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CLINICAL AND DIAGNOSTICAL ASPECTS IN DAIRY MEDITERRANEAN BUFFALO MASTITIS

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INDEX:

ABSTRACT

The goal of the present study was the verify the qualitative and quantitative numerical linear correlation between the most common diagnostics test (electrical conductivity, California Mastitis Test, somatic cells count and bacteriological milk culture) employed for detection of mastitis in Mediterranean Buffalo (*Bubalus Bubalis*).

Four hundred and eighteen primiparous Mediterranean Buffaloes, randomly chosen between December 2011 and September 2012, were enrolled in the study. All the subjects with traumatic evidences of mastitis were discarded. A composite milk sample (pool of the 4 quarts) was collected from each animal, between days 30 and 150 after calving, during evening milking and send to laboratory for somatic cell count and bacteriological milk culture. Electrical conductivity and California Mastitis Test were recorded and performed, respectively, in farm after milking.

Low correlation values between the most of variables were found, but a moderate strong coefficient between California Mastitis Test and somatic cells count was detected (0,657). The lowest correlation's values were recorded between electrical conductivity and the other test because it showed a low ability to rightly identify buffalo udder health status.

The diagnostic characteristic of electrical conductivity and California Mastitis Test measurements cannot adequately reflect Mediterranean Buffalo's udder bacteriological status. A few informations, depending by selected threshold, can be reached by somatic cells count (sensibility and specificity of 41% and 88%, respectively, with cutoff of 100'000 cells/ml; 25,6% and 100% with that one of 200'000 cells/ml). At present, between the investigated diagnostic test, no association can be considered an efficient alternative to the *gold standard* for mastitis diagnosis, created for dairy cow and also used in Mediterranean Buffalo, represented by combined used of somatic cells count and bacteriological milk culture.

ABSTRACT

L'obiettivo del presente studio è stato verificare la correlazione lineare numerica, qualitative e quantitativa, tra i più comuni test diagnostici (conduttività elettrica, California Mastitis Test, conta delle cellule somatiche, esame batteriologico del latte) usati per l'individuazione delle mastiti nel Bufalo Mediterraneo (*Bubalus Bubalis*).

Quattrocentottanta bufale primipare, allevate nel sud Italia e scelte con un campionamento casuale random, tra Dicembre 2011 e Settembre 2012 sono state incluse nello studio. Tutti i soggetti segni di mastite di origine traumatica sono stati scartati. Un campione di latte dei quattro quarti è stato raccolto da ciascun animale, tra i 30 ed i 150 giorni dopo il parto, durante la mungitura serale e mandato in laboratorio per la conta delle cellule somatiche ed esame batteriologico. Conduttività elettrica e California Mastitis Test sono stati rispettivamente registrati ed eseguiti in azienda, dopo la mungitura.

Bassi valori di correlazione sono stati trovati tra la maggior parte delle variabili, però un coefficiente moderatamente forte è stato invece individuato tra California Mastitis Test e conta delle cellule somatiche (0,657). I più bassi valori di correlazione sono stati registrati tra conduttività elettrica e gli altri test perché ha quest'ultima ha dimostrato scarsa abilità ad individuare correttamente i diversi stati di salute della mammella del bufalo.

Le caratteristiche diagnostiche delle misurazioni della conduttività elettrica e del California Mastitis Test, non possono riflettere adeguatamente lo stato batteriologico della mammella di Bufalo Mediterraneo. Poche informazioni, dipendenti dai valori soglia selezionati, possono essere ottenute dalla conta delle cellule somatiche (sensibilità e specificità rispettivamente del 41% ed 88% considerando il limite delle 100'000 cellule somatiche, mentre del 25,6% e 100% con quello di 200'000 cells/ml). Al momento, tra i test inclusi nello studio, nessuna associazione può essere considerata un alternativa efficiente al *gold standard* nella diagnosi di mastite, pensato per la vacca da latte ed utilizzato anche nel Bufalo Mediterraneo, rappresentato dall'uso combinato di conta delle cellule somatiche ed esame batteriologico.

INTRODUCTION

In Europe, there are approximately 440000 Mediterranean Buffaloes (FAO, 2011), and Italy is the most important country where these ruminants are bred (374000 animals) (FAO, 2011; Fagiolo and Lai, 2007). The reasons for the increasing interest in buffalo breeding, over recent years, are the popularity of buffalo Mozzarella cheese (D.O.P.) and the absence of milk quotas in Europe Community for its production (Fagiolo and Lai, 2007).

Mediterranean Buffaloes (*Bubalus bubalis*) are typically reared in central and southern Italy. 80% of Italian milk productions is centred in the Campania region around Latina, Caserta, Salerno districts where there are intensive farms (Fagiolo and Lai, 2007). In Italy, dairy Mediterranean Buffaloes and bovine breeding systems are very similar: unifeed feeding systems, twice daily milking, mid-lactation production levels of between 14-16 litres/day/animal, similar gestation lengths, similar postpartum and calf management practices, as well as

similar herd problems. All of these factors contribute to the occurrence of mastitis in both species.

Buffalo mastitis is one of the most costly disease in the dairy industry. It is the result of the interaction between a combination of microbiological factors, host responses in the udder, and management practices. Predisposing factors for mastitis include environmental aspects, such as poor hygiene, poor husbandry, overcrowding, bad ventilation, poor milking technique and malfunction of milking machines (Fagiolo and Lai, 2007). Early detection of mastitic animals is absolutely important for dairy farmers to reduce production losses (Sharma at al., 2010). It is almost always caused by bacteria (Galiero, 2002). The most important contagious pathogens are Stapylococcus aureus and Streptococcus agalactiae, while common environmental pathogens include Streptococco uberis, dysagalactiae, Coliforms, Pseudomonas, Prototheca and yeasts (Fagiolo and Lai, 2007) . Even though the buffalo has been traditionally considered less susceptible to mastitis than cattle (Wanasinghe, 1985), some studies showed similar mastitis frequencies for the two species (Kakra and Rizvi, 1964; Badran, 1985; Basal at al.,1995).

In mastitic milk, elevated somatic cell count (SCC), changes in composition, impair coagulation, loss in cheese yield were associated with the production of a cheese with poor quality and high moisture content. In addition, it may result in the presence of bacteria and other infectious agents which may be harmful to humans and contaminate food safety and quality (Munro et al., 1984; Grandison and Ford, 1986; Politis and Kwai-Hang, 1988a, 1988b, 1988c)

According to severity, duration, nature of the exudate and primary cause mastitis, can be classified into clinical and subclinical forms (Fagiolo and Lai, 2007). Diagnosis of clinical mastitis is based on the local and systemic reactions and changes in milk (e.g off-color, watery, bloody appearance and presence of flakes, clots and pus) both in dairy buffalo and cow. The amount udder swelling, severity of pain and the overall appearance of the animal will indicate the severity of infection and serve as a guide for the course of treatment. The diagnosis of subclinical mastitis is more problematic since the milk and the udder appear normal but usually has an elevated somatic cell count. (Sharif and Muhammad, 2008; Sharma at al., 2010). Early diagnosis of mastitis is essential because change in the udder tissue take place much earlier than they became apparent (Sharma at al., 2010). Various methods, based on physical and chemical changes of milk and cultural isolation of organisms, are used for diagnosis of subclinical disease (Batra and Mcallister, 1984; Emanuelson et al., 1987). The diagnosis of mastitis according to the International Dairy Federation (IDF, 1971) guidelines is based on the somatic cell count (SCC) and bacteriological milk culture (BC). At present, several field screening test, such us California Mastitis Test (CMT) and the electrical conductivity (EC), not requiring any complex laboratory equipment, were developed (Sharma et al., 2010). Every test present different sensibility and specificity rate, but no one show values of 100% so, their combined used is often recommended (Rubén N. 2002).

Although these test were created for dairy cow, they are commonly used in Mediterranean Buffalo because the mastitis management is often based on knowledge transferred from bovine scientific

evidences. The two species were considered similar for a long time, and buffalo world, as consequence, has been affected by poor scientific and cultural knowledge. All the studies presents in literature, showed some difference in test behaviour when applied in buffalo. These findings have to always considered to performed a right mastitis diagnosis.

The aim of this study was to evaluate and compare the ability of several diagnostic test commonly used in the diagnosis mastitis of cow, (electrical conductivity, California mastitis Test, somatic cell count, bacteriological milk culture results) to classify correctly udder health status of individual primiparous Mediterranean buffalo throughout the assessment of numerical qualitative and quantitative linear correlation.

The experimental data will be preceded by an update on mastitis in buffalo cow based on the literature data.

GENERAL ASPECTS OF MASTITIS IN DAIRY MEDITERRNEAN BUFFALO

UDDER ASPECTS AND MILK FLOW

Buffalo breeding had an important develop in several countries of the world, in the last twenty years. This positive trend was mainly recorded in India, Middle East, New Zealand and Brazil and Europe where the Italy is the country in which it has a remarkable zootechnical importance. The increasing interest, in dairy buffaloes, allowed to perform a lot of studies to improve the knowledge about udder anatomy and physiology and to optimize its use as dairy animals. This aim was only recently reached, because the characteristics of dairy buffalo and cow were associated for long time.

The particular morphology of buffalo udder is associated to milking difficulties and a successful machine milking is much more complex than in cows (Borghese et al., 2007). Several studies are performing to understand the buffalo udder anatomy and physiology with the aim to make new ideal milking cluster, following its characteristic and reducing the incidence of mastitis.

Mediterranean Buffalo udder has some anatomical and physiological analogies with cow but it have also several peculiarities. The first difference regards the cisternal area and milk fraction available. The amount of cisternal milk in buffaloes is less than 25% of what is reported for cows (Thomas et al., 2004b; Bruckmaier et al., 1994). The cisternal area can be measured two-dimensionally with ultrasound and Bruckmayer and Blum (1992) found a total cisternal area (teat and gland) of around 22 cm^2 for a single buffalo quarter which is less than half of that seen for cows (40-45 cm^2) and its lumen is collapsed post milking (Ambord et al., 2010). The Mediterranean Buffalo has cisternal volumes of 75 to 220 cm^3 and the volume of the alveolar tissue varied from 3000 to 4000 cm^3 (Borghese, 2007). The volume of teat and gland regions is about the same size (Thomas et al., 2004b) in contrast to cows that have more volume in the gland cistern. The quantitative cisternal milk restrained is only to about 5% in Mediterranean Buffalo (Thomas et al. 2004b) lesser than dairy cows (20%), goats and sheep (approximately 50%) (Bruckmaier et al., 1992, 1998; Rovai et al., 2008; Salama et al., 2004). Furthermore, cisternal milk in cows, goats and sheep is immediately available for the milking machine (Bruckmaier and Blum, 1998) while in buffaloes, the small cisternal milk fraction is therefore available if milk ejection does not occur before the start of milking. However, even if cisternal milk is present, there is often no visible milk flow prior to milk ejection if the milking cluster is attached and vacuum applied. Only a manual pre-stripping instead allowed the removal of cisternal milk also before milk ejection. This peculiarity also indicates that the teat closure is much tighter in buffaloes than in dairy cows where the

cisternal milk is always easily obtained by machine milking before milk ejection (Bruckmaier et al., 1994; Weiss et al., 2004).

In buffalo 95% of the milk is tored in the secretory tissue even after a milking interval of 10 to 12 hours. It is only possible to extract this alveoli milk with an active milk ejection (Thomas et al., 2004b). In buffalo, it is imperative that this milk is removed as completely as possible milk ejection and an efficient milking technique. Incomplete milk removal causes immediate production losses and apoptosis in the mammary epithelium (Stefanon et al., 2002)

Buffalo udder is also characterized by extremely long teat canals. According to Thomas et al. (2004) the teat canal length, determined by b-mode ultrasound, before pre-stimulation is around 37 mm in rear teat and 30 mm in front (Thomas et al., 2004). This characteristic can be considered a cause of lesser incidence of mastitis in buffalo than in cow because it provides to reduce the possibility of intramammary infections (Krishnaswamy et al., 1965). Obviously, a major length of buffalo teat canal needs higher values of mechanic vacuum to milking the animals. Considering this characteristic, a vacuum of up to 45 kPa in buffaloes is generally ineffective unless alveolar milk ejection occur to open the teat canal (Ambord et al., 2009) while in dairy cows 20 kPa are usually enough.

