolution images. The sensitivity and resolution of any particular camera depend upon the size and specification of the detector.

The thermal imaging camera is very similar to an optical camera but has optics for infrared instead of the optical elements commonly used for general photography. Optical glass is not suitable for the purpose since it has a high absorbance in the wavelengths typical of the infrared. Germanium is the commonly used material because it is transparent to infrared radiation.

By contrast, the atmospheric transparency to infrared radiation is one of the primary factors that make the thermographic technique possible.

The emission peak moves towards shorter wavelengths as the temperature increases, so a material superheated to about 3000 K has a maximum emission in the area of near infrared (1  $\mu\text{m}$ ), while objects at room temperature (300 Kelvin, 27 °C) have the corresponding emission peak at 10  $\mu\text{m}$ .

The detector of the camera, on which the IR radiation is focused, transforms the incident energy into an electrical signal to be supplied to the amplification circuit. The detector output signal is amplified and converted from analog to digital and sent to a computerized system that displays images and processes the data that has been collected.

Another important characteristic is the ability to distinguish between two neighbouring points, called spatial resolution, which is usually expressed in mrad. An infrared thermography (IRT) IRT system with a spatial resolution of 1.4 mrad is able to distinguish the temperature of two points 1.4 mm apart at a distance of 1 m. A modern thermal imaging camera with a 320x240 pixel microbolometric detector and 35 mm standard optical lens, allows a spatial resolution of a few centimetres at 10 m distance. At that distance the smallest object that can correctly have its temperature measured must be 14 mm wide (1.4x10) (Luzi et al., 2013).

The advantage of the thermographic technique is the representation of the results in the form of digital images which can subsequently be appropriately edited to meet the specific requirements using image processing software. Thermal images are initially represented in grayscale, each level corresponding to the different intensity of radiation detected (Pajani, 1989). The false colour images are obtained by ascribing, to each grey level, a colour specifically chosen to better highlight thermal information.

#### *3.4.1 Applications of thermography in Veterinary Medicine*

Thermographic imaging has been recently applied in veterinary medicine and in animal production as a potential diagnostic and preventive tool.

One of the first report of application of thermography is an experiment performed by Smith (1964) in horses. Smith reported an increase in temperature in bruised hocks compared to uninjured legs or hocks[70](70). Since then several applications of thermography has been reported.

Infrared thermography (IRT) is a non-contact, and therefore remote, method of measuring the surface temperature of humans and animals. This fact gives rise to the concept of IRT as a method of disease surveillance.

Surveillance by IRT can be used on a static population where a group of subjects is continuously or repeatedly monitored. In these situations temperature measurement can be made on the group as a whole, or on individuals within the group. This type of surveillance is most appropriate for livestock animals in confined settings such as poultry, swine and dairy barns, or feedlot settings for beef cattle. In these contexts, IRT is used to measure surface temperature and infers pathological conditions from detection of aberrant temperature or to variation in temperature measurements.

However, the number of temperature measurements, whether single or multiple, places different emphases and expectations on the diagnostic utility and efficacy of IRT.

Multiple temperature measurements permit a more accurate assessment of thermal regulation in response to physiological challenges. Thus, changes in radiated temperature may reflect an organism's attempts to maintain core temperature in the face of those challenges. As a consequence, radiated temperature may not necessarily reflect core temperature but nonetheless be a superior indicator of thermal challenge because it reflects an animal's attempts at thermoregulation. This is important in the early diagnosis of disease prior to the onset of other symptoms such as fever.

In the bovine species, infrared thermography has been used as a diagnostic tool for disease detection. It may, for example, be used to reveal inflammation or subclinical pathological signs detecting an increase in surface temperature before the disease become evident (Stelletta et al., 2012).

In feedlot cattle, IRT has potential to detect changes in temperature before the appearance of clinical symptoms. In a small group of animals (n = 65) with a Bovine Respiratory Disease (BRD) prevalence of 14% the system demonstrated the potential to predict febrile disease approximately 2 days prior to

the onset of clinical symptoms (Schaefer et al., 2012). In dairy cows it has found applications also for the rapid detection of Digital Dermatitis. Setting the threshold for disease at 27 °C for dirty feet identified 80% of feet with lesions and 73% of feet without lesions correctly (Stokes et al., 2012).

#### 3.4.2 Infrared thermography applied to mastitis

Perhaps the best example of repeated temperature measurements by IRT of individual animals within a group is in the detection of mastitis in dairy cattle. Mastitis is the most important disease of dairy herds, incurring substantial costs to the dairy industry through loss of milk production and treatment costs. The severity of the disease ranges from sub-clinical to acute inflammation and is usually caused by bacterial infection of the teat canal. Sub-clinical infection is the most difficult to detect due to the absence of visible clinical symptoms but the costs to milk production are very high. It is important to diagnose mastitis as early as possible to increase the opportunity for a favourable treatment outcome and reduce the losses associated with a reduction in the quality and quantity of milk. Consequently, on-farm testing is a necessary requirement to reduce the economic impact of the disease. The search for a rapid, accurate and inexpensive test that can be conducted on-farm and is diagnostic of sub-clinical disease has been described as “the holy grail” for mastitis detection (Hillerton, 2003). These requirements have driven research efforts to include temperature measurements of the udder by IRT (Viguier et al., 2009).

In dairy cows, IRT has the potential to be a rapid, non-invasive, real-time method of detecting mastitis since the surface temperature of the skin reflects the underlying blood circulation and the metabolism of the tissue (Paulrud et al., 2005), some types of mastitis may cause an increase in the skin temperature of the udder (Colak et al., 2008; Polat et al., 2010), especially if mastitis is accompanied by fever (Hovinen 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). Thus, studies supporting the use of IRT for diagnosis of mastitis have shown a response in udder surface temperature to stimulation with LPS , correlation with CMT and diagnostic value compared to CMT.

Martins et al. (2013) evaluated the use of infrared thermograph for mastitis diagnosis in sheep, discovering higher temperatures in subclinical mastitis group than in control and clinical mastitis group.

There have been only a few detailed studies of IRT as a method for diagnosing mastitis (Paulrud et al., 2005 and Pezeshki et al., 2011), mainly due to the high technical and financial input required even for minimal standards of quantification. Obtaining reliable IR data from a live animal is challenging and to date temperature patterns required interpretation by trained persons (Jiang et al., 2005).



## **4. EVALUATION OF THE UDDER HEALTH STATUS IN SUBCLINICAL MASTITIS AFFECTED DAIRY COWS THROUGH BACTERIOLOGICAL CULTURE, SOMATIC CELL COUNT AND THERMOGRAPHIC IMAGING**

### *4.1 Aim*

The aim of this project was to evaluate the potential application of thermographic imaging during the milking routine to evaluate udder health status in cow's quarters with high Somatic Cell Count and possibly to discriminate between different pathogens.

For this purpose Udder Surface Temperatures measured before the milking were compared to SCC, SCS and bacteriological culture for the detection of bacterial infection.

### *4.2 Materials and methods*

98 Holstein Friesian cows were selected in 4 medium sized farms (80-200 lactating cows) in Veneto Region, Italy.

#### *4.2.1 Selection of animals*

Basing on the farm records of the last test-day performed by the Regional breeder associations (Associazione Provinciale Allevatori di Padova e Vicenza) cows with high SCC (>200.000 cells/ml) were selected.

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.

#### *4.2.2 Farm characteristics*

All the selected farms had high producing cows with mean milk production above 9000 kg per lactation. 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.

No ventilators or water dropping was active at the time of sampling.

Milking parlor type were Side opening ("Tandem") in farm A and Herringbone ("Fishbone") for farm B, C and D.

The sampling had been conducted from October to December 2014.

#### *4.2.3 Collection of thermographic images*

Milking-room temperature (14-22°C) 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 Systems<sup>TM</sup>, as described by Berry et al. (2003). In brief, according to the type of milking parlor, 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 images were taken before udder and teat end preparation for collection of milk samples in order to not alter udder surface temperature.

The software used for image analysis was ThermaCam Researcher Basic 2.08, Flir System<sup>TM</sup>. 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.

#### 4.2.4 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 and sphincters were cleaned externally first with commercial pre-milking disinfectant solutions then dried with individual paper 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), the SCC analysis was conducted by an external lab (Eptanord, Conselve, Italy).

Milk samples were stored at 2-6 °C and cultured until 24 hours.

Figure 1: Material used for collection of milk samples



#### 4.2.5 Bacteriological cultures and identification

Bacteriological cultures were carried out as described (Hogan et al., 1999) at the Diagnostic Laboratory of the Istituto Zooprofilattico Sperimentale delle Venezie, Legnaro, Italy. In summary, an approximate 10 µl 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  $37^{\circ}\pm 1^{\circ}\text{C}$ .

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.