As in cows, milk ejection in buffaloes occurs only in response to oxytocin release, which is induced by teat stimulation (Bruckmaier and Blum, 1996; Bruckmaier, 2005; Thomas et al., 2005). If in cow, a good teat stimulation, is provided by the normal pulsation of the teat cup liner, this is not always true in buffalo because the only attachment of milking machine rarely induced oxytocin release and milk ejection (Thomas et al., 2005). Several studies addressing

machine milking of buffaloes showed that careful teat preparation and pre-stimulation are important preconditions for successful milk removal (Thomas et al., 2005). After a 3-min pre-stimulation the alveolar milk was ejected in all buffaloes and milk flow occurred immediately after cluster attachment without interruptions until the end of milking. As repeatedly shown in dairy cows, omitting the pre-stimulation causes a transient reduction in milk flow after that the cisternal milk is removed and before the milk ejection occurs (Mayer et al., 1984; Bruckmaier and Blum, 1996; Weiss and Bruckmaier, 2005). According to Ambord et al. (2010), during machine milking, both the frequency of delayed milk ejection and the time until delayed milk ejection decreased, with increasing duration of pre-stimulation up to 3 min. The induction of milk ejection requires tactile stimulation of the teats and/or the udder which causes the release of oxytocin and hence myoepithelial contraction and alveolar milk ejection (Bruckmaier and Blum, 1996; Bruckmaier, 2005). Administration of exogenous oxytocin before milking did not significantly affect milk flow parameters. The latency period of milk ejection in buffaloes in response to exogenous oxytocin is around 25 -30s (Thomas et al., 2004), i.e. very similar to the latency period in dairy cows (Bruckmaier et al., 1994).

Other buffalo characteristic, influencing milk flow, is the different teat closure due to the presence of muscle tissue above the teat canal. This morphological peculiarity provides additional teat closure before milk ejection. Histological studies performed on buffalo teat tissue assessed that, although the amount of muscle cells and teats were similar to cow, buffalo teats seem to contain qualitatively stronger muscles in the proximal part of the teat above the teat canal than cows.

Obviously this additional teat closure can be overcome by positive pressure such as pre-stripping, but not by the application of a vacuum to the outer side of the teat (Ambord et al., 2010). The morphology of the teat closure change in response to milk ejection and increased intra cisternal pressure during teat stimulation. In fact, a study performed to verify the interrelation between teat anatomy and machine milking in dairy Mediterranean Buffalo showed that the length of the teat closure structure, observed by ultrasound, diminished dramatically during a 3-min prestimulation (Ambord et al., 2010). This buffalo characteristic can be also considered another reason of lesser incidence of mastitis in buffalo than in cow (Krishnaswamy et al., 1965).

The particular morphology of buffalo udder is traditionally associated to milking difficulties. To better investigate the characteristics of milk ejection Bava et al. (2007) performed a study on 184 Mediterranean Buffalo in an intensive farming system in whitch milk flow profile were measured with electronic mobile milk flow meters. The results showed that during the first 3 minutes of milking 73% of total milk yield was milked; analogue researches in cow detect a lesser percentage (67%) (Sandrucci et al., unpublished data). Like in dairy cows (Sandrucci et al., 2007), time of milk ejection (period of time between milk flow > 0.5 kg/min and cluster removal) significantly decreased during lactation because of the reduction of milk production.

A long lag time (1.94 min ± 1.57) before milk ejection (period of time between teat cup attachment and the start of milk ejection) was also describe, probably due to the lack of teat stimulation before milking that induced a delay in milk ejection reflex (Sandrucci et al., 2007;Bruckmaier and Blum, 1998). The period tended to increase,

from the 17,5%, to 28.3% of total milking time, with increasing stage of lactation (calculated as the sum of time of milk ejection and lag time) This phenomenon is explained by the reduction of cistern size and milk yield as lactation progressed (Thomas et al., 2004) and by the delay of alveolar milk ejection due to the decrease of udder filling (Bruckmaier and Hilger, 2001).

Finally, a mean and maximum milk flow rates 0.92 Kg/min ± 0.37 and 1.42 Kg/min ± 0.60, respectively were found. These value are low in comparison with that one describe in cows (Sandrucci et al., 2007) but they usually decreased during lactation as a consequence of the reduction of milk yield (Bava et al., 2007).

MEDITERRANEAN BUFFALO MASTITIS

Mastitis is defined as an inflammatory reaction of parenchyma of the mammary gland that can be of an infectious, traumatic or toxic nature (International Dairy Federation, 1987). It is a highly prevalent disease in dairy buffalo and cattle, and one of the most important diseases affecting the world's dairy industry causing reduced milk yields and having deleterious effects on the chemical and cytological composition of milk. In addition, it may result in the presence of bacteria and other infectious agents which may be harmful to humans. Mastitis therapy often results in the presence of antibiotic residues in milk rendering it unsuitable for human consumption or further processing (Costa et al., 1997a). Mastitis generally leads to inflammation of one or more quarters of the mammary gland and it is often affecting not only the individual animal but the whole herd or at least several animals within the herd. If left untreated, the condition can lead to deterioration of animal welfare resulting in culling of affected cows, or even death (Fagiolo et al., 2007).

Classification

According to severity, duration, nature of the exudate and primary cause mastitis, can be classified into clinical and subclinical forms.

Clinical mastitis is most frequently infectious and classified, according to its severity, rapidity of onset and duration, into per-acute, acute, sub-acute and chronic forms (Du Prez et al. 1994). It is characterized by physical, chemical and usually bacteriological changes in the milk such as presence of blood, water, pus containing clots, flakes and shreds consisting of fibrin and cellular debris and by pathological changes in the glandular udder tissue, e.g. in the chronic form there is progressive fibrosis which leads to enlargement and asymmetry of the gland. Moreover, a portion of the gland may become eventually atrophic. Clinical acute mastitis start suddenly, usually accompanied by systemic effects as swollen and painful quarters, fever, inappetence, dehydration, a remarkable decrease in milk production and evident milk alterations. Sub-acute mastitis are characterized by lack of clinical evidences, few udder clinical signs and some milk alterations. Chronic mastitis show lack of clinical signs, no milk production and udder healings (Fagiolo and Lai, 2007).

Subclinical mastitis are observed more frequently, characterized by normal gland and milk appearance and increase of somatic cell count. They are often not diagnosed and consequently their alterations are only detected by using direct diagnostic test (Fagiolo and Lai, 2007).

Effects on milk quality

Mammary gland inflammation affects milk yield and quality and can lead to great economic losses for dairy farmers and cheese makers, in dairy buffalo and cow. In fact, only milk from healthy

udder produces had milk of a physiologically normal composition (Hamann, 2002).

Recent studies confirmed that the mean percentages values of normal buffalo milk of fat and protein were 8,58 ± 1,22% and 4,76 ± 0,55% respectively (Bava et al., 2007) and that milk yield per milking significantly decrease during lactation, while fat and protein percentages significantly increased: fat percentage changed from 8,29% to 9,14% ($P<0,05$) and protein percentage from 4,61% to 5,04% ($P<0,05$) in the stage of lactation between 90 and 180 day after calving (Bava et al., 2007; Ceròn-Munoz et al., 2002).

In mastitis milk, the changes in composition impair coagulation, cheese yield, and composition lead to poor quality cheese and elevated somatic cells count (SCC) associated with the production of a cheese with high moisture content (Munro et al., 1984; Grandison and Ford, 1986; Politis and Ng-Kwai-Hang, 1988a; Politis and Ng-Kwai-Hang, 1988b; Politis and Ng-Kwai-Hang, 1988c; Barbaro et al., 1991). Significant changes in lactose (4,87 % vs 4,64% in animals with SCC > 200’000 and SCC < 100’000 cell/ ml, respectively, $P<0,05$) and chloride content (0,882 mg/ml vs 0,650 mg/ml in animals with SCC > 200’000 and SCC < 100’000 cell/ ml, respectively, $P<0,05$) were observed with increasing SCC values. Higher SCC was also associated with impaired rennet coagulation properties: the clotting time increasing, while the curd firming time ($P \leq 0,05$) and firmness decreased (Tripaldi et al. 2010). High somatic cells counts (SCC) affects the quality and shelf-life of pasteurized milk (Ma *et al*., 2000) and is indeed able of deeply degrading milk proteins, so influencing the produced mozzarella cheese and its quality (Fagiolo and Lai, 2007).

Somatic cells count is a measure widely used to asses mammary health status (Smith, 2002; Tripaldi et al., 2010; Schwart et al., 2011). The somatic cells are physiologically composed by leucocytes and epithelial cells (Lee et al., 1980). Usually, mastitis can influence the quantitative and percentages of leukocytes in milk, and the number and distribution of leukocytes are important for the success of udder defenses against invading pathogens (Schwart et al., 2011; Leitner et al., 2003). Milk includes all types of white cells: polymorphonuclear leukocytes (PMNL), macrophages and lymphocytes. They play an important role in inflammatory responses within the mammary gland (Kelly et al., 1998; Paape et al., 1979; Sordillo and Nickerson,1988).

The induction and suppression of immune responses are regulated by lymphocytes (Nickerson, 1989). They recognize antigens through membrane receptors specific for invading pathogens (Sordillo et al., 1997).

Macrophages are active phagocytes cells in the mammary gland and capable of ingesting bacteria, cellular debris, and accumulated milk components (Sordillo and Nickerson,1988). Milk or tissue macrophages recognize the invading pathogens and initiate an immune response by the release of chemo attractants inducing the rapid recruitment of PMNL into the mammary gland (Paape et al., 2002; Oviedo-Boyso et al., 2007).

The main task of PMNL is to defend against invading bacteria at the beginning of an acute inflammatory process (Paape et al., 1979; Oviedo-Boyso et al., 2007). Their primary function is ingestion and destruction of invading microorganisms, as well as secretion of inflammatory regulators (Kelly et al., 1998). Usually, an increase in SCC is due to an increase in PMNL. Some researchers view the

PMNL count as an earlier and more specific indicator than SCC (Kitchen, 1981; O' Sullivan et al., 1992).

The distribution of leukocyte types varies in normal cow milk without any symptoms of mastitis. Some recent studies, about differential cell count, found lymphocytes proportions between 14 and 80%, macrophage proportions between 12 and 46%, and those of PMNL between 6 and 50% (Rivas et al., 2001a; Merle et al., 2007; Koess and Hamann, 2008). In mastitic milk, the proportions between the white cell changes and the PMNL can reach 95% (Paape et al., 1979; Kehrli and Shuster, 1994).

The differential cell count, in normal buffalo milk, have different finding. The literature describes leucocytes percentage, in Mediterranean Buffalo, between 1 and 84% for lymphocytes, between 1 and 71% for macrophage and between 8 and 98% for PMNL (Tripaldi et al., 2010; Piccinini et al., 2006; Dhakal, 2004; Thomas et al., 2004; Silva and Silva, 1994;). Recent studies, about the effects of mastitis on Mediterranean Buffalo milk quality, showed mean values of lymphocytes, macrophages and PMNL of 38 ± 18, 13 ± 11, 49 ± 20 (% ± SD), respectively (Tripaldi et al., 2010). The study assessed also that in milk samples, positive for udder-specific bacteria, polymorphonuclear leukocytes made up greater than 50% of the cells and that the correlation between SCC and PMNL was stronger (0,70). It also described a significantly increased ($P \leq 0{,}05$) of PMNL percentages in the following class: 38% ± 2 SD for SCC < 100'000 cell/ml , 56% ± 4 SD for SCC between 100'000 and 200'000 cell/ml and 64% ± 3 SD for SCC > 200'000 cell/ml (Tripaldi et al., 2010).

Bacteriological Mastitis

Mastitis generally results from interaction between a variety of microbial infections and host responses in the udder, and it is influenced by management practices. Factors which predispose to mastitis include mostly environmental aspects such as poor hygiene, poor husbandry, overcrowding, bad ventilation, poor milking technique and malfunction of milking machines. Besides, factors which adversely affect the normally efficient barriers to infection of the udder such as teat skin, teat canal and mammary cistern, predispose udder to mastitis. Moreover, among pathogenic mechanisms, Gram positive and Gram negative bacteria seem to use different adhesion mechanisms to epithelial cells of the mammary gland (Fagiolo and Lai, 2007). Besides several enzymes, ialuronidase, lecytinase, haemolysine, collagenase and kinase act as aggressive factors invading host tissues. In the buffalo species too, mastitis is the most costly disease in the dairy industry even though buffalo has been traditionally considered less susceptible to mastitis than cattle (Wanasinghe, 1985), but some studies, performed on Murrah buffalo, showed similar mastitis frequencies for the two species (Bansal, 1995; Badran, 1985; Kalra, 1964). Nevertheless, as in any raw milk, microorganisms can multiply rapidly in buffalo milk due to its high nutrient content. However, in comparison with cattle, buffaloes have some characteristics that may contribute to greater risk of mastitis such as more pendulous udder and longer teats. Conversely, they have a long narrow teat canal, which may be expected to prevent the invasion of microorganism. Krishnaswamy et al. (1965) instead stated that teat sphincter of buffalos have smoother muscular fiber in such a

way it constitutes a better barrier to microorganism invasion than cows teat sphincter.

In buffalo cows mastitis is quite always caused by bacteria (Galiero, 2002). Mastitis-causing bacteria can be classified in contagious as Streptococcus agalactiae, Stapylococcus aureus, Arcanobacter piogenes, Micoplasma; environmental as Streptococcus uberis, and dysgalactiae, Escherichia coli, Enterobacteriacee, yeasts and moulds (Prototheca zoophii) and opportunist as coagulase negative Staphilococcus (Fagiolo and Lai, 2007).

Usually, the majority of the infections is caused by the contagious pathogens *Staphylococcus aureus*, *Streptococcus agalactiae*, and by the environmental pathogens *Streptococcus uberis, disgalactiae,* Coliforms (E. coli, Klebsiella, Enterobacter) Pseudomonas, Prototheca, yeasts and *Escherichia coli*. These pathogens infect the udder generally through the ductus papillaris, which is the only opening of the udder to the outside world. Staphylococcus aureus is the most problematic and significant pathogen in contagious mastitis due to the occurrence of pathogen strains particularly resistent to antibiotics. Staphylococcus typically colonize the breaked skin. Abrasion of the teat end and faulty milking encourages the transfer of bacteria into the udder. It can cause mostly subclinical mastitis and it remains for long time in farms. Streptococcus agalactiae is an udder strict parasite being transmitted exclusively during milking procedure. This infection remains in the milk ducts as superficial. It causes clinical and mostly subclinical mastitis inducing a remarkable increase in somatic cells count and a significative decrease in milk secretion. Clinical symptoms can vary from mild to moderate and clot occurs in

the milk. Streptococcal mastitis is not usually fatal nor causes a special problem for therapy (Fagiolo and Lai, 2007).

Regarding environmental pathogens, they are present in the faeces, in the soil, on the structures, milking equipment and in the litter. The milking equipment, particularly, plays a pathogenetic role in determining teat lesions which allow microorganisms penetration and invasion in the udder whereas bedding contamination represents the main source of infection of Coliforms since they do not colonize the milk duct and the most critical time is just after milking or during interval between two milkings (Fagiolo and Lai, 2007).

Regarding opportunist pathogens, these are usual guests of the animal skin and they need predisposing factors such as skin lesions, unproper milking, inadequate disinfection (Fagiolo and Lai, 2007).

Galiero et al. (1996) examining 13 farms have observed that contagious pathogens as coagulase-positive Staphylococcus aureus and Streptococcus agalactiae were mostly responsible of mastitis onset, whereas Streptococcus disgalactiae seemed to have a marginal role.

In Latina district, Zottola et al. (2005) have carried out a study in 10 farms in order to investigate water buffalo mastitis in this area. In almost 50% of positive animals, just one microbial strain was detected whereas in the rest of them it was observed more than one microbial strain. In the same study, among positive buffalos the most frequently detected pathogen strain were Streptococcus uberis and Str. agalactiae followed by few account of Streptococcus disgalactiae, Aerococcus viridans, Enterococcus faecalis and Streptococcus bovis.

Mediterranean Buffalo breeding has been recently introduced in northern Italy also. In northern districts Moroni et al. (2006) have

examined lactating water buffalos of 2 farms and have found that the predominant microorganism is *coagulase-negative Staphylococcus*, whereas *Streptococcus* accounted for 15 %, mostly *Str. Uberis* (80%). Among other pathogens were *Bacillus spp.*

THE DIAGNOSIS OF MASTITIS

Mastitis influences the quality and quantitative of milk. Penetration of pathogenic microorganisms in the teat canal irritates and invades the delicate mammary tissue, causing an inflammatory response and consequent milk changes. Degree of these changes depends by infecting agent and the inflammatory response (Sharif and Muhammad, 2008). The diagnosis of mastitis, both in dairy buffalo and cow, is not always simple because the inflammatory process is characterized by different clinical findings relate to its gravity. Early detection of mastitic cows is important for dairy farmers to reduce production losses. Diagnosis of clinical mastitis is based on the local and systemic reactions and changes in milk (e.g. off-color, watery, bloody appearance and presence of flakes, clots and pus) both in dairy buffalo and cow. The amount udder swelling, severity of pain and the overall appearance of the animal will indicate the severity of infection and serve as a guide for the course of treatment. The diagnosis of subclinical mastitis is more problematic since the milk and the udder

appear normal but usually has an elevated somatic cell count. (Sharif and Muhammad, 2008; Sharma et al., 2010). Early diagnosis of mastitis is vital because change in the udder tissue take place much earlier than they became apparent (Sharma et al., 2010). Various methods, based on physical and chemical changes of milk and cultural isolation of organisms, are used for diagnosis of subclinical mastitis (Batra and Mcallister, 1984; Emanuelson et al., 1987). The diagnosis of mastitis according to the International Dairy Federation (1971) guidelines is based on the somatic cell count (SCC) and microbiological status of the quarter, although, several simple test, which do not require any complex laboratory equipment and considering field screening test, were developed (Sharma et al., 2010). Results of these studies can be considered the born of California Mastitis Test (CMT) and the electrical conductivity (EC). At present, each of these (SCC, bacteriological milk culture (BC), CMT and EC) present different sensibility and specificity rate but no one had values of 100% so, their combined used is often recommended (Rubén, 2002).

At present, diagnosis of mastitis in buffalo is based on clinical signs and laboratory tests as described for cow. Although these test were created for dairy cow, they are commonly used in Mediterranean Buffalo because the mastitis management is often based on knowledge transferred from bovine scientific evidences. The two species were considered similar for a long time, and buffalo world, as consequence, has been affected by poor scientific and cultural knowledge. All the studies, present in literature, shown little difference in test behaviour when applied in buffalo mastitis so, these conditions have to always considered to performed right diagnosis.

Somatic Cell Counts (SCC)

The mammary gland is made up of a remarkably sensitive tissue which has the capability of producing a large volume of secretion, milk, under normal or healthy conditions. When bacteria enter the gland and establish an infection, inflammation is initiated accompanied by an influx of white cells from the bloodstream, altered secretory function, and changes in the volume and composition of secretion. Since cell numbers in milk are closely associated with inflammation and udder health, these somatic cell counts (SCC) are accepted as the international standard measurement of milk quality. Unfortunately, although this cytological examination is considered the most reliable and specific to measure udder inflammation available to every dairy farmer in the most of the developed countries (Harmon, 2002), it cannot identify the etiologic agent (Hamann, 2002; Kitchen. 1981). It can be performed on quarter, composite (pool of 4 quarts) or bulk thank milk samples (Rubén, 2002). Milk somatic cells are primarily constituted by leukocytes or white blood cells, which include macrophages, lymphocytes, and polymorphonuclear neutrophils. Studies identifying cell types in milk have shown that epithelial cells or the cells which produce milk are infrequently found in udder secretions, including those from the dry gland, with a range from 0 to 7% of the cell population (Lee et al., 1980).

The somatic cell count can be considered a valid indicator of udder health and an excellent marker for mastitis also in dairy buffalo. According to several studies in Mediterranean Buffalo the normal values of SCC can be included between 191.800 and 221.280 cell/ml (APA Latina District, 2000; Tripaldi et al., 2003). In fact, several works indicate the values of 200.000 cell/ml as threshold for early

identification of buffalo udder inflammation (Tripaldi et al., 2010). The importance of this limit was also confirmed by Moroni et al. (2006) throughout a study to assess the phenotypic relationship among udder infection, SCC and milk production. During the study the 100% of quarters with SCC >200’000 cell/ml had intramammary infection, whereas 98% of quarters with SCC below this threshold were uninfected. In addition, the associations between high SCC level (SCC > 200’000) and significantly decreased milk yield and changes in buffalo milk composition and coagulating properties were detected. Other works confirmed this negative relationship (Tripaldi et al. 2003, 2010). Actually, cow’s quarters instead producing milk with SCC >200’000 cells/ml, without clinical signs, is defined as subclinically mastitic, while bacteriologically negative quarters with SCC < 100’000 cells/ml are considered healthy (Smith, 2002; Pyorala, 2003). Schepers et al. (1997) confirmed an increase in SCC from <200’000 to >200’000 cells/ml was optimal for the prediction of new intramammary infections (IMI) (Sensitivity (Se), 38.8%; Specificity (Sp), 91.9%), while Dohoo and Leslie (1991) found a Se of 83,4 % and Sp of 58,9% to detect quarters infected with major pathogens using an SCC threshold of 200’000 cells/ml in cow.

The changes leading to progressive increase in somatic cell level and loss in milk productions due to mastitis are documented both in dairy buffalo and cow (Tripaldi et al., 2010; Moroni et al., 2006; NMC, 1996). In Nili-Ravi buffaloes, mastitis shortens lactation period of each animal by 57 days on an average and reduces 438 Kg of milk per lactation were described (Candy et al., 1983). Unfortunately, although an correlation between IMI, SCC and decrease productions was found also in Mediterranean Buffalo (Tripaldi et al., 2010,

Moroni et al., 2006), no work is so complete and able to quantify the loss due to high levels of SCC as in cow, where Koldeweij el al. (1999) found a linear relationship between production and Log_{10} of SCC. In general, more exactly data are available in cow. Jones in 1986, suggested that SCC of 0,6 to 1 million cells/ml were associated with an 8 to 12% reduction in herd milk production. National Mastitis Council (1996) indications also showed that 6% of cow's quarters could be infected when the bulk tank milk has a SCC of 200'000 cells/ml and that a herd with a bulk tank SCC of 500'000cells/ml would have 16% infected quarters with 6% reduction in milk yield. It also recorded the 32% of infected quarters and milk loss of 18%, when a herd had a bulk tank SCC of one million/ml.

Traditionally, physiological factors affecting SCC in cow, can be considered stage of lactation and milking frequency (Harmon, 1994). The logarithm of SCC was high at the beginning of the lactation, dropped to a minimum between 40 and 80 days postpartum, and then steadily increased until the end of lactation (from 83'000 at 35 days after calving to 160'000 by day 285) (Berkema et al., 1999; Scheper et al., 1997). A plot of monthly SCC inversely correlates to lactation curve was describe by Reneau (1986). The influence of number of lactation, stage or seasons, on SCC in buffalo are mainly describe in Murrah buffaloes but, they did not affect the somatic cells and low mean of SCC. Values below the threshold of 200'000 cells/ml were also found during the whole milking time (136'000/ml in July-August, 10'800/ml in May-June and 76'000/ml in December-January) (Singh and Ludri, 2001). A seasonal pattern were instead observed in cow by Schukken et al. (1992) that showed expected lowest mean SCC

occurred in April and expected highest mean SCC occurred in October.

Time and frequency of milking are also considered affecting milk SCC in cow, but scientific evidences showed a different situation in buffalo. A study performed on Murrah buffalo detected no difference in SCC values between the morning and evening milk thereby suggesting that no diurnal variation existed in somatic cell secretion in milk of buffaloes milked at equal intervals during morning and evening hours (Singh and Ludri, 2000). Obviously, different situation was described in cow because Kukovics et al., (1996) found higher SCC in afternoon than in morning milking, and also increased with age, year and lactation number. Moreover, a shift a SCC decrease in bulk milk and change in cell proportion was detected with a shift from two times a day to three times a day milking (Hogeveen et al., 2001), while too much short milking intervals (4 h and less) or too long increase their levels (Hamann, 2001; Pettersson et al., 2002).

Bacteriological Milk Culture (BC)

Bacterial culture is routinely used to diagnose mastitis both in dairy buffalo and cow. It can use to obtain informations about herd problem when performed on bulk tank milk level or individual problem when performed in quarter o composite (pool of 4 quarters) milk samples. Microbiologic exam of milk samples may be used for control programs or detection of new pathogens. Culturing is also used to determine antibiotic susceptibility of mastitis pathogens. The microbiologic examination of milk samples is considered to be the gold standard for identification of infected quarters (NMC, 2004).

According to National Mastitis Congress (2004) guidelines a proper collection of milk samples is of paramount importance for identification of mastitis pathogens. At present, all these guidelines about milk sampling technique and the interpretation of milk cultures' results are considered useful also for dairy buffalo. Aseptic technique is an absolute necessity when collecting milk samples to prevent contamination by organisms found on the skin, udder, and teats; hands of the sampler; and in the barn environment. Contaminated samples result in misdiagnosis, increased work and expense, confusion, and frustration. Contamination can be avoided by following the procedures described below:

- label tubes prior to sampling (date, farm, buffalo, quarter);
- brush loose dirt, bedding, and hair from the udder and teats. Thoroughly wash and dry grossly dirty teats and udders before proceeding with sample collection (udders should be washed as a last resort);
- discard several streams of milk from the teat (strict foremilk) and observe milk and mammary quarters for signs of clinical mastitis. Record all observations of clinical signs;
- dip all quarters in an effective pre-milking teat disinfectant and allow at least 30 seconds contact time;
- dry teats thoroughly with an individual towel.
- beginning with teats on the far side of the udder, scrub teat ends vigorously (10 to 15 seconds) with cotton balls or gauze pledgets moist (not dripping wet) with 70% alcohol. Teat ends should be scrubbed until no more dirt appears on the swab or is visible on the teat end. A single cotton ball or alcohol swab

should not be used on more than one teat. Take care not to touch clean teat ends. Avoid clean teats coming into contact with dirty tail switches, feet, and legs. In herds where animals are not cooperative, begin by scrubbing the nearest teat until clean, obtain the sample, and move to the next teat;

- begin sample collection from the closest teat and move to teats on the far side of the udder. Remove the cap from the tube or vial but do not set the cap down or touch the inner surface of the cap. Always keep the open end of the cap facing downward. Maintain the tube or vial at approximately a 45 degree angle while taking the sample. Do not allow the lip of the sample tube to touch the teat end. Collect one to three streams of milk and immediately replace and tightly secure the cap. Do not overfill tubes, especially if samples are to be frozen;
- to collect a composite sample (milk from all four quarters in the same tube), begin sample collection with the nearest teats and progress to the teats on the far side of the udder. One to 2 ml of milk should be collected from each quarter of the udder;
- when samples are taken at the end of milking or between milkings, teats should be dipped in an effective germicidal teat disinfectant following sample collection;
- store samples immediately on ice or in some form of refrigeration. Samples to be cultured at a later date (more than 48 hours) should be frozen immediately.