CAMP reaction was observed on a 5% defibrinated sheep blood (Allevamento Blood, Teramo, Italy) agar base (Biolife, Milano, Italy) using a reference strain of *S. aureus* (ATCC<sup>®</sup> 25923) using the technique described by Darling (1975).

A presumptive identification of *Corynebacterium* spp. was based on colony morphology after 48 h, a positive catalase reaction and Gram stain appearance at 1000X observation.

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).

Plates with absence of growth at 24 hours were incubated again and checked again at 48 and 72 hours before being considered negative.

#### 4.2.6 Mycoplasma culture and identification

Mycoplasma culture and identification was performed at the Diagnostic Laboratory of the Istituto Zooprofilattico Sperimentale delle Venezie, Legnaro, Italy. 2 ml of milk of the same samples used

for bacterial culture 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  $37 \pm 1$  °C under 5% CO<sub>2</sub> 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 7<sup>th</sup> 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 15<sup>th</sup> 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 (DGGE) method were performed as described by McAuliffe et al. (2005). Example of a DGGE gel with the bovine Mycoplasma is shown in Figure 2.

Figure 2. DGGE of bovine mycoplasmas. Lanes: 1 and 9, *E. coli* marker; 2, *M. bovis*; 3, *M. bovoculi*; 4, *M. bovirhinis*; 5, *M. californicum*; 6, *M. canis*; 7, *M. bovigenitalium*; 8, *M. dispar*.

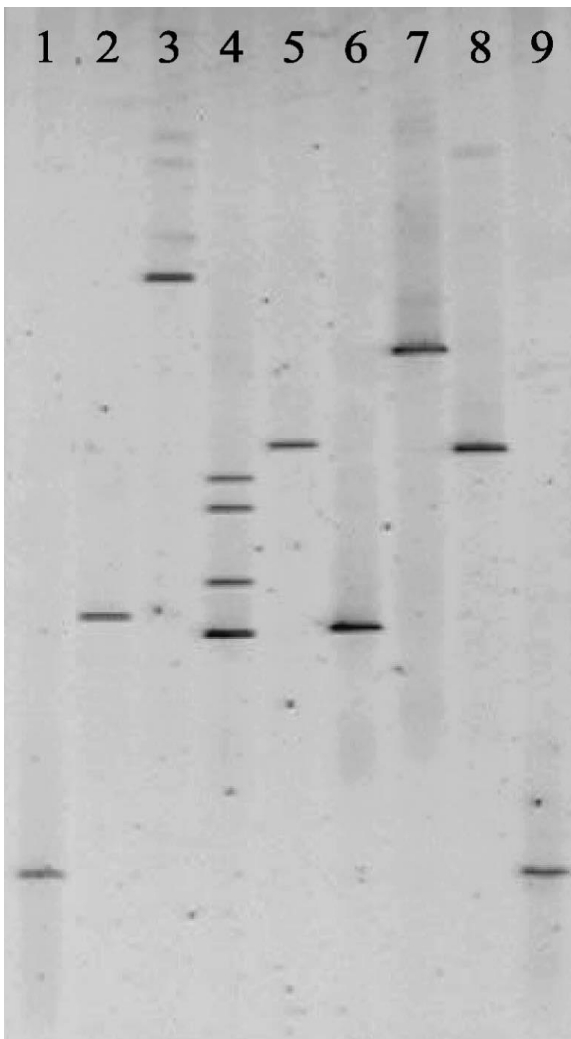


Image taken from: McAuliffe et al. (2005) Differentiation of *Mycoplasma* Species by 16S Ribosomal DNA PCR and Denaturing Gradient Gel Electrophoresis Fingerprinting.

#### 4.2.7 Statistical analysis

SCS was calculated as described in literature:  $\text{Score} = \log_2(\text{SCC}/100.000) + 3$  (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.

Roc analysis on Temperature data according to the results of the microbiological exams were done according to the procedure described by (DeLong et al., 1988). 95% Confidence interval (CI) were used when calculating sensitivity and specificity.

Significance was defined at  $p < 0.05$ . All the data were analyzed with SAS software (Littell 2006).

### 4.3 Results

#### 4.3.1 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 respectively.

Coagulase negative Staphylococci were present in all the farms investigated in this study.

Other pathogens as Group D Streptococci, *E. coli* and *Corynebacterium spp.* were isolated from the farms involved in the study.

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

Farm	Quarters (cows)	Negative	<i>S. aureus</i>	<i>S. agalactiae</i>	<i>S. uberis</i>	CNS	<i>Mycoplasma bovis</i>	Other 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 ( <i>E. coli</i> ) 1( <i>Corynebacterium spp.</i> )
C	80 (20)	21	36	8	0	14	0	1( <i>Corynebacterium spp.</i> )
D	63 (16)	41	0	17	2	3	0	0
Total	380	179	84	25	26	54	7	7

CNS: Coagulase Negative Staphylococci.

#### Farm A

82 samples didn't produced any growth and were considered negative. The most prevalent pathogen isolated were CNS, present in the 19% of the samples. *S. aureus* was present in 5 samples belonging to three different animals. 4 animals resulted positive for *S. uberis* (8 samples). Seven quarters resulted positive for *Mycoplasma spp.* subsequently identified as *Mycoplasma bovis* with the technique previously described and belonged to three different animals.

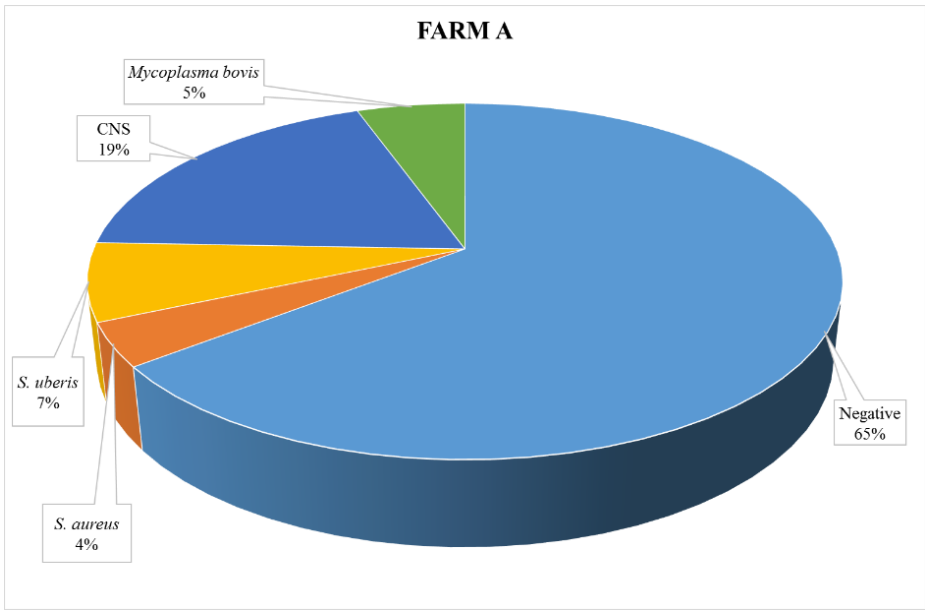


Chart 1: Farm A prevalence of mastitis pathogens and negative results.

**Farm B**

35 samples didn't produced any growth and were considered negative. The most prevalent pathogen isolated were *S. aureus* isolated from 42 samples belonging to 18 cows of 30 tested (60%). *S. uberis* and CNS had similar prevalence (respectively 13 and 12%). *E. coli*, *Corynebacterium spp.* And Group D streptococci were also isolated in this farm.

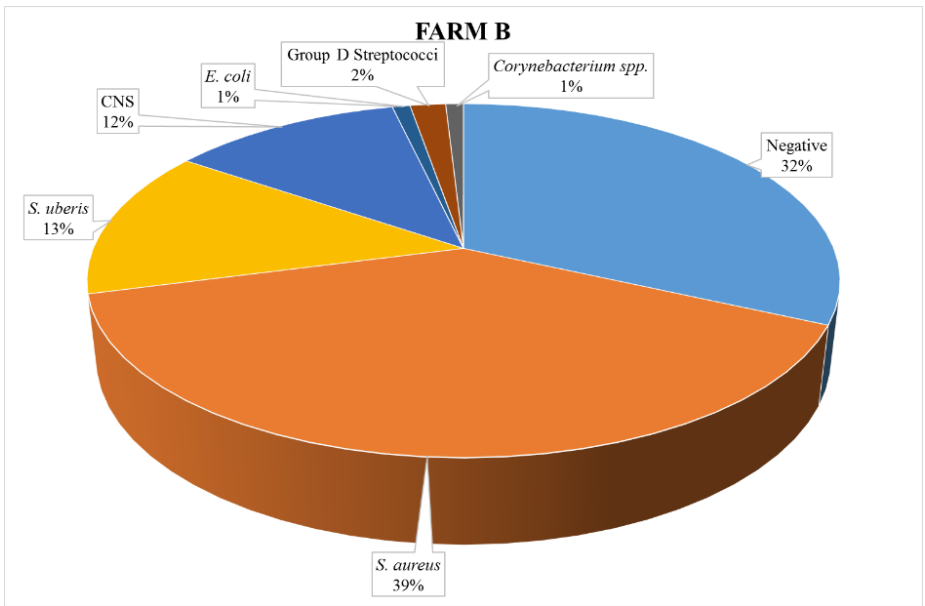


Chart 2: Farm B prevalence of mastitis pathogens and negative results.