Diagnosing intramammary infection can be subject to errors. Culture of milk samples generally can results in: no bacterial growth, growth of a pure culture and growth of multiple colony types. Any of

the three outcomes may not represent the true infection status of the quarter. This is the reason because diagnosis of intramammary infection status based on multiple samples is more reliable than diagnosis based on a single sample Therefore, strict adherence to aseptic sampling technique and proper storage and handling of milk samples are absolutely essential (NMC, 1999).

The timing of collection of the milk samples can influence the recovery of mastitis organisms. It was demonstrated that, more colonies of *Staph aureus* were recovered from samples obtained before milking (91%) as compared to samples obtained after milking (81%) (Sears et al., 1991).

False positive (FP) and false negative (FN) results are commonly observed performing milk sample cultures.

FP results are present when a pathogen is isolated in pure culture but the quarter is truly not infected. It often occurs as a result of contamination during sample collection and/or processing. When intramammary infection status is based on culture of a single sample, samples get interpreted as an infected quarter, in fact, a frequent assumption is that the recovery of the contagious pathogens such us Staphylococcus aureus or Streptococcus agalactiae from a single milk sample is evidence of intramammary infection (NMC, 1999).

The results are considered FN when no microbial growth is detected following microbiological culture but the quarter is truly infected. Reasons for such samples include: low number of microbial colony-forming units, special media or growth conditions are required, inhibitors in the milk sample, such as antibiotics, have interfered with the growth of the pathogen, sample mishandled during storage resulting in death of the pathogen (Sears el al., 1990). False-negative

samples are more likely to occur with coliform and Staph. aureus infections than infections caused by Strep. agalactiae in cow. Attempts to reduce the number of false negative samples by using enrichment techniques or a period of preliminary incubation should be avoided (NMC, 1999). Bacteria do not grow in conventional culture in a substantial proportion of mastitic milk samples. According to the literature, no bacterial growth is detected in at least 20 to 30% of milk samples taken from udder quarters with clinical mastitis; in a study in the United Kingdom, the figure was 26.5% (Bradley et al., 2007), in an earlier study in the United States, 27.2% (Hogan et al., 1989), and in Finnish studies 23.7% (Koivula et al., 2007) and 27.1% (Nevala et al., 2004). An exceptionally high figure of 43.9% was recently reported in Canada (Olde et al., 2008). This incongruence was possible because clinical signs may be present but the pathogen has been eliminated by the animal's immune system (NMC. 1999).

Considering all the causes of FN results, also in subclinical mastitis, samples with no bacterial growth are generally even more common. Several studies had showed this condition with variable percentage between 28,7% (Koivula et al., 2007) and 38.6% (Bradley et al., 2007). Finally, Makovec and Ruegg (2003) reported a figure of 49.7% without differentiating between subclinical and clinical mastitis. A negative result for a milk sample is not only frustrating for the farmer and the veterinarian submitting the sample, and laboratory responsible for mastitis diagnostics.

The opposite condition, quarter milk sample resulting in the culture of three or more dissimilar colony types, can be possible. In this situation the sample is considered contaminated and a "low level" and

a “gross level” of contamination are generally recognized (NMC. 1999).

When a low level of contamination is detect dissimilar colonies are present on the plats. These may be the only colonies on the streak or they may be present in the streak of an otherwise pure culture of a pathogen. This contamination should be recorded together with the pathogen.

When a gross level of contamination is instead observed three or more colony types are present on the milk streak, often in relatively heavy growth. Such samples should be declared contaminated and no attempt should be made to identify potential pathogens within the mix of microbial growth and the quarter should be resampled. Common contamination sources include dirty teat ends, milk touching fingers before entering the tube, non-sterile tubes or inoculating needles, streaking milk samples on contaminated media, excess alcohol on teats or hands, contaminated cotton swab container, and the container lid not sealed tightly resulting in alcohol evaporation from cotton swabs (NMC. 1999).

No complete studies are present about SE and SP rate of milk culture in Mediterranean Buffalo although it is accepted as “gold standard” test for detection of the causative agent of IMI. A different situation characterizes the cow were several studies were performed on this argument. In fact, for quarter milk samples and a milk inoculum of 0,1 ml milk samples found Se of 90.9% and Sp of 99.8% (1). For single composite milk samples with the same milk inoculum Se was 92% and Sp 86% (11).

Recent studies in cow were performed to examine the potential benefits of using different combinations of multiple quarter milk

samples compared with a single sample for diagnosing IMI due to coagulase-negative staphylococci and Streptococcus spp in dairy cattle. Series interpretation of duplicate or consecutive samples resulted in the highest specificity (Sp; CNS Sp = 92.1–98.1%;Streptococcus spp. Sp = 98.7–99.6%), but lowest sensitivity (Se; CNS Se = 41.9–53.3%; Streptococcus spp. Se = 7.7–22.2%). Parallel interpretation of duplicate or consecutive samples resulted in the highest Se (CNS Se = 70.8–80.6%; Streptococcus spp. Se = 31.6–48.1%), but lowest Sp (CNS Sp = 72.0–77.3%; Streptococcus spp. Sp = 89.5–93.3%) (Dohoo, 2011).

Many forms of herd culture programs have been suggested and implemented (Leslie, 1994) in cow. Practice experience suggested that they are also applied in Mediterranean but few have been formally evaluated. These programs include: periodic culture of all milking cows in the herd; strategic culturing of herd additions and all cows and heifers at dry-off and/or freshening; culturing all clinical cases of mastitis identified in the herd; and periodic culture of bulk tank milk samples (NCM. 2000).

California Mastitis Test*.(CMT)*

The California Mastitis test was developed in 1957 and for 50 years the only reliable cow-side screening test for subclinical mastitis. Its reaction is an indirect measure of SCC in milk (Barnum and Newbould, 1961), it reflects the SCC level quite accurately and is a reliable indicator of the severity of infection (Schalm and Noorlander, 1957; Barnum and Newbould, 1961; Dohoo and Meek, 1982). It is used to identify quarters that have subclinical mastitis but does not identify the type of bacteria. It was developed to test milk from

individual quarters but has also been used on composite milk samples and bulk milk samples detecting abnormal milk (Schalm and Noorlander, 1957). Fresh, unrefrigerated milk can be tested using the CMT for up to 12 hours, reliable readings can be obtained from refrigerated milk for up to 36 hours. If stored milk is used, the milk sample must be thoroughly mixed before testing because somatic cells segregate with the milk fat. The CMT reaction must be scored within 15 seconds of mixing because weak reactions will disappear after that time. The test is based upon a reaction between the amount of cellular nuclear protein present in the milk sample represented by inflammatory and a reagent represented by 3% sodium lauryl sulphate and bromocresol. This is usually carried out, according to the method described by Schalm and Noorlander (1957), at cow-side by mixing an equal volume of milk with reagent. Each quarter milk sample from the cow was placed in one clean well of a black plastic test paddle divided into four separate wells, one for each quarter sample. As the plate was rotated gently, any colour changes or formation of a viscous gel were interpreted (Bhutto A. L. et al. 2012). The relationship between SCC values and CMT is not precise because of the high degree of variability in SCC values of each CMT (Jasper, 1967). According to Jasper (1967). Scores, correlated with SCC values were, given to the results within the range 0–3 as reported in Table 1

CMT Score	Somatic Cell Range	Visible Reaction
N	0 to 200’000	Mixture remains liquid, no evidence of precipitate
T	200’000 to 400’000	Slight precipitate, best seen by tipping, disappears with continued movement
1	400’000 to 1’200’000	Distinct precipitate but no tendency toward gel formation
2	1’200’000 to 5’000’000	Mixture thickens immediately, moves toward center
3	over 5’000’000	Gel forms and surface becomes convex

Table 1. Relationship between CMT Score and Somatic cell counts.

The CMT with score N equates with SCC level of 200’000 cell/ml or less which is considered to be the physiological level for milk from uninfected cows. CMT Score T (trace) corresponds to a SCC level of 200’000 to 300’000 cells; a level at which mastitis pathogens are likely to be isolated. The use of the CMT to identify quarters infected with contagious mastitis has been extensively evaluated (Barnum and Newbould, 1961, Brookbanks, 1966, Painter and Schnepper, 1965, Wesen et al., 1968). In general, as CMT reactions increase, the likelihood of recovering pathogenic bacteria increases. The ability of the CMT to detect infected quarters of fresh cows has been recently reported by Sargeant et al. (2000). They described a percentage of 57% of infected quarters accurately identified (43% were missed) when a positive reaction (CMT>1) was present. Another study recorded CMT value of trace or greater in 92% of infected cows while CMT value of >1 in only 72% of case. The conclusion of the research was that, to minimize the number of false negative results, the test should be read as positive when at least a trace reaction is apparent (Brookbanks, 1966). Recently, a study performed by Salvador et al. (2012) about the prevalence and risk factors of subclinical mastitis as determined by the California Mastitis Test in water buffaloes (Bubalus

bubalis) detected mean values of sensitivity (82.4%) and specificity (80.6%) more higher then cow.

It can be used to screen animals or quarters to withhold from the bulk tank. More common and routine uses include screening of suspected new mastitis infections, and animals quarters to be sampled for SCC determination of bacterial culture. In many herds the test is used to screen fresh cows and cows at dry off period. This information is useful for selecting cows for further evaluation (milk culture), culling, selective dry cow treatment or extended therapy.

It was describe that in 10-20% of positive CMT reaction, the correspondent milk samples for bacterial culture have no bacteriological growth. This is due to a number of factors including short lived infections that have been cleared by the cow or infections that are characterized by intermittent shedding of bacteria (*Strep ag, Staph aureus*, mycoplasma) (N.Y. State - Cattle Health Assurance Program).

This test can be also used indicator of IMI during dry period. It was considered able to identify the 80% of all major IMI by Poutrel and Rainard (1981), although other works recorded a decrease of the Se (55 to 69%) and Sp (57-86%) values when used to identify IMI after calving (Sargeant et al., 2001; Dingwell et al., 2003b; Wallace et al., 2004). All studies reported that the CMT was not sensitive enough to be used alone as a screening test for IMI (Cole et al., 1965; Middleton et al., 2004; Ruegg and Sekito, 2004). The CMT can also be used to evaluate composite milk samples as well as bulk tank milk. Test criteria for commingled milk are less severe than that for quarter samples because of the dilution effect of milk from normal quarters or cows. The appropriateness or value of CMT evaluation of bulk milk

decreases as herd size increases. Finally, CMT can be used to monitor udder health trends over time (Rubén et al., 2002).

Electrical Conductivity (EC)

Electrical conductivity of milk has been introduced as an indicator trait for mastitis over the last decade (Hamann and Zecconi, 1998). It is introduced also in buffalo breeding for detection of mastitis. The EC is determined by the concentration of anions and cations. If the cow suffer from mastitis, the milk concentration of lactose and K^+ are decreased and concentrations of Na^+ and Cl^- are increased because of changes in the permeability of cells and blood vessels. This situation leads to increased EC, measured in milliSiemens (**mS**), because it is directly correlate with the concentration of these solutes (Kitchen, 1981). Most automatic milking systems have EC sensors incorporated for measuring EC during milking (in-line), and with the increasing use of such systems. Today, the role of the EC in dairy Mediterranean Buffalo mastitis is not completely clear and more complete research should be performed on its changes in animals with different health status. Boselli et al. (2004) found mean values of 4,11 mS/cm in Mediterranean Italian buffalo, while Dhakal et al. (2008) described in Murrah values of 3,75 ± 0,52, 4,17 ± 0,68, 5,35 ± 1,44 (EC ± SD, $P < 0,01$) in healthy, subclinically and clinically mastitic milk, respectively. Different situation was found by Haman and Zecconi (1998) in cow, describing values between 4,0 and 5,0 mS and 5,0 and 9,0 mS, in healthy and clinically infected quarters, respectively. Several authors found instead different range of EC, from 6,45 to 6,85 mS (Woolford et al., 1998) and from 4,83 to 7,03 mS (Isaksson et al., 1987), in animals with subclinically mastitis.