### Farm C

26% of the samples resulted negative. 36 samples obtained from 17 cows yielded the growth of *S. aureus*. *S. agalactiae* were isolated from 5 cows. CNS were present in 18% of the samples. *Corynebacterium spp.* was isolated from only one sample.

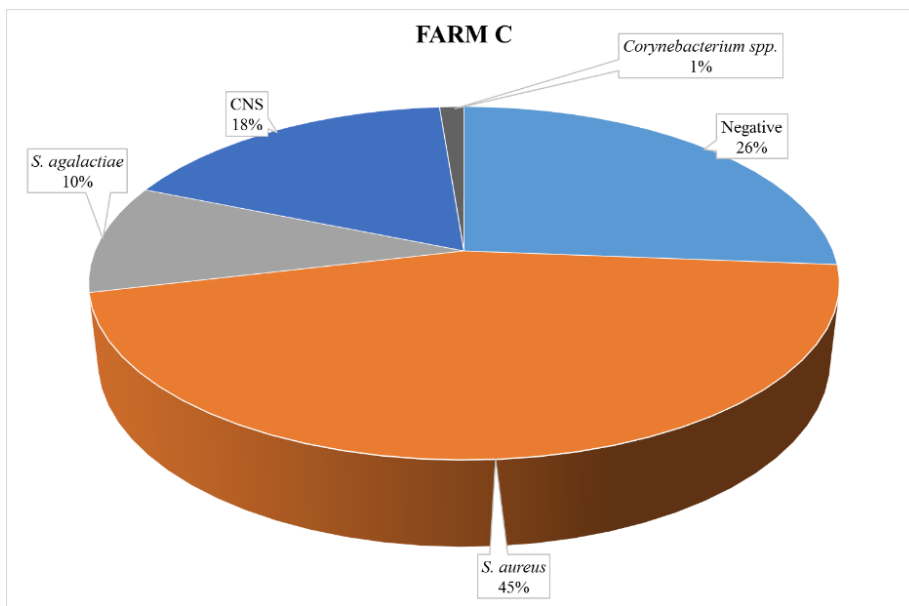


Chart 3: Farm C prevalence of mastitis pathogens and negative results.

### Farm D

65% of the samples failed to produce any growth and were considered negative. The most prevalent pathogen isolated was *S. agalactiae* present in 17 samples. *S. uberis* and CNS had similar prevalence (respectively 3 and 5% of the samples).

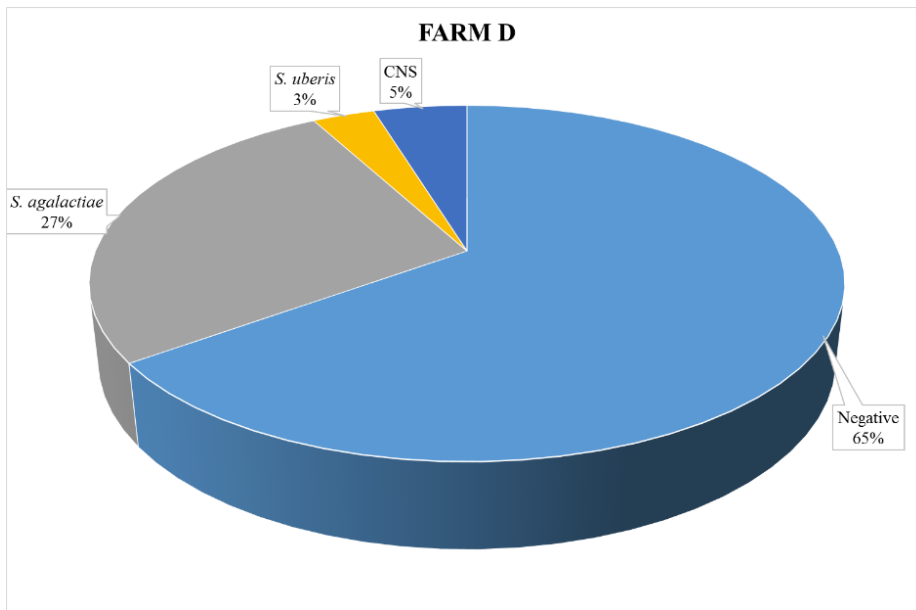


Chart 4: Farm D prevalence of mastitis pathogens and negative results.

#### 4.3.2 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  $4.24 \times 10^6$  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  $1.97 \times 10^6$  cells/ml respectively) but *S. aureus* positive quarters had a higher mean SCS ( $5.8 \pm 0.3$  vs  $4.8 \pm 0.4$ ).

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

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

<sup>a,b,c,d</sup>: 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.

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.

SCC below 200 000 cells/ml was recorded in 138 samples. 34 of them (24.6%) were positive for at least one pathogen (in one case a quarter was positive for *S. aureus* and *M. bovis*) leading the sensitivity of SCC to the 83.08% (77.17% - 87.99%, 95% CI).

In the remaining 243 samples with high SCC (>200 000 cells/ml) 76 samples (31.3%) were negative for bacterial culture with a specificity of 57.78 (50.21% to 65.09%, 95% CI).

### 4.3.3 Udder surface temperatures

Figure 3 and 4 show examples of the images taken in this study and subsequently analyzed to obtain USTs. In order to obtain the temperatures collected in this study 2 to 4 pictures were taken from each cow. The total amount of time required for the picture caption was less than 1 minute.

Figure 3: Thermographic imaging of the Front Left and Right Quarters

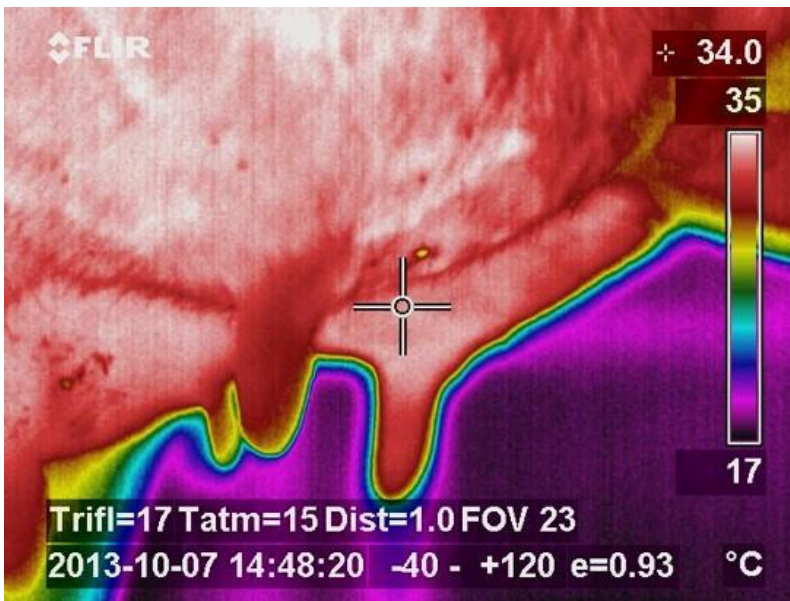
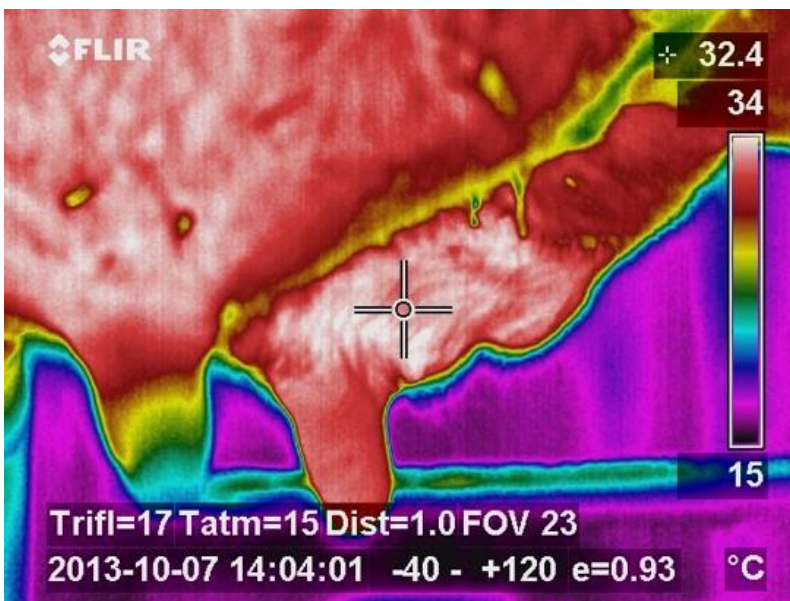
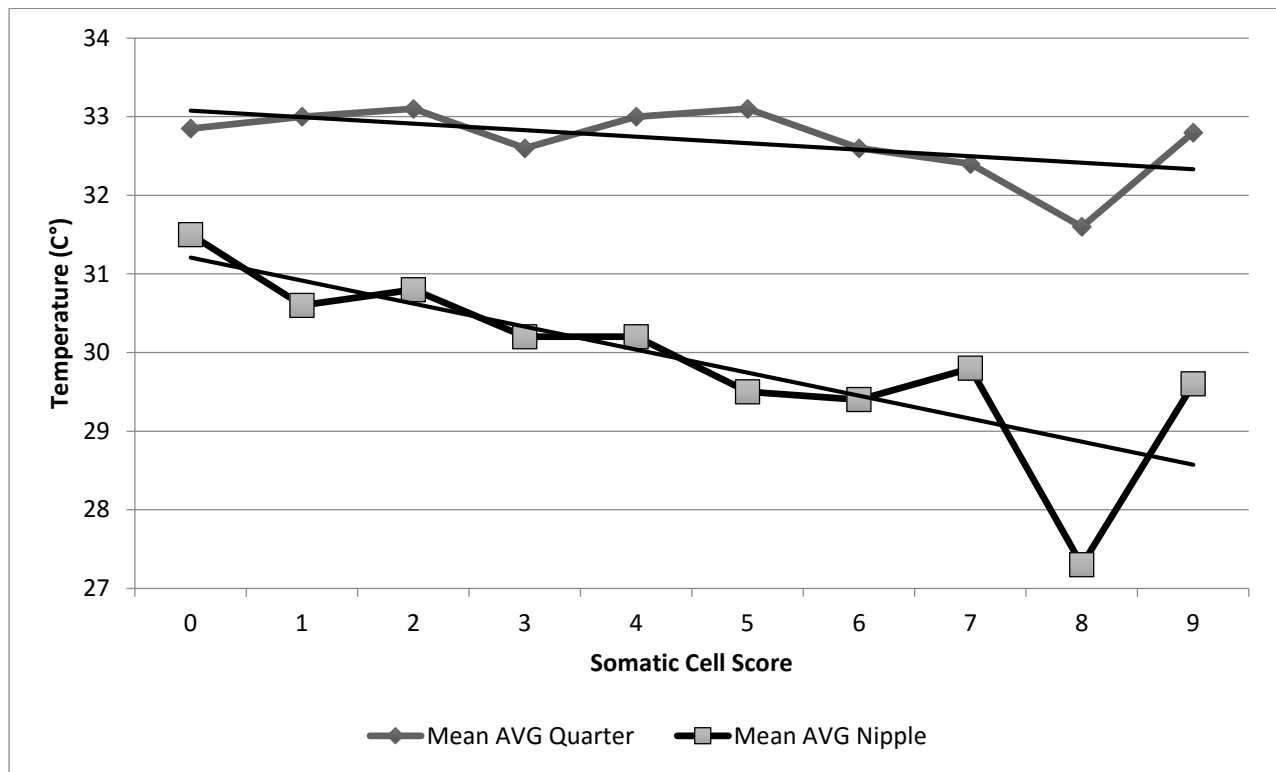


Figure 4: Thermographic image detail of a Front Left Quarter



The mean temperatures measured both at quarter and nipple levels at increasing SCS are shown in Figure 5.

Figure 5: Mean Average (AVG) temperatures at Quarter and Nipple level according to SCS.



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 ( $30.42 \pm 0.26^{\circ}\text{C}$  at nipple level and  $33.08 \pm 0.17^{\circ}\text{C}$  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.



Table 3: Minimum, Maximum and Average temperatures  $\pm$  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 $\pm$ 0.42	33.34 $\pm$ 0.17	30.42 $\pm$ 0.26	30.82 $\pm$ 0.31	34.32 $\pm$ 0.14	33.08 $\pm$ 0.17
<i>S. aureus</i>	84	23.83 $\pm$ 0.58	32.49 $\pm$ 0.22	28.75 $\pm$ 0.33	28.70 $\pm$ 0.45	33.52 $\pm$ 0.20	31.95 $\pm$ 0.23
<i>S. agalactiae</i>	25	24.47 $\pm$ 0.93	32.58 $\pm$ 0.33	28.86 $\pm$ 0.51	30.67 $\pm$ 0.73	34.10 $\pm$ 0.31	32.93 $\pm$ 0.36
<i>S. uberis</i>	26	24.10 $\pm$ 0.83	32.23 $\pm$ 0.30	29.47 $\pm$ 0.35	29.29 $\pm$ 0.47	33.21 $\pm$ 0.20	31.85 $\pm$ 0.24
CNS	54	25.35 $\pm$ 0.61	33.04 $\pm$ 0.23	30.10 $\pm$ 0.45	30.56 $\pm$ 0.65	34.40 $\pm$ 0.28	32.90 $\pm$ 0.33

CNS: Coagulase Negative Staphylococci.

A graphical representation of the mean temperatures at nipple and quarter level according to the isolated pathogen is shown in Chart 5.

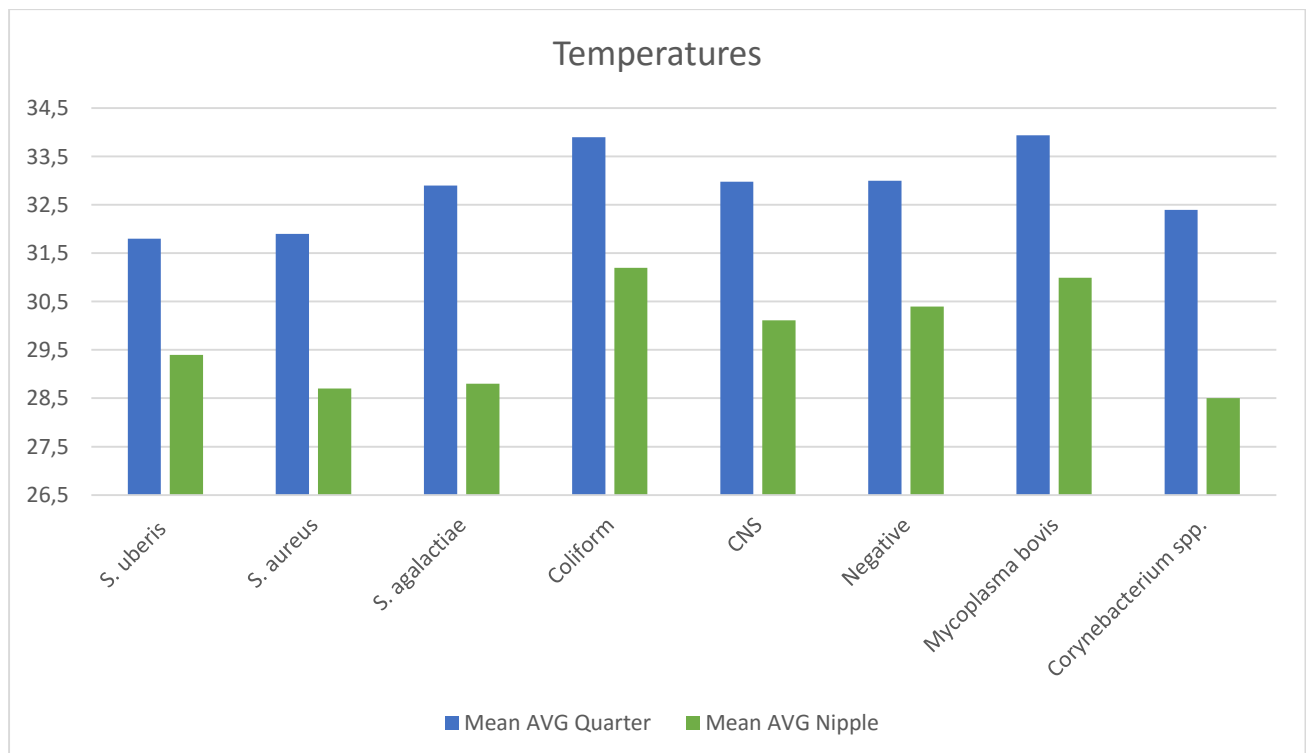


Chart 5: Average temperatures means at Quarter and Nipple level according to the pathogen isolated.

#### 4.3.4 Roc analysis

ROC analysis performed on mean temperature of the nipple (Figure 6) considering as positive the cows positive for pathogens established a threshold of 29.7 °C obtaining a sensitivity of 61.80% (50.9 – 71.9%, 95% CI).

ROC analysis performed on mean quarter temperatures (Figure 7) obtained a threshold of 31.2 °C but with a sensitivity of 23.90 % (17.5 - 31.3, 95% CI) and a specificity of 91.43% (85.5 - 95.5, 95% CI).