Electrical conductivity has mainly been expressed as a maximum value for each quarter or each milking (Maatje et al., 1992; Lansbergen et al.,1994; De Mol et al., 1999). Several detection models based on maximum values, showed difficulties to correctly detect sick cows (considerate healthy) and healthy cows (considerate sick) (Norberg. et al., 2004; De Mol et al., 1999). It has been suggested that by extracting only the high EC measurements from a milking, valuable information about EC pattern may be lost (Lake et al., 1992; Nielen et. al., 1995). A cow suffering from mastitis may not always show an increased EC of milk from the infected quarter, but the within-milking variation in EC of milk from an infected quarter may be larger than variation in EC. It is important to remember that a lot of non-mastitis factors influencing EC and including milk temperature, stage of lactation, fat percentage, milking interval, and breed were influence the test results(Timsit and Bairelle, 2008; Norberg et al., 2004).

The International Dairy Federation performed a meta-analysis of EC (using absolute thresholds) from a selection of published papers (Hamman and Zecconi, 1998). The EC did not perform well as a screening test for either clinical or subclinical mastitis. Of 100 positive EC tests only 58 would truly have clinical mastitis and 15-30% of animals identified as mastitis free would be truly infected. These results led the IDF panel to conclude that: "The published information is too varied to justify a claim that mastitis, especially subclinical mastitis, can be detected by means of electrical conductivity measurements in milk". A recent research, various EC traits were investigated for their association with udder health. Four EC traits were defined; the inter-quarter ratio (IQR) between the

highest and lowest quarter EC values, the maximum EC level for a cow, IQR between the highest and lowest quarter EC variation, and the maximum EC variation for a cow. Values for the traits were calculated for every milking throughout the entire lactation. All EC traits increased significantly ($P<0.001$) when cows were subclinically or clinically infected. Traits reflecting the level rather than variation of EC, and in particular the 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).

EXPERIMENTAL DATA

MATERIALS and METHODS

Animals and Sampling

The present study was carried out on 480 primiparous Mediterranean Buffaloes (*Bubalus Bubalis*), reared in a breeding of 2000 buffaloes, placed in south Italy. All the animals were randomly chosen between December 2011 and September 2012. A complete udder clinical examination was performed in each animal and those one with traumatic evidences of mastitis were discarded.

A composite milk sample (pool of the 4 quarts) of 50 ml was collected from each animal, between days 30 and 150 after calving, during evening milking and placed in sterile test-tubes (*Nuova APTACA, Italy*). All the samples were collected aseptically according to National Mastitis Congress guidelines (2004). After teats' dry cleaning, discharged of the first milk squirts, drying teats thoroughly with an individual towel, apex disinfection throughout with gauze and

alcohol 70%, the composite milk samples were collect using sterile gloves.

Clinical signs (CS)

Mastitis was diagnosed by clinical examination before sampling. Local and systemic reactions and changes in milk appearance were observed. All the animals showing clinical signs of mastitis were recorded and classified. In these animals, a complete (teat and gland) udder ultrasonographic examination was performed with an 12-MHz linear probe (General Electrics, Logiq E) to confirm the presence of intramammary abnormality due to mastitis. The teats were immersed in warm water to improve image quality, as described by Sendag and Dinc (1999). The presence of clinical signs and ultrasound evidences of mastitis were considered in end point phase.

California Mastitis Test (CMT)

CMT on each composite sample was directly performed in farm, after collection. Two millilitres of milk were mixed with an equal quantitative of apposite reagent (*CMT-TEST, Bovi-vet, Danmark*). According to range establish by Jasper D.E. (1967), all CMT reactions were scored in: 0 (negative), +/- (trace), 1 (weak positive), 2 (distinct positive), 3 (strong positive). All the reactions were proved by the same investigator (Figure 1). The results ranged between trace and strong positive were considered positive cut-off. According to Brookbanks E.O. (1966) indications, to minimize CMT false negative results, trace (+/-) of reaction were considered as positive (+1) during the statistical analysis.

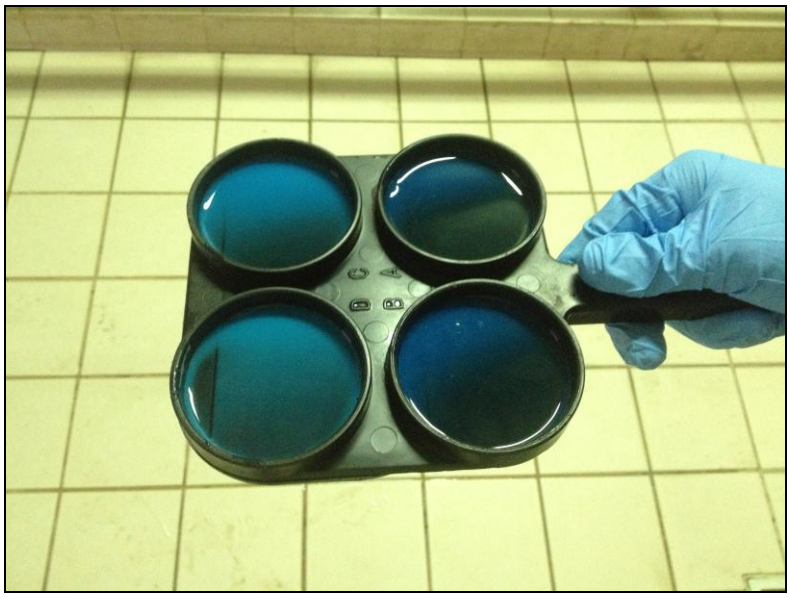


Figure 1. California Mastitis Test performed, after milking, in farm.

Measurement of Electrical Conductivity (EC)

An automatic digital mastitis detector (*Afifarm™, Afimilk® Israel*) was used for electrical conductivity measurement during milking process. Sensors for measuring EC were incorporated in the milking unit. The EC in buffalo milk was expressed as millisiemens per centimeter (mS/cm). Absolute evening EC values were obtained reading directly from EC meter and recorded manually after milking (Figure 2). All the equipment were monthly calibrate. EC > 8,5 mS/cm were considered in end point phase.

Figure 2. Manual electrical conductivity records.

Somatic Cells Count (SCC) and Bacteriological Milk Cultures (BC)

All the samples were cooled in a cool box (4°C), and brought to reference laboratory within 1 h. Each of these was submitted to somatic cells count and bacteriological examination within 2 hours after collection. SCC was determined using a Fossomatic 5000 (*Foss Electric, Hillerod, Denmark*).

The bacteriological milk culture of the composite samples was performed according to the IDF (1981) guidelines. Ten microliters of milk were streaked onto a quadrant of a 7% bovine blood agar plate containing 0,05% esculin (*Merck KGaA, Darmstadt, Germany*), incubated for 48 h at 37°C, and examined. If less than five identical colonies were present on the plate, the sample was not included in the analysis because of the possibility of contamination. SCC>200.000 cells/ml and BC positive for pathogens-specific of clinical mastitis were considered as positive cut-off.

Definition of Udder Health Status

The buffalo included in the study were defined as healthy, infected, affected by subclinical or clinical mastitis according to SCC values (above and below 200'000 cells/ml), microbiological status and clinical signs presence as indicated in Table 2.

Health status	SCC values (Cells/ml)	Microbiological status	Clinical signs
Healthy	SCC ≤ 200'000	Negative	Negative
Infected	SCC ≤ 200'000	Positive	Negative
Subclinical mastitis	SCC > 200'000	Positive	Negative
Clinical mastitis	SCC > 200'000	Positive	Positive

Table 2. Definition of udder health status used in the study.

Statistical Analysis

EC and SCC measurements were analysed by standard descriptive statistics, and normality was assessed using histograms, normal probability (QQ) plots and Shapiro Wilk tests. Both variables were highly right-skewed, and log10 transformations were used to normalize the respective distributions. Untransformed and log-transformed EC and SCC values were compared between animals with and without clinical symptoms using box plots and parametric (students t) and non-parametric (Mann-Whitney U) tests. EC and SCC measurements between animals in the four different CMT classes were compared using box plots and one way Analysis of Variance (ANOVA) and Kruskal Wallis ANOVA on Ranks followed by posthoc multiple comparisons with Bonferroni correction.

Numerical correlation between EC, CMT, SCC, BC and CS results were assessed using Spearman rank correlation test, first in all animals

and subsequently (a) in animals with SCC < 200’000 cells/ ml (clinically healthy, potentially infected) and (b) animals with SCC > 200’000 cells/ml (subclinically or clinically diseased). The diagnostic characteristics of EC and SCC measurements to reflect the buffalo’s udder bacteriological status (BC status positive or negative) were assessed using Receiver-Operator characteristic (ROC) curves and cross tabulations. Cases where the test were positive (based on a preselected cutoff) and coincided with a positive bacteriological milk cultures were true positives (TP) and cases where they failed to predicted an observed BC positive were considered false negative (FN). True negatives (TN) represented occasions when no BC positive was predicted, and the buffaloes were healthy. Cases where healthy buffaloes were classified as infected, based on EC or SCC results, were considered false positives (FP). The sensitivity is the percentage of infected cows that were classified as infected ((TP/(TP + FN)) × 100), and the specificity is the percentage of uninfected cows that were correctly classified as healthy ((TN/(FP + TN)) X 100).

RESULTS

The 20,4% (96/470) of the subjects enrolled in the study were considered affected by mastitis [clinical mastitis(CM) or subclinical mastitis (SCM)], while the 79,6% (374/470) were considered not affected by mastitis [infected (IMI) or healthy (H)].

The 23,2% of buffalo (109/470) were positive to EC while the 43,4% (204/470) to CMT. The 56,6 % of CMT performed was negative, the 25,1% widely positive, the 14,3% distinctly positive and 4,1% strongly positive (Table 3). The subjects positive to bacteriological milk culture were the 84,0% (395/470) while those one to SCC the 20,4% (96/470). The percentage of positive BC above and below 200’000 cells/ml were 100% (96/96) and 63,6 % (299/374), respectively. No contaminated composite milk samples were found.

Logarithmic of electrical conductivity (LogEC) and logarithmic of somatic cells count (LogSCC) values were ranged from 0,58 to 1,15

mS/cm (0,92 ± 0,06, mean ± SD) and from 3,60 to 7,13 cells/cm (5,48 ± 6,01).

CMT frequency distribution	Count	Percent
0	266	56,6
1	118	25,1
2	67	14,3
3	19	4,1

Table 3. Frequency distribution of CMT of all the buffaloes enrolled in the study.

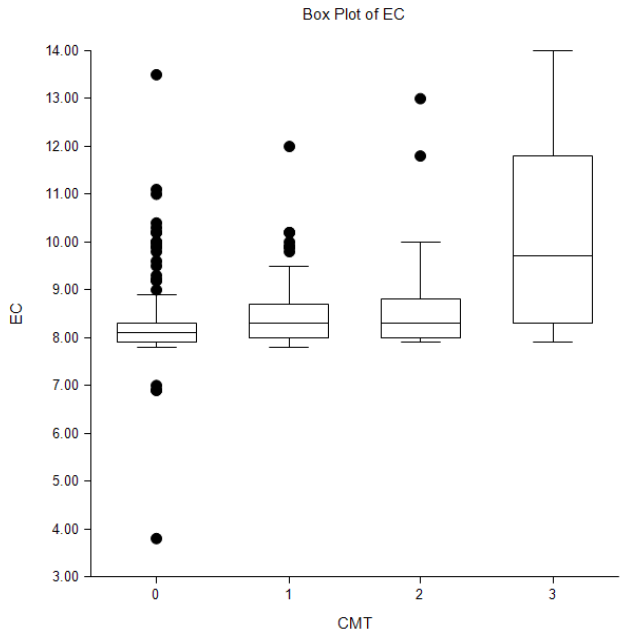
The means values of LogEC and LogSCC were 0,91 ± 0,14, 0,92 ± 0,18, 0,93 ± 0,18, 1,01 ± 0,33 mS/cm and 4,77 ± 4,63, 5,14 ± 5,08, 5,95 ±6,26, 6,43 ± 6,40 cells/ml related to CMT results 0, 1, 2, 3, respectively. The percentage of positive bacteriological milk culture instead were, 75,56%, 91,52%, 100% and 100%. The clinical signs were present only when the CMT results were strongly positive (Table 4).

Significative difference was recorded between mean EC measurements in animals with strongly positive CMT and the others comparison between EC and CMT ($P<0.0001$); furthermore, it was also found between SCC values in subject with strongly and distinctly positive CMT results and the others comparison between these two variables ($P<0.0001$) (Table 4 and Graph 1, 2). No significative difference was recorded between EC and SCC means measurements in animals with positive and negative BC (Graph3, 4).