Figure 6: Roc analysis Mean Nipple temperature according to the results of the microbiological exams.

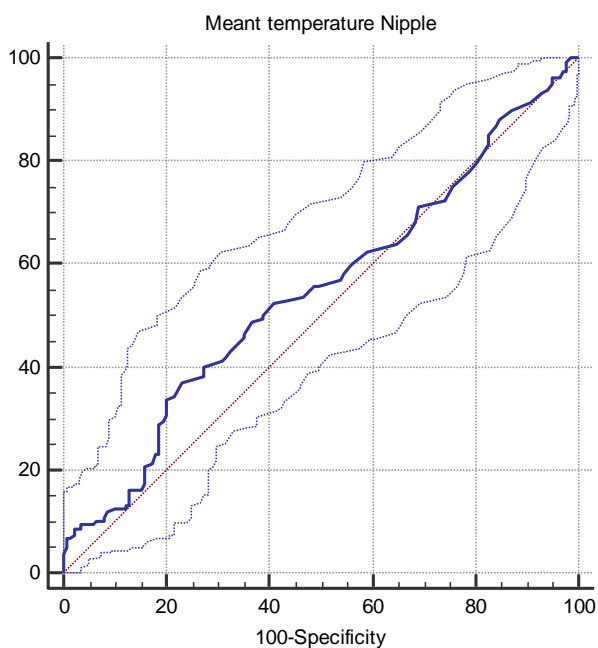
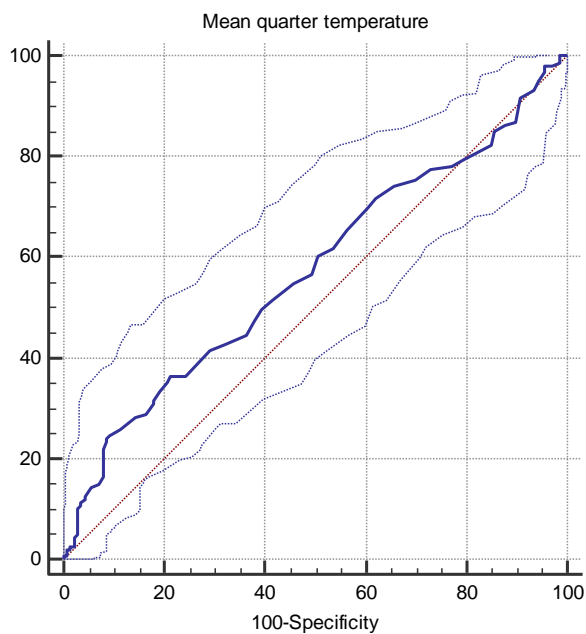


Figure 7: Roc analysis of Mean Quarter Temperature according to the results of the microbiological exams.



#### 4.4 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. The prevalence inside the positive farms was highly variable and reflected the use of measures for the control of the spread of contagious mastitis pathogens, in farm A and Farm D (where *S. agalactiae*, another contagious pathogen, was isolated) individual paper towels were used to remove the pre-milking disinfectant and high SCC were milked separately after the healthy cows. Another well described *S. aureus* feature that we confirmed, is the ability to colonize the udder without causing an inflammation, in fact *S. aureus* was present in 16 quarters with cells below the traditional threshold of 200 000 cells/ml. It has been reported that only 60 percent of the infections were found in cows producing milk with SCC greater than 200,000/ml (Pettersson-Wolfe et al., 2010). This ability allows *S. aureus* to spread inside the heard without being recognized that a problem is present.

*S. agalactiae* caused the highest raise both in SCC and SCS ( $P < 0.05$ ) thus causing the highest level of inflammation. *S. agalactiae* is a well-known pathogen that before the modern strategies for udder health control was the most widespread worldwide. But since the 1960s *S. aureus* became the most prevalent pathogen. This is mostly due to the better ability of *S. aureus* to colonize cow's udder escaping from the immunitary system and for the worst response to treatment. *S. agalactiae* is actually still very sensitive to penicillin based antibiotics and has a higher cure rate when compared to *S. aureus*.

The high rise in SCC is caused by the extensive damage done to udder secretory tissues as is well documented in literature.

*S. uberis* was present in 12.9% of the positive samples and this is in line with previous findings that ranks it among the main causes of mastitis in countries around the world, including Australia, Brazil, Canada, The Netherlands, New Zealand, the United Kingdom and the United States (Zadoks et al., 2003). *S. uberis* does often have an environmental source, but it may also spread from cow to cow (Zadoks et al., 2003). *S. uberis* is also known to cause subclinical mastitis making and subclinical infections, more difficult to recognize, may act as a source of infection for other animals, resulting in spread of a mastitis problem in the herd.

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). Moreover CNS were the only pathogen isolated in all the farms and had similar prevalence, which are similar to what was previously reported by Schukken et al. (2009) who reviewed the data of northeastern USA finding an overall prevalence of 15% (SD 12%). CNS are common environmental bacteria that are usually present on the skin of the cows but the preventive measures used for contagious microorganism such as post-milking teat disinfectant have proven to the number of IMI caused by CNS (Hogan et al., 1987). 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. Test and culling is often the

strategy suggested in countries where *Mycoplasma mastitis* is a recognized problem. However this strategy may not be necessary but should be controlled by full milking time hygiene practices that include disinfectant in the udder pre-milking wash, single service towels used to clean and dry udders pre-milking, use of clean gloved hands by milkers, milking unit backflush, and post-milking teat disinfection (Fox, 2012). The presence of IMI caused by *Mycoplasma bovis* in one farm reported in this study confirm the presence of this pathogen as a cause of mastitis also in Veneto Region. 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 Djabri 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). If we consider only SCC without taking into account the bacteriological and thermographic findings, 138 quarters had low SCC, with a mean of 69 225 cells/ml which is close to previous findings that set the mean for healthy quarters to 60 000 cells/ml (Djabri et al., 2002).

Sargeant et al. (2001) in a study investigating fresh cows found a lower sensitivity (56.7%) and a higher specificity (72.3%) compared to our study (respectively 83.08 and 57.78). This is probably caused by differences in the choice, lactation stage and microbiological culture methods.

The presence of an increased risk for a positive bacteriological culture in cows with high SCC has been previously reported 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 and Zachary, 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).

The results obtained in this work 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 and Zachary, 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 33.42°C and 33.45°C. 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. The surprisingly higher sensitivity and specificity found by Polat et al. (2010) is however calculated with a SCC threshold much higher (400 000 cells/ml) and without taking into account the presence of pathogens. Moreover 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 cow infected with CNS. More importantly it has been able to differentiate between *S. aureus* and Streptococcal mastitis, suggesting the use of SCS in the choice of the therapy.

The SCS seems to have better capability than SCC to discriminate between different etiological agent thus this value should be implemented in the test results performed by breeder's associations, after that farmers and veterinarians should be trained in understanding and using this value. The lack of a significant association between aetiological agent and udder temperatures confirm the poor diagnostic value of thermography 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 also in cows suffering of subclinical mastitis.





# **5. METHICILLIN RESISTANT STAPHYLOCOCCUS AUREUS (MRSA) PREVALENCE IN DAIRY FARMS OF THE VENETO REGION**

## *5.1 Aim*

As a part of a broader research conducted by the Istituto Zooprofilattico Sperimentale delle Venezie (Legnaro, Padova) aimed to investigate the presence and prevalence of MRSA in livestock and companion animals in the three Regions that constitute the North-East part of Italy (Triveneto), in this study we evaluated the prevalence of MRSA in dairy cows farms of the Veneto Region. To evaluate the presence of MRSA also as colonizer of the upper respiratory tracts of the animals and to investigate if the presence of MRSA in the nasal cavities of the animals was a risk factor for MRSA as a cause of mastitis also nasal swabs were collected in the same farms. The isolated strains were furtherly characterized in order to understand the genetic characteristics and the aspects of antimicrobial resistance.

## *5.2 Materials and methods*

### *5.2.1 Definition of sampling size and selection of the farms*

The data about dairy industry were provided by the National Database (Banca Dati Nazionale). The farms to object of the investigation were selected according to the size. Only farms housing more than 100 animals were considered. The sampling parameters used were:

- Prevalence of the disease: 50%
- Accuracy 10%
- IC 95%

Hypothesizing a prevalence of the disease of 10% inside the farms, 28-30 animals were sampled in order to find at least one positive animal.

Due to the lack of actual data in the investigated area the parameters used were selected to provide the largest sampling size.

According to the parameters used we selected 70 farms in the Veneto Region taking also into consideration the distribution of the farm inside the different Provinces that constitutes the Veneto Region.