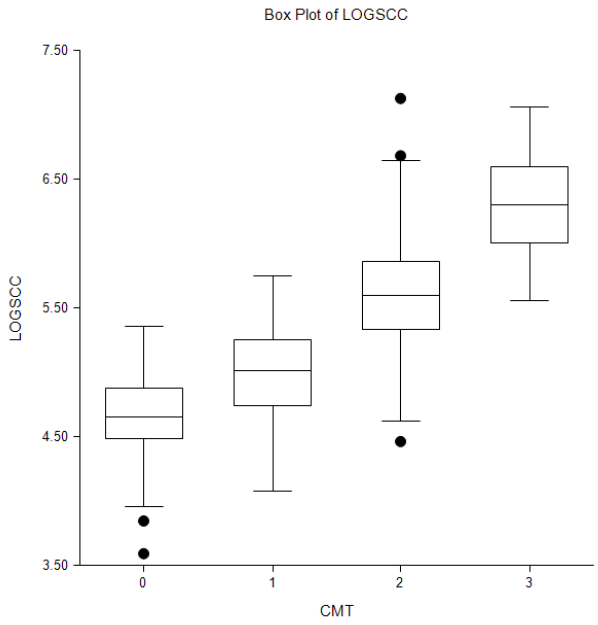
TEST	CMT (0)	CMT (1)	CMT (2)	CMT (3)
EC (mS/cm)	0,91 ± 0,14 [b]	0,92 ± 0,18 [b]	0,93 ± 0,18 [b]	1,01 ± 0,33 [a]
SCC (cells/ml)	4,77 ± 4,63 [b,b*]	5,14 ± 5,08 [b,b*]	5,95 ± 6,26 [b,a*]	6,43 ± 6,40 [a]
BC + (%)	75,56	91,52	100	100
CS (%)	-	-	-	100

Table 4. [a,a*b,b*]Mean and standard deviation (SD) and percentage of LogEC, LogSCC, BC and CS related to CMT levels (P<0.0001).

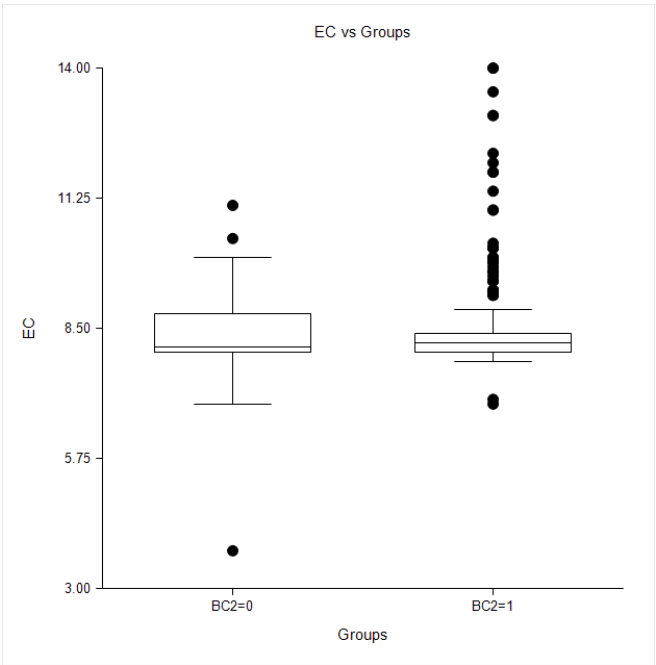
No significative difference was recorded between means EC measurement in animals with positive and negative CS, while it was detected (P< 0.0001) between SCC means values (Graph. 5).



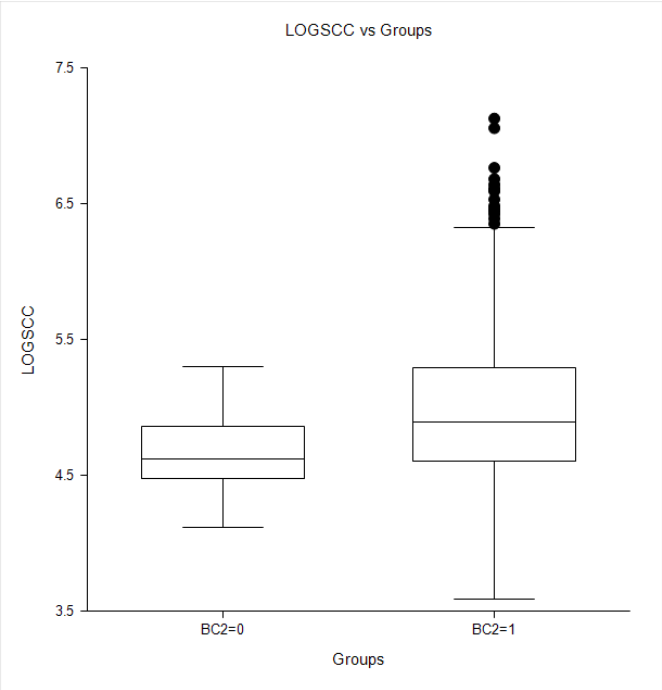
Graph 1. Box plot comparing the measurements of EC in animals with different CMT status (KW ANOVA on Ranks, p < 0.0001; group 3 is significantly higher than all other groups)



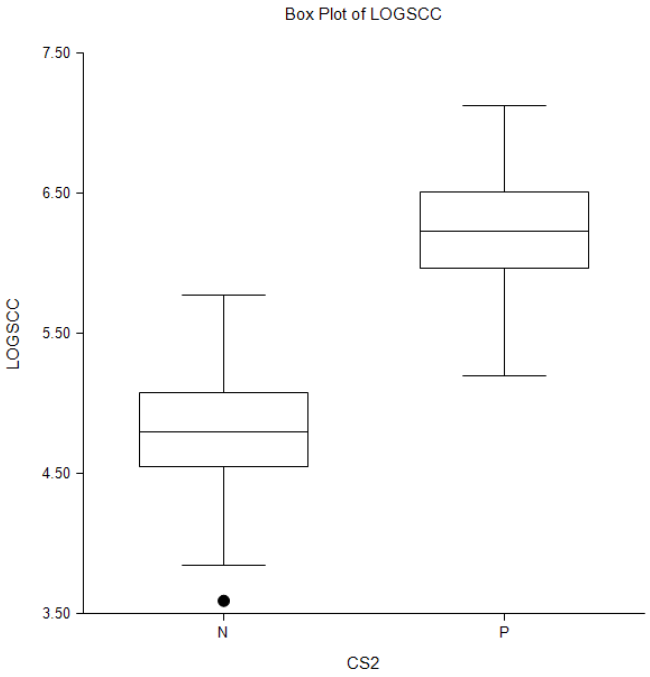
Graph 2. Box plot comparing the measurements of LogSCC in animals with different CMT status (KW ANOVA on Ranks, $p < 0.0001$; all differences except between groups 2&3 are statistically after Bonferroni correction for multiple comparison.



Graph 3. Box plot comparing the measurements of EC in animals with negative (0) and positive (1) BC status (Mann-Whitney U test; $p = 0.957$)



Graph 4. Box plot comparing the measurements of LogSCC in animals with negative (0) and positive (1) BC status (Mann-Whitney U test; $p < 0.0001$).



Graph 5. Box plot showing the distribution of LogSCC in animals with (6,24 ± 0,39, mean ± SD) and without (4,84 ± 0,40) clinical signs. The difference was highly significant (Two sample t-test, $p < 0.0001$).

Spearman Test

Low numerical correlations of 0,256 and 0,246 were observed between EC-CMT, EC-SCC while a moderate correlation of 0,657 was seen between CMT and SCC.

Correlation coefficients between numerically coded qualitative variables BC2, EC2, CMT2, SCC2 and CS2 were low to moderate (Table 5). A low correlation of 0,220 was also found between SCC values, above and below 200'000, and positive and negative BC.

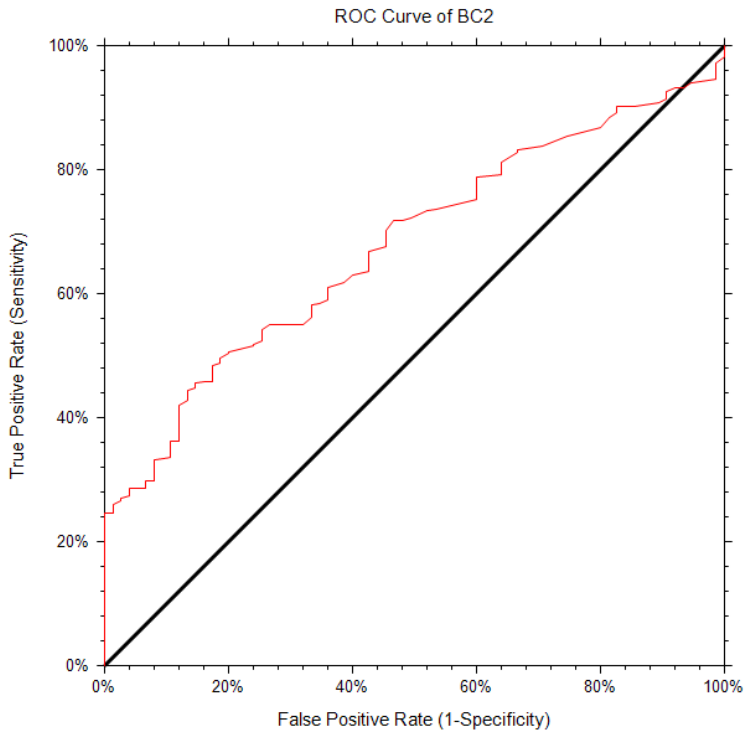
	EC	CMT	SCC	BC2	EC2	CMT2	SCC2	CS2
EC	1	0.256	0.246	-0.002	0.730	0.243	0.227	0.208
CMT	0.256	1	0.657	0.274	0.199	0.959	0.675	0.481
SCC	0.246	0.657	1	0.224	0.161	0.588	0.698	0.461
BC2	-0.002	0.274	0.224	1	-0.079	0.264	0.220	0.127
EC2	0.730	0.199	0.161	-0.079	1	0.174	0.174	0.197
CMT2	0.243	0.959	0.588	0.264	0.174729	1	0.546	0.333
SSC2	0.227	0.675	0.698	0.220	0.174	0.546	1	0.557
CS2	0.208	0.481	0.461	0.127	0.197	0.333	0.557	1

Table 5. Spearman rank correlation coefficients between different quantitative and qualitative measurements of disease status- Values > 0.2 indicate a low and values > 0.5 a moderately strong linear correlation.

Diagnostic test characteristics of SSC when compared to bacterial culture

The ROC analysis indicates that there is little diagnostic information in the SSC measurements towards the bacteriological status of the animal (Graph 6). Depending on the selected threshold (cutoff value) for SSC, sensitivity-specificity combinations of 41% SE and 88% SP (SSC cutoff 100'000 cells/ml) and 25.6% SE and 100% SP (cutoff 200'000 cells/ml) could be reached. The diagnostic validity

of EC measurements towards the bacterial culture status was poor (details not shown).



Graph 6: Reveiver-Operator-Characteristic curve of SSC measurements against bacterial culture status.

Clinical mastitic animals (CM)

After the udder clinical examination 10 animals were discarded due to clinical evidences of traumatic mastitis. Of 470 primiparous Mediterranean Buffaloes enrolled in the study, the 7,9 % (37/470) showed several clinical signs of mastitis characterized by off-color and watery appearance milk, presence of flakes, clots and pus. Udder swelling and pain, such us overall appearance of depression, were also recorded in some of these. Recognized mastitic pathogens affecting dairy buffaloes isolated in clinically mastitic animals were summarized in the Table 6.

Bacteria
Staphylococcus aureus
Staphylococcus haemoliticus
Aerococcus viridans
Staphylococcus chromogens
Streptococcus dysgalactie
Staphylococcus xylosus
Staphylococcus warnery
Streptococcus uberis
Escherichia coli

Table 6. Pathogens-specific for clinical mastitis in buffalo isolated in clinically sick animals.

In the clinically disease buffaloes, the EC was positive in 56,8% of cases (Table 7) while the CMT was always positive. It was distinctly positive (+2) in 54,1 % of cases while the remaining 45,9% was strongly positive (+3) (Table 8). LogEC and LogSCC values were ranged from 0,88 to 1,15 mS/cm (0,98 ± 0,27 mean ± SD) (Table 9) and from 5,78 to 7,13 cells/ml (6,41 ± 6,44), respectively (Table 10). The highest number of subjects were observed between 7,5 and 8,5 mS/cm (Table 9) and between 5 X 10^5 and 1 X 10^6 cells/ml (Table 11). Significative difference were recorded in means LogEC and LogSCC levels between clinically mastitic animals and subclinically mastitic, infected or healthy ($P<0.0001$) (Table 11). Finally, ultrasonographic evidences of clinical mastitis were also found in all the animals examined (Figure 1, 2).