#### *5.2.2 Selection of the animals and collection of samples*

28 to 30 lactating cows were randomly selected inside the farms. All the animals that was showing sign of mastitis as described by Radostits et al. (2006) or had previous history of high SCC (above 200 000 cells/ml) were included. Collection of mammary secretion was done aseptically according to National Mastitis Council guidelines (Hogan et al. 1999). In brief, teat ends and sphincters were cleaned externally first with commercial pre-milking disinfectant solutions then dried with individual paper 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.

The nasal swabs were collected with the animals placed in the self-locking head gate after the milkings when usually all the animals goes to the food trough. One bacteriological swab was inserted in both nostrils of each sampled animal and placed in Amies medium (Copan Innovation, Brescia, Italy).

#### *5.2.3 Culture and identification of MRSA*

All the analyses were performed at the Laboratory of Clinical Diagnostic of the Istituto Zooprofilattico Sperimentale delle Venezie.

1 ml of milk was inoculated in 9 ml of broth containing 6.5 % sodium chloride (Mueller-Hinton broth, Biokar Diagnostics, Alonne, FR) as described by Battisti et al. (2010). After an incubation period at  $37\text{ }^{\circ}\text{C} \pm 1\text{ }^{\circ}\text{C}$  for 18–24 h 10  $\mu\text{l}$  of broth were plated on a commercial selective plate for MRSA , CHROMagar® MRSA II (BD BBL™, New Jersey, USA). The selective plates were incubated at

35 °C ± 1 °C in aerobic conditions and read at 24 and 48 hours. Growth on CHROMagar® MRSA II as pale pink colonies represented the first sign of methicillin resistance. Coagulase positivity was also tested.

Identification of *S. aureus* and confirmation as MRSA was performed by a conventional multiplex PCR with the primers listed in Table 4. *Staphylococcus aureus* DSMZ 11729 was used as a control.

Table 4 . Primers and products size used for confirmation of MRSA

Primer	Target	Sequence(5'-3')	Product size (bp)	Reference
<i>mecA_F</i>	mecA	AAA ATC GAT GGT AAA GGT TGG C	533	Louie et al., 2002
<i>mecA_R</i>		AGT TCT GCA GTA CCG GAT TTG C		
16S_for	16S	AGA GTT TGA TCA TGG CTC AG	798	
16S_rev	rDNA	GGA CTA CCA GGG TAT CTA AT		
nuc_for	nuc	GCG ATT GAT GGT GAT ACG GTT	270	
nuc_rev		AGC CAA GCC TTG ACG AAC		

#### 5.2.4 Antimicrobial susceptibility testing

Colonies grown on CHROMagar® MRSA II were tested for susceptibility to sixteen antimicrobial agents. The test was performed by the disk diffusion method on Mueller-Hinton agar (KIMA S.A.S, Padova, Italy) according to the guidelines of the Clinical Laboratory Standards Institute (CLSI). Oxacillin was used as a marker to detect *mecA*-mediated methicillin resistance.

Discs of penicillin G (10 IU), ampicillin (10 µg), amoxicillin-clavulanic acid (20 + 10 µg), oxacillin (1 µg), cefalotin (30 µg), spiramycin (100 µg), erythromycin (15 µg), tetracycline (30 µg), tilmicosin (15 µg), tylosin (30 µg), enrofloxacin (5 µg), lincosamides (clindamycin CC 2 µg), tiamulin (30 µg), trimethoprim-sulfamethoxazole (1.25 + 23.75 µg) (BD BBL, New Jersey, USA), and cefquinone (30 µg) (Oxoid Ltd, Basingstoke, UK) were used for the antimicrobial sensitivity test. Interpretive criteria for the inhibition zone diameters provided by CLSI, or alternatively by the manufacturers (i.e., tylosin), were followed.

#### 5.2.5 MRSA characterization

*spa*- typing was performed according to the scheme developed by Moodley et al. (2006) using sequence signatures 5'-AATAATTCA and 3'-GACAAGCG. Multilocus Sequence Typing (MLST) was performed according to (Enright et al., 2002).

Alleles at the seven loci were assigned by comparing the sequences at each locus to those of the known alleles in the *S. aureus* MLST database. The allele numbers at each of the seven loci define the allelic profile of each isolate. An allelic profile is defined as a sequence type (ST) that provides a convenient and unambiguous descriptor for each *S. aureus* genotype.

121 isolates representing all the positive farms (1 to 4 isolates per farm) were tested by PCR for the presence of some antibiotic resistance genes. The determination of antibiotic resistance genes to vancomycin (*vanA*, *vanB*), tetracycline (*tetL*, *tetM*, *tetK*), erythromycin (*ermA*, *ermB*, *ermC*) was performed with gene-specific primers (Ng et al., 2001; Poeta et al., 2005; Jung et al., 2009).

The Panton-valentine leukocidin PVL gene was amplified according to (McClure et al., 2006).

### 5.3 Results

#### 5.3.1 MRSA prevalence

MRSA was present in 13 composite milk samples from 5 farms and in 2 swabs belonging to a different farm.

A summary of the sampling scheme and of the positive farms is provided in table 5.

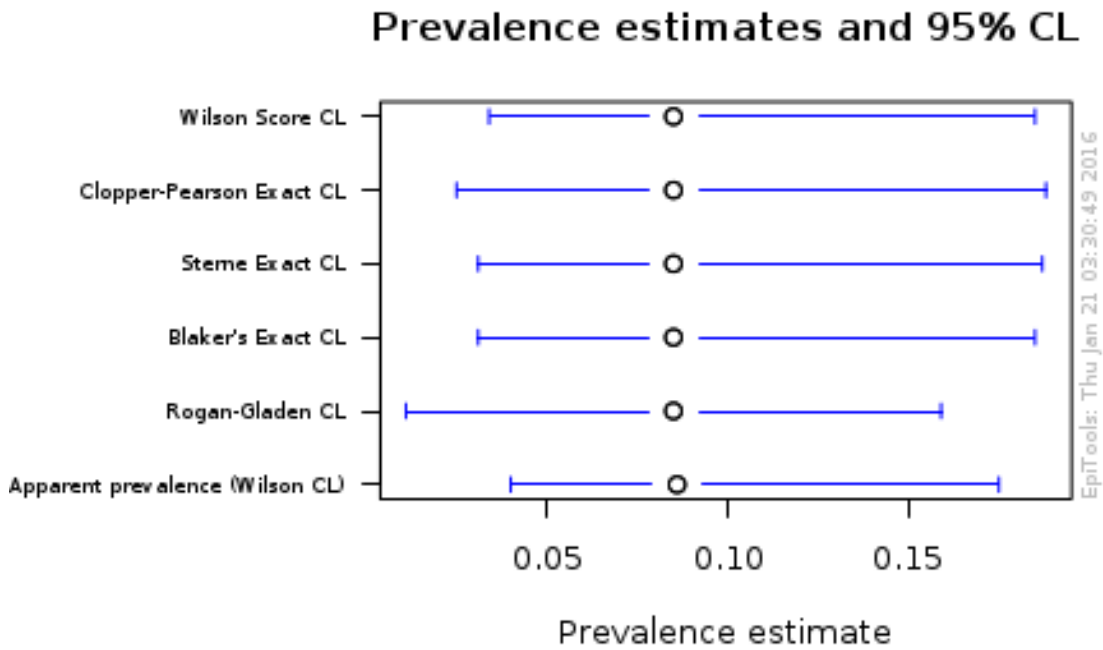
Table 5: Details of localization and size of the farms, number of samples collected and samples positive for MRSA.