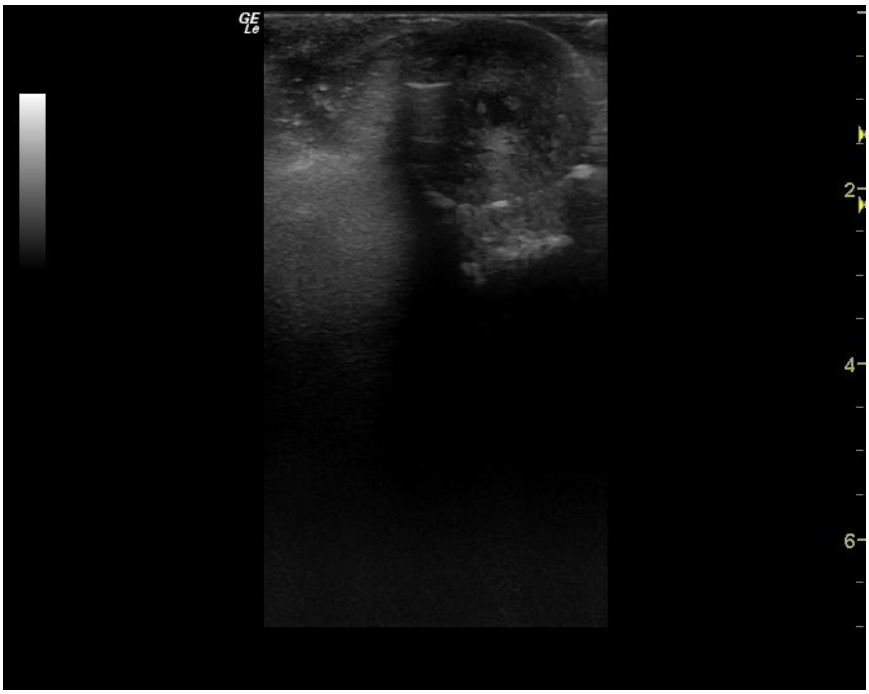


Figure 3. Ultrasonographic transversal teat view. Partial teat channel occlusion due to fibrin presence during clinical mastitis.

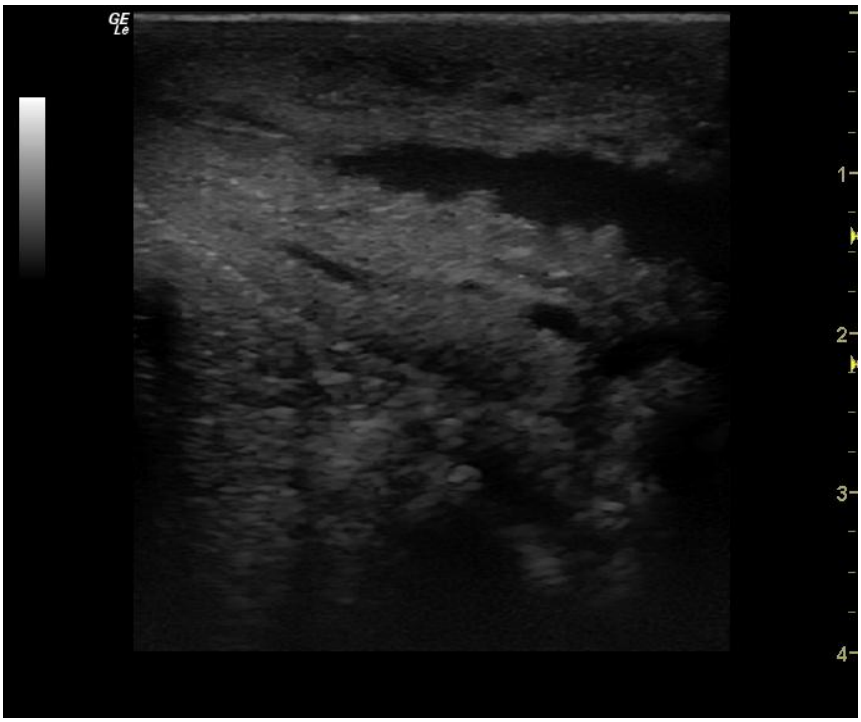


Figure 4. Ultrasonographic longitudinal teat view. Irregular profile of teat channel during clinical mastitis.

Subclinical Mastitic Animals (SCM)

Subclinical mastitis were observed in 12,6 % of subjects (59/470). The 28,8% of EC measurements were positive (Table 7) while the CMT in 94,6%. It was negative in 5,1% of case, weakly positive 35,6%, distinctly positive 55,9%, while strongly positive 3,4% (Table 8). LogEC and LogSCC values were ranged from 0,90 to 1,11 mS/cm (0,93 ± 0,11 mean ± SD) (Table 9) and from 5,31 to 5,75 cells/ml (5,55 ± 5,03), respectively (Table 10). The highest number of subjects were observed between 7,5 and 8,5 mS/cm (Table 9) and between 2 X 10^5 and 2,50 X 10^5 cells/ml (Table 10).

Significative difference was recorded in means LogSCC measurements between subclinical mastitic animals and infected or healthy ($P<0.0001$) while considering the LogEC it was only found between subclinical and infected buffalo ($P<0,05$) (Table 11).

Animals with intramammary infection (IMI)

Infected udder were observed in 63,6 % of the buffalo investigated (299/470). The 16,4 % animals showed positive EC values (Table 7) while the 34,8% positive CMT results. They were negative in 66,2% of times, weakly positive 29,1%, distinctly positive 4,7% and never strongly positive (Table 8). LogEC and LogSCC values were ranged from were ranged from 0,84 to 1,30 mS/cm (0,92 ± 0,72, mean ± SD), respectively (Table 9), and from 4,85 to 5,30 cells/ml (4,84 ± 4,66), respectively (Table 10). The highest number of subjects were observed between 7,5 and 8,5 mS/cm (Table 9) and between 2 X 10^4 and 4 X 10^4 cells/ml (Table 10).

No significative difference was recorded in means EC and SCC levels between infected and healthy subjects (Table 11)

Healthy Animals (H)

Healthy udder were observed in 16 % of the animals examined (75/470). The EC was positive in 30,6% of animals (Table 7) while the CMT of healthy composite milk samples were negative in 86,7% of case, weakly positive 13,3% but never distinctly positive or strongly positive (Table 8). LogEC and LogSCC values were ranged from were ranged from 0,58 to 1,04 mS/cm (0,92 ± 0,03, mean ± SD) (Table 9) and from 4,11 to 5,30 cells/ml (4,78 ± 4,63), respectively (Table 10). The highest number of subjects were observed between 7,5 and 8,5 mS/cm (Table 9) and between 2 X 10^4 and 4 X 10^4 cells/ml (Table 10).

EC values	Healthy buffaloes (N°)	%	Infected buffaloes (N°)	%	Subclinical buffaloes (N°)	%	Clinical buffaloes (N°)	%
Positive	23	30,6	49	16,4	17	28,8	21	56,7
Negative	52	69,4	250	83,6	42	71,2	16	43,3

Table 7. Distribution of healthy, infected and affected by subclinical and clinical mastitis buffaloes (N° and %) considering EC values.

CMT values	N° healthy buffaloes	%	N° infected buffaloes	%	N° Subclinical buffaloes	%	N° clinical buffaloes	%
0	65	86,7	198	66,2	3	5,1	-	-
1	10	13,3	87	29,1	21	35,6	-	-
2	-	-	14	4,7	33	55,9	20	54,1
3	-	-	-	-	2	3,4	17	45,9

Table 8. CMT values and percentage of healthy, infected and affected by subclinical and clinical mastitis buffaloes.

Range (< EC ≤)	N° healthy buffaloes	%	N° infected buffaloes	%	N° Subclinical buffaloes	%	N° clinical buffaloes	%
3,5-4,5	1	1,3	-	-	-	-	-	-
4,5-5,5	-	-	-	-	-	-	-	-
5,5,-6,5	-	-	-	-	-	-	-	-
6,5-7,5	1	1,3	2	0,7	-	-	-	-
7,5-8,5	50	66,6	248	83,0	42	71,2	16	43,3
8,5-9,5	15	20,0	36	12,1	14	23,7	9	24,3
9,5-10,5	7	9,5	10	3,3	2	3,4	4	10,8
10,5-11,5	1	1,3	1	0,3	-	-	2	5,4
11,5-12,5	-	-	1	0,3	-	-	3	8,1
12,5-13,5	-	-	1	0,3	1	1,7	1	2,7
13,5-14,4	-	-	-	-	-	-	2	5,4

Table 9. EC values (mS/cm) and percentage of healthy, infected and affected by subclinical and clinical mastitis buffaloes.

N° healthy buffaloes	Range (< SCC ≤)	N° infected buffaloes	Range (< SCC ≤)	N° Subclinical buffaloes	Range (< SCC ≤)	N° clinical buffaloes	Range (< SCC ≤)
5	0-2 (X 10^4)	27	0-2 (X 10^4)	15	2-2,50 (X 10^5)	10	5-1 (X 10^5)-(X 10^6)
30	2-4 (X 10^4)	77	2-4 (X 10^4)	4	2,50-3 (X10^5)	7	1-1,5 (X 10^6)
13	4-6 (X 10^4)	58	4-6 (X 10^4)	12	3-3,5 (X 10^5)	2	1,5-2 (X 10^6)
14	6-8 (X 10^4)	42	6-8 (X 10^4)	7	3,5-4 (X 10^5)	5	2-2,5 (X 10^6)
4	8-10 (X10^4)	31	8-10 (X 10^4)	6	4-4,5 (X 10^5)	3	2,5-3 (X 10^6)
1	10-12 (X 10^4)	27	10-12 (X 10^4)	7	4,5-5 (X 10^5)	2	3-3,5 (X 10^6)
2	12-14 (X 10^4)	12	12-14 (X 10^4)	6	5-5,5 (X 10^5)	2	3,5-4 (X 10^6)
-	14-16 (X 10^4)	5	14-16 (X 10^4)	2	5,5-6 (X 10^5)	2	4-4,5 (X 10^6)
3	16-18 (X 10^4)	6	16-18 (X 10^4)	-	6-6,5 (X 10^5)	1	4,5-5 (X 10^6)
3	18-20 (X 10^4)	14	18-20 (X 10^4)	-	6,5-7 (X 10^5)	3	> 5 (X 10^6)

Table 10. SCC values (cells/ml) of healthy, infected and affected by subclinical and clinical mastitis buffaloes.

TEST (means values)	Healthy Buffaloes	Infected buffaloes	Subclinical buffaloes	Clinical Buffaloes
EC (mS/cm ± SD)	0,92 ± 0,03[b,]	0,92 ± 0,72[b,d*]	0,93 ± 0,11[b,c*]	0,97 ± 0,27[a]
SCC (cells/ml)	4,78 ± 4,63[b, b*]	4,84 ± 4,66[b, b*]	5,55 ± 5,03[b, a*]	6,41± 6,44[a]

Table 11. [a,b,a*,b*]Mean and standard deviation (SD) of LogEC and LogSCC in healthy, infected and affected by subclinical and clinical mastitis buffaloes (P<0.0001). [c*,d*] Statistical significativity of P<0,05.

DISCUSSIONS

General considerations

According to our knowledge this is the first report of comparison between direct and indirect screening test in Mediterranean Buffalo mastitis. The numerical correlation between electrical conductivity, California Mastitis Test, somatic cells count, bacteriological milk culture and clinical signs was assessed in animals with different health status. Only primiparous buffaloes, from days 30 after calving, were enrolled in the study because the highest incidence of mastitis in buffalo was recorded after the first month of milking and the first parturition (Dhakal et al., 2008). Ten animals showing clinical evidences of traumatic mastitis were discarded because the goal of the study was to verify the correlation between screening test in animals with or without bacteriological mastitis.

Although the mastitis represents the most costly disease in buffalo dairy industry, poor and not complete data are available in literature

regarding to prevalence of mastitis, intramammary infection and about the common test employed (Fagiolo And Lai, 2007; Tripaldi et al., 2010).

In the present study, the frequency of clinical mastitis recorded was lower (7,9%) than data observed by other Authors in buffaloes but similar to those reported in cow. Dhakal et al. (2008) found higher percentages (31,3%) in Murrah Buffalo, while a prevalence of CM of 9,9 % has been reported in cow (McDougall, 1999) although values of 19,8 % and > 30 % was indicated by others Authors in heifer within the first 14 day after calving (Sargeant et al., 1998; Berkema et al.1998).

Moreover, a lower (12,6%) prevalence of subclinical mastitis (SCM) was observed in this study than reported in in Murrah buffaloes (21,7%) (Dhakal et al., 2007) or cow, in which, Plozza et al. (2011) described a SCM prevalence of 29%, Giannecchini et al. (2002) of 52,4%, and Östensson et al. (2013) of 88,6%. This difference can be explain by the longer teats and narrow teat canal, sphincter with smoother muscular fiber then cow; this can prevent the invasion of microorganism and reduce the incidence of mastitis in Mediterranean Buffalo (Krishnaswamy et al. 1965; Borghese et al. 2007).

The prevalence of intramammary infection (animals BC positive and SCC < 200’000 cells/ml) was instead of 63,6%. A few data are available about the occurrence of IMI in buffalo composite milk samples. Hokmabad. et al. (2011) found values of 13.87% in Azeri buffaloes, while at present, the only data useful about Mediterranean Buffalo were showed by Moroni et al. (2006) that found 100% of quarts affected by intramammary infection in milk samples with SCC

> 200’000 cells/ml. In the present study, it was supposed that the high prevalence of IMI detected was due to several weak points of buffalo livestock management increasing the possibility of animal infections and bacteria pollution (breeding based on open land, presence of paddocks, swimming pool pre-milking room and sprinklings with water from the floor) (Borghese at al. 2007).

Finally, the percentage of healthy buffalo, detected (16%), were similar with data present in literature on Mediterranean Buffalo (12%) (Moroni et al. 2006).

Considering the several categories of animals (H, IMI, SCM, M), higher means EC values were observed than in Murrah buffalo (Dhakal et al., 2008) and cow (Norberg et al., 2004; Woolford et al. 1998). Several authors described an influence of milk temperature on EC findings in cow. Higher EC measurements were recorded at milking time when the milk leaves the teat cistern with a temperature of approximately 38° C (Uhler, 2009; Wong, 1988). In our study, measurements performed when the milk temperature was still warm, due to the use of an automatic digital mastitis detector for electrical conductivity incorporated in the milking unit, can explain the differences.

As described for Murrah buffalo and cow significative difference was recorded in means SCC levels between CM and SCM animals, and between CM-SCM with IMI or H animals ($P<0.0001$) (Table 11).The means SCC values observed in CM, SCM, IMI and H animals were comparable with data present in literature about Murrah buffalo (Dhakal et al., 2008). The sensibility (SE) and specificity (SP) of the test observed, were 41% and 88% at the cut-off of 100’000 cells/ml level and 25.6% and 100% at that one of 200’000 cells/ml. Using the

fixed threshold of 100’000 an higher % of false negative can be detected than cow in which were reported values of SE and SP of 83,2 and 80,5, respectively. Considering the threshold of 200’000 cells/ml, SE and SP of 74,5% and 89,6 were described Schepers et al. (1997), respectively, while in buffalo by an additional increased of false negative animals and the disappearance of false positive was observed.

The CMT test were always positive in subject with CM, while in SCM and IMI animals only in the 94,6 and 33,8 % of cases, respectively. The low (3,1%) and high (52,9%) percentage of false negative results observed with SCC > 200’000 and SCC< 200’000 cells/ml, respectively, confirm the same trend observed in other study on buffalo (Salvador et al., 2012) and cow (Sharma et al., 2010).

Relationship electrical conductivity-bacteriological milk culture

No statistical correlation was found between numerically coded qualitative variables EC and BC. The diagnostic characteristics of EC measurements cannot adequately reflect buffalo’s udder bacteriological status. The 16,4% (49/299) and 28,8% (17/59) of the animals were rightly identified as IMI or SCM, respectively. A low significative statistic difference (P<0,05), in means EC values between SCM and IMI buffaloes was found because the highest number of animals (198/299) showed normal values EC (Table 9). Probably, in the two categories no serious tissue udder tissue damage or important changes in milk composition significantly influencing EC values, as instead described during the clinical disease (Uhler, 2009), were found. The ability to classify correctly healthy buffalo classified was higher (69,9%) than in IMI or SCM animals.

Relationship electrical conductivity-clinical signs

Regards the qualitative statistical correlation found between the several variables, EC and CS a very low coefficient qualitative (0,197) was recorded. Although, the mean EC value was above the threshold in animals with clinical signs (0,98 ± 0,27), only the 56,7% of these subjects (21/37), was correctly identified were.

In subjects affected by mastitis, the milk concentration of lactose and K^+ decreased while those one of Na^+ and Cl^- increased because of changes in the permeability of cells and blood vessels. The knowledge of a direct relationship between the disease's gravity and changes in milk composition, both in buffalo (Tripaldi et al., 2010) and in cow (Kitchen, 1981), can explain the particularly high EC values recorded when clinical evidence of mastitis were found. In fact, highly significative differences, in means EC values, were recorded between subjects with clinical evidences of mastitis and SCM, IMI or H animals ($P<0.0001$).

Relationship electrical conductivity-California Mastitis Test

A low moderate quantitative relationship between EC and CMT values (0,256) was recorded. In fact, only with CMT strongly positive (3) high values of EC were observed (1,01 ± 0,33) while for CMT weak (1) and distinct (2) positive values no increased of EC levels and no significative statistic differences were found. In buffalo enrolled, values of CMT 1 and 2, could be associated with weak inflammatory process leading poor changes in milk composition and increase in EC values. Both the test showed in Mediterranean Buffalo a good ability to identify healthy animals (CMT: 86,7%; EC 69,4%), according to

cow's findings reported in literature (CMT: 74,1%, Sharma et al., 2010; EC: 74,8%, Norberg et al., 2012).

Relationship electrical conductivity-somatic cells count

A low qualitative correlation coefficient of 0,246 was found between EC and SCC. In fact, only the 56,8% (21/37) and 28,8% (17/59) of clinical and subclinical cases (SCC>200'000), were classified correctly by EC because the most of them were ranged below its threshold (<8,5 mS/cm) (Table 9). A direct correlation between the disease's gravity, increase in somatic cells count and changes in milk composition leading increase in EC values was described both in buffalo (Tripaldi et al., 2010; Dhakal et al., 2008) and in cow (Hamann and Zecconi, 1998). The hypothesis was confirmed also by the presence of significative difference in means EC and SCC levels between CM-SCM and IMI-H animals (P<0.0001) and by its absence between H and IMI animals. (Table 12).

Relationship somatic cells count-clinical signs

Obviously, the Spearman test showed a moderately strong qualitative correlation between SCC and CS (0,557). In fact, a differences highly significative (P<0.0001) were found in means SCC values in animals with (6,24 ± 0,39) and without (4,84 ± 0,40) clinical signs (Graph 5). Serious udder inflammations, with clinical evidences, always lead to evident increase of SCC in all dairy species (Tripaldi et al., 2010, Sharif and Muhammad, 2008; Paapea, 2007; Schukken, 2003).

Relationship somatic cells count-bacteriological milk culture

Tripaldi et. al. (20l0) described significative difference of SCC values (P<0,05) between udder infected by specific pathogens and healthy buffalo. In the present study, low qualitative correlation between SCC and BC was found (0,220) and significative statistic differences between means SCC values were detected between CM-SCM and H animals (P<0.0001), but not between IMI and H. This phenomenon was supposed possible considering the high number of IMI as the consequence of a contact between microorganism and animals not causing udder damage or cellular reaction. The poor environmental hygiene and the high levels of bacterial contaminations typical of buffalo breeding conditions could promote the mechanism (Borghese at al., 2007).

At present, according to the international Dairy Federation (IDF) only the combined used of these two test can guarantee the right identification of udder health both in Mediterranean buffalo and cow.

The SCC evaluation without any informations about udder microbiological status produces an high percentage of false negative results because all the subject affected by IMI are not identify, while the milk culture establish the presence of mastitis pathogens but doesn't provide a measure of the degree of inflammation associated with infection (Sharma et al., 2010; Viguier et al, 2009; Schukken, 2003). Useful informations could be reach, as in cow, throughout a milk differential cells count for the evaluation of role and quality of the cells involved in the early phases of inflammatory reaction. Analyzing udder quarters classified as healthy by having SCC < 100'000 cells/ml, inflammatory reactions were detectable at an SCC level of ≥ 9'000 cell's/ml due to predominating PMNL (Schwarz et al.

2011). No data were actually available in Mediterranean Buffalo about these evaluations.

Relationship somatic cells count-California Mastitis Test

Between SCC and CMT were instead found moderately strong quantitative (0,657) and qualitative (0,546) correlation. In buffaloes enrolled, lower means values of SCC, related to CMT results, were recorded than those one described by several authors in cow. Furthermore, unlike the literature in cow the threshold of 200'000 was exceed only for CMT results distinct (2) and strongly positive (3) (Kaşikci et al., 2012; Jasper, 1967). The CMT is an indirect measure of SCC in milk (Harmon, 2001; Barnum and Newbould, 1961), it reflects the SCC level quite accurately and is a reliable indicator of the severity of infection (Schalm and Noorlander, 1957; Barnum and Newbould, 1961; Dohoo and Meek, 1982). In fact, a significative statistic difference, between means SCC values in animals with CMT 3-2 and 1 or 0 was found ($P<0.0001$), but not between 1 and 0, when a less serious inflammatory reaction was present.

Relationship California Mastitis Test-bacteriological culture

A qualitative coefficient of correlation of 0,264 was detected between CMT and BC. The relationship can be explain considering that only when the inflammatory process started and the udder reaction due to bacteria was present (high SCC levels), the CMT became positive. This hypothesis was confirmed by a sensibility of 94,4% to identify the SCM, decreased to 33,7% with IMI due to a high number of false negative (198/299). A low CMT sensibility to detect IMI without SCC evident reaction was also confirmed by

analogous results found in cow (Cole et al., 1965; Middleton et al., 2004; Ruegg and Sekito, 2004). As described, the CMT is an indirect measure of SCC in milk (Harmon, 2001; Barnum and Newbould, 1961) in fact, significative difference were observed between means SCC values in animals with CMT 3-2 and 1 or 0 animals ($P<0.0001$).

A 13,3% of false positive was found in healthy animals (Table 8) and 10-20% of positive CMT reaction with a correspondent bacterial culture negative was described also in cow. Short lived infections that have been cleared by the cow or infections that are characterized by intermittent shedding of bacteria can justify the situation (Sanford C.J. 2006).

Relationship California Mastitis Test-clinical signs and Bacteriological milk culture-clinical signs

Considering as the gravity of the inflammatory process can influence the CMT values, the low qualitative correlation (0,333) between this test and CS can be explained. The CMT was always positive, only when the CS were present but, an high percentage of CMT positive not correlated to clinical evidences of mastitis was recorded (83,4%).

Finally, no significative qualitative correlation coefficient between BC-CS was found (0,127). The low result was possible because the 76,2 % of infected animals (all the SCM and IMI subjects) were asymptomatic.

CONCLUSIONS

Mastitis is the most costly disease both in dairy buffalo and in cow because it is associated with significant milk loss, qualitative and quantitative changes in its composition. Its early detection is important for dairy farmers to reduce economic loss and to optimize drugs administrations.

A low linear correlation coefficient was found between the most of the test investigated, while a moderately strong correlation coefficient between CMT and SCC was found. As in cow, also in Mediterranean Buffalo, CMT can be considered an indirect measure of SCC in milk and a reliable indicator of the severity of infection so, its use as screening test to detect mastitis in farm is recommended. The electrical conductivity instead showed the lowest correlation coefficients respect to the other test because less influenced by buffalo udder health status.

Moreover, a lower prevalence of clinical and subclinical mastitis was recorded in Mediterranean Buffalo then cow probably due to anatomical differences between the two species and different activity in innate defence against pathogens. Several Authors, described higher lysozyme levels in buffaloes then cow, suggesting a critical non-specific defence role of this enzyme in these ruminants (Piantedosi et al., 2012; Lombardi P. et al., 1996). The percentages of intramammary infection were instead higher, probably favourite by Mediterranean Buffalo breeding system. The diagnostic characteristics of EC and CMT measurements cannot adequately reflect buffalo's udder bacteriological status (BC status positive or negative). A few informations, depending by selected threshold, can be reached by somatic cells count (sensibility and specificity of 41% and 88%, respectively, with cutoff of 100'000 cells/ml; 25,6% and 100% with that one of 200'000 cells/ml).

Early IMI, not causing udder damage and a significative increase in number of inflammatory cells can justify the inadequate test's ability to detect intramammary infection. Further research, concentrate on the examination of the role and quality of immune cells milk in buffalo affected by intramammary infection, should be performed to promote an early diagnosis of the disease and improve buffalo udder health status, decreasing economic loss and public health risk.

In conclusion, our data suggest that a value of 2×10^5 cells/ml could be used as the threshold value for early identification of animals affected by subclinical mastitis, such us, according to International Dairy Federation (IDF) guidelines establish for cow, also in Mediterranean Buffalo, the gold standard for mastitis diagnosis is still represented by combined used of somatic cells count and

bacteriological milk culture because, no one association between the others diagnostic investigated in this study represent an efficient alternative.

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