Farm	Province	Date of sampling	Size of the farm	Nasal swabs collected	Milk samples collected	Nasal swabs positive	Milk samples positive
1	Padova	06-Dec-12	>501	30	30	0	0
2	Vicenza	07-Dec-12	201-500	30	30	0	0
3	Padova	05-Sep-13	201-500	30	30	0	0
4	Belluno	15-Oct-13	201-500	28	40	0	0
5	Verona	04-Oct-13	100-200	28	30	0	0
6	Verona	04-Oct-13	100-200	29	30	0	0
7	Vicenza	23-Jan-13	201-500	30	30	0	0
8	Padova	18-Nov-13	201-500	30	30	0	2
9	Treviso	21-Nov-13	100-200	30	36	0	3
10	Vicenza	04-Dec-14	201-500	30	32	0	0
11	Vicenza	30-Jan-13	201-500	30	30	0	0
12	Vicenza	13-Feb-13	201-500	30	30	0	0
13	Vicenza	20-Feb-13	100-200	30	30	0	0
14	Vicenza	26-Feb-13	100-200	30	39	0	0
15	Rovigo	07-Mar-13	201-500	30	30	0	0
16	Padova	25-Mar-13	201-500	30	30	0	0
17	Padova	14-Mar-13	100-200	30	30	0	0
18	Padova	03-Apr-13	>501	30	30	0	0
19	Padova	08-Apr-13	>501	30	30	0	0
20	Verona	06-Jan-13	100-200	30	30	0	0
21	Venezia	27-Jun-13	100-200	30	30	0	0
22	Venezia	02-Jul-13	>501	30	30	0	0
23	Treviso	16-Jul-13	100-200	30	30	0	0
24	Vicenza	15-Jan-13	201-500	30	20	0	0
25	Padova	08-Aug-13	100-200	30	30	0	0
26	Verona	28-Oct-13	100-200	28	30	0	0
27	Vicenza	10-Mar-14	100-200	30	30	0	0
28	Vicenza	11-Mar-14	201-500	30	30	0	0
29	Vicenza	19-Mar-14	100-200	30	31	0	0
30	Vicenza	27-Mar-14	100-200	30	30	0	0
31	Vicenza	02-Apr-14	100-200	30	30	0	0
32	Vicenza	04-Apr-14	100-200	30	30	0	0
33	Vicenza	30-Apr-14	100-200	30	30	0	0
34	Vicenza	09-May-14	201-500	30	30	0	0
35	Vicenza	14-May-14	100-200	30	31	0	0
36	Padova	15-May-14	100-200	30	30	0	0
37	Verona	13-Jun-14	100-200	30	30	0	0
38	Verona	04-Jul-14	100-200	30	30	0	0
39	Verona	16-Jul-14	100-200	30	30	0	2
40	Verona	27-Sep-13	100-200	32	25	0	0
41	Verona	04-Oct-13	100-200	30	32	0	0
42	Verona	04-Oct-13	100-200	30	30	0	0
43	Verona	21-Oct-13	100-200	30	30	0	0
44	Verona	24-Jan-13	100-200	30	30	0	3
45	Verona	28-Oct-13	100-200	30	31	0	0

Farm	Province	Date of sampling	Size of the farm	Nasal swabs collected	Milk samples collected	Nasal swabs positive	Milk samples positive
47	Verona	30-Jan-13	100-200	30	30	0	0
48	Verona	28-Mar-13	100-200	30	30	0	0
49	Verona	28-Mar-13	>501	30	30	0	0
50	Verona	7-Sep-13	201-500	30	30	0	0
51	Venezia	9-Aug-13	100-200	30	30	0	0
52	Venezia	20-Sep-13	201-500	30	30	0	0
53	Verona	4-Oct-13	100-200	32	30	0	0
54	Treviso	3-Sep-13	100-200	30	30	0	0
55	Padova	16-Sep-13	201-500	30	30	0	0
56	Padova	4-May-13	201-500	31	30	0	0
57	Padova	12-Jul-13	201-500	30	30	0	0
58	Padova	16-Apr-13	201-500	30	30	0	0
59	Padova	16-Apr-13	>501	30	30	0	0
60	Padova	15-Apr-13	201-500	30	30	0	0
61	Padova	27-Mar-13	201-500	30	30	2	0
62	Padova	14-Mar-13	100-200	30	30	0	0
63	Padova	30-Nov-12	100-200	30	18	0	0
64	Padova	30-Nov-12	201-500	30	14	0	0
65	Rovigo	19-Jul-13	100-200	30	30	0	0
66	Treviso	28-Aug-13	100-200	30	30	0	0
67	Treviso	29-Aug-13	100-200	30	29	0	0
68	Vicenza	7-Mar-13	201-500	28	30	0	0
69	Verona	17-Sep-13	100-200	30	30	0	0
70	Treviso	13-May-14	100-200	30	17	0	3
<b>Total</b>				<b>2096</b>	<b>2075</b>	<b>2</b>	<b>13</b>

6 farms resulted positive for MRSA with an apparent prevalence (Wilson CL) of 8.6% (4-17.5%, 95% CI). Figure 8 shows Blaker's, Sterne, Clopper-Pearson and Wilson confidence limits, calculated as described by Reiczigel et al. (2010).

Figure 8: MRSA prevalence estimates



The prevalence inside the herds was low varying from 0.0% to 4.7%. And no farm was positive for both nasal swabs and milk.

### 5.3.2 Antimicrobial resistance

Antimicrobial resistance was tested as described before. All the isolates were resistant to three or more antimicrobials and were subsequently classified as multi-resistant.

The percentage of resistance for each antimicrobial are provided in Chart 7.

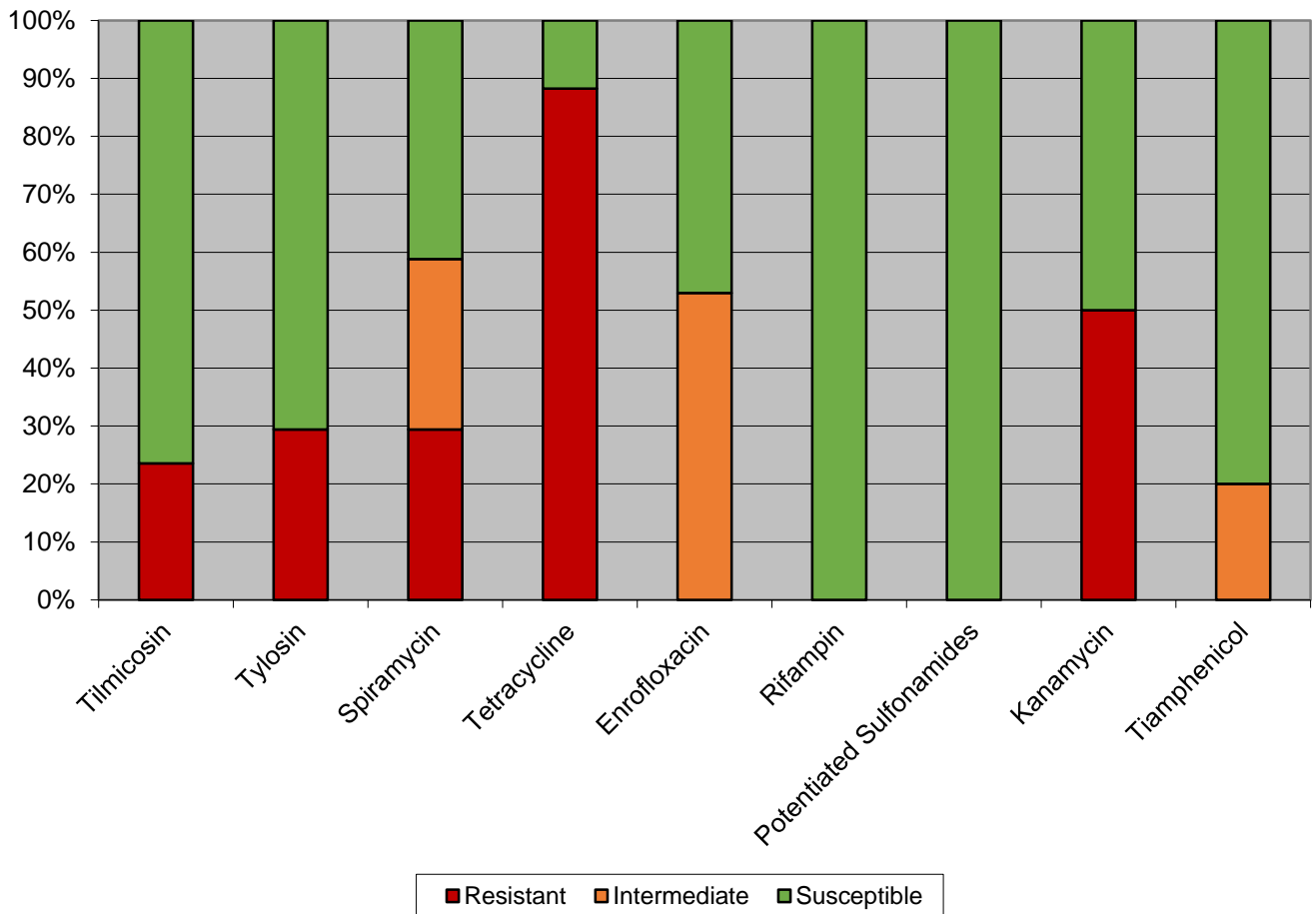


Chart 7: Antimicrobial resistance characteristics of the MRSA strains.

### 5.3.3 Genetic characteristics

All the isolates were confirmed as MRSA by PCR as previously described.

Presence of antimicrobial resistance genes and strain type characteristics are summarized in table 6.

In brief, tet genes were present in all the isolates (100%), erm genes were present in 66% of the isolates. No isolate resulted positive for vanA and vanB or Panton-Valentine leukocidin (PVL).

8 different *spa* types were found grouped in 3 major Clonal complexes.



Table 6: Genetic characteristics of the strains isolated.

Farm	Sample	tetk	tetL	tetM	vanA	vanB	ermA	ermB	ermC	PVL	<i>Spa</i> type	Clonal Complex
9	Milk	1	0	1	0	0	0	1	0	0	t9305	CC97
9	Milk	1	0	1	0	0	0	1	0	0	t4795	CC97
9	Milk	1	0	1	0	0	0	1	0	0	t1730	CC97
44	Milk	0	0	1	0	0	0	0	0	0	t034	CC398
44	Milk	0	0	1	0	0	0	0	0	0	t034	CC398
44	Milk	0	0	1	0	0	0	0	0	0	t034	CC398
8	Milk	1	0	0	0	0	0	0	1	0	t127	CC1
8	Milk	1	0	0	0	0	0	0	1	0	t127	CC1
39	Milk	1	0	1	0	0	0	0	0	0	t13289	CC398
39	Milk	1	0	1	0	0	0	0	0	0	t13289	CC398
61	Nasal swab	0	0	1	0	0	1	0	0	0	t034	CC398
61	Nasal swab	0	0	1	0	0	1	0	0	0	t034	CC398
70	Milk	1	0	0	0	0	0	0	1	0	t177	CC1
70	Milk	1	0	0	0	0	0	0	1	0	t177	CC1
70	Milk	1	0	0	0	0	0	0	1	0	t177	CC1

#### 5.4 Discussion

The prevalence of MRSA in this work is similar to what was reported in other countries. In Belgium (Vanderhaeghen et al., 2010) reported a herd prevalence of approximately 10%. In Turkey, Turutoglu et al. (2006) found 18 out of 103 (17.5%) *S. aureus* isolates from mastitis milk samples to be MRSA. However, they did not mention whether all strains were collected from different farms experiencing *S. aureus* mastitis. In addition, their detection method was limited to phenotypic disk diffusion testing. Performing only phenotypic tests has previously been shown to lead to false positive or false negative results (Murakami et al., 1991). In a study conducted in Italy (Luini et al., 2015), but limited to herds experiencing *S. aureus* mastitis, 15% of the affected herds resulted positive for MRSA. The prevalence within the herd resulted low in most of the studies and our results confirm those findings. Therefore, if the low within-herd prevalence of MRSA could reduce the zoonotic potential of these strains, yet the presence of MRSA infected cows in dairy farms could remain unobserved, since

bacteriological analysis of milk is usually performed only when a clear problem of mammary infections affects the herd.

There are no study at our knowledge that have investigated simultaneously the presence of MRSA in milk and nasal cavities in dairy cows. But MRSA nasal colonization is common in many food producing animal species also in Italy. In veal calves farming, where are raised male calves of dairy breeds, in Netherland in a study of 2010 28% of the samples collected were positive and 88% of the veal calves farms were positive (Graveland et al., 2010). The lower prevalence in nasal swabs compared to the prevalence in milk is, because of these reasons, surprising. The main explanation could be the lower use of systemic antibiotic treatment and lower length of treatments in dairy cows compared to veal calves. In dairy cows due to the financial loss caused time of withdrawal of the milk following a systemic antibiotic treatment, the antibiotic therapy are reduced to the minimum extent. However local antibiotic treatment for the therapy of mastitis is a common practice in advanced dairy farming and most of the farms apply Dry Cow therapy. The use of antimicrobials with effects mostly in udder environment, and especially cephalosporins (Sawant et al., 2005) that have no effect on MRSA, could have cleared competitive flora in the udder, thus creating the conditions for a stable colonization or infection by MRSA, while in nasal cavities even if the animal comes in contact with MRSA, the natural and undisturbed microbial flora prevent a stable colonization of MRSA.

The phenotypic antibiotic resistance of MRSA mostly reflected the strains genomic pattern, with high prevalence of tetracycline resistance. Even if 66.6% of the isolates were carrying genes for resistance to macrolides, only about 30% of the strains demonstrated it phenotypically. The presence of *erm* genes however may lead to constitutional or inducible resistance (Lina et al., 1999). In addition to its resistance against all  $\beta$ -lactam antibiotics, which are still the most used antimicrobial agents in the treatment of mastitis, the typical antibiotic resistances of LA-MRSA also include some other antibiotics used to treat or prevent mastitis, such as aminoglycosides and macrolides (Sawant et al., 2005). This could lead to serious treatment problems.

No resistance to Vancomycin was detected in these strains, neither the Panton-Valentine Leukocidin that is one of the major virulence factors present in Community Acquired MRSA (CA-MRSA). The absence of PVL, *vanA* and *vanB* genes, together with the resistance to tetracyclines are typical characteristics of LA-MRSA (Pantosti and Venditti, 2009).

The *spa* typing of the isolates revealed different *spa* types inside the same farm. This may suggest the entrance of different variants of the same MRSA clone inside the farms or multiple episodes of contact with different MRSA strains. This should lead to investigations on the source of MRSA in dairy cows, presence of pigs on the same farm, shared equipment or vehicles with pig farms and Veterinarians could represent a risk factor but further studies are needed.

The majority of the strains belonged to CC398 that has been described as a lineage with limited virulence and ability to spread between humans, but severe clinical manifestations, such as wound infections and endocarditis, have been recently attributed to this clone (EFSA, 2009; Van Rijen et al., 2008). Cases of nosocomial ventilator-associated pneumonia have also been reported in Germany (EFSA, 2009). Moreover, an outbreak of infection with MRSA ST398 occurred in a surgical ward of a hospital in the Netherlands in 2007 (Wulf et al., 2008).

Like clonal lineage CC398 also MRSA CC1/ST1 seems to have a wide host range. ST1 are more frequently detected among MRSA from Italian hospitals (Monaco et al., 2010). MRSA ST1 were described from an outbreak of subclinical mastitis in Hungary (Juhász-Kaszanyitzky et al., 2007), from pigs in a slaughterhouse in Italy (Battisti et al., 2010).

*S. aureus* ST97 has been described as associated with bovine mastitis in many parts of the world (Cuny et al., 2010). More recently it was also reported from pigs in Germany (Meemken et al., 2008) and in Spain where it has been found among isolates from nasal carriers and with low frequency also among isolates from nosocomial infections (Menegotto et al., 2012). ST97 are infrequent among MRSA clonal lineages from infections in humans. Data from a livestock-intense region in North-Western Germany show that a total of 21 isolates characterized by MRSA CC9 (*spa* type t1430) and CC97 (*spa* type t3992) were found among 14,036 MRSA isolates from screenings and clinical

specimens obtained from human in- and outpatients typed (0.15%) within a 54-month period. This demonstrates that despite the relatively frequent contamination of food items with MRSA belonging to these clonal lineages, they have spread only rarely in the population. This argues for rather limited relevance of food as a vector for MRSA toward human colonization.

In conclusion the risk for spread of MRSA from dairy cow sources into the human population is low in our area because of the low prevalence and especially the low prevalence in nasal swabs. Generally, persons are not at risk as long as raw milk is not consumed. However, persons in close contact with MRSA-infected cattle, including Veterinarians, farmers, milking personnel, and persons working at slaughterhouses, may become colonized from the bovine source, and MRSA is an emerging mastitis pathogen that dairy Veterinarians of will have to fight in the next years.

## 6. Conclusions

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 results obtained in this work that suggest the use of SCS as an indicator of a herd infection caused by a specific bacteria or a group of bacteria.

SCC has proven in this work to have a reasonable sensitivity for being used as a selection method for cows to be submitted to bacteriological culture.

A correct selection of the animals to be further tested is of crucial importance in infectious disease investigation, in order to reduce the costs of the laboratory analysis and to improve the quality of the results.

The results obtained in this study may suggest the use of thermography for the evaluation of the functionality of cow's udder but there is still much to learn about loss of functionality in subclinical mastitis affected cows before it can be demonstrated.

More research in this field is needed before we can implement the monitoring for udder health with infrared thermography but further advances in the technology and the continuous request for instruments that may reduce the incidence of subclinical mastitis justify our efforts.

The confirmation of the presence of MRSA in dairy cows of the Veneto Region, even if at a low prevalence, represent a new challenge for Veterinarians that are involved in udder health management. If the cure rate of *S. aureus* infections is low in case of mastitis, there are even less chances of curing an MRSA mastitis since they are usually resistant to the majority of the antimicrobials commonly used for mastitis therapy.

MRSA is also a potential zoonotic pathogen and the strains isolated in this study has already proven their zoonotic abilities and are able to cause infections in humans, thus MRSA positive cows represent a risk for the farm personnel and the Veterinarians that comes in contact with them or with unpasteurized milk products.

The higher prevalence of MRSA in milk samples compared to nasal swabs may also indicate the antibiotic treatment as a risk factor for MRSA infection, thus more strict rules for antibiotic therapy should be applied. In particular the practice of choosing a therapy following culture and antimicrobial susceptibility results should be applied more often if the presence of multiresistant bacteria is suspected.

Because of the low prevalence of MRSA in cows, its apparent low ability to spread rapidly in positive farms and its antimicrobial resistance characteristics a policy of test and cull should be applied to clear the farms from positive animals.

A continuous surveillance program should be applied also in dairy cows to monitor the evolutions in the epidemiology of MRSA to assess the risk for udder health and for the people in contact with dairy cows.

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