

Diagnostic methods of subclinical mastitis in bovine milk: an overview

Métodos de diagnóstico de mastitis subclínica en leche bovina:
una descripción general

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ABSTRACT

Keywords:

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Diagnostic
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Bovine mastitis is defined as inflammation of the udder caused mainly by bacterial pathogens and depending on the degree of inflammation it is classified as subclinical and clinical. Particularly in the subclinical form, there are no alterations in milk, udder or animal, but it does affect its components, impairing its use in the dairy industry, and leading to significant economic losses due to discard and decrease in production. Therefore, the detection of subclinical mastitis is based on field and laboratory tests. Currently, there are several methods, mostly based on the measurement of somatic cells present in milk because of the inflammatory process. In this paper, an approach is made on the different methods of detection of subclinical mastitis in milk from conventional or traditional to alternative methods with greater precision.





RESUMEN

Palabras clave:

Mastitis bovina
Diagnóstico
Conteo de células
somáticas
Ultrasonido

La mastitis bovina se define como la inflamación de la ubre causada principalmente por patógenos bacterianos y dependiendo del grado de inflamación se clasifica en subclínica y clínica. Particularmente en la forma subclínica no se presentan alteraciones en leche, ubre o animal, pero sí afecta sus componentes, impidiendo su aprovechamiento en la industria láctea, y conllevando a pérdidas económicas importantes por concepto de descarte y disminución en la producción. Por ello, la detección de mastitis subclínica se basa en la realización de pruebas en campo y en laboratorio. Actualmente existen diversos métodos, en su mayoría basados en la medición de las células somáticas presentes en leche como resultado del proceso inflamatorio. En el presente artículo se realiza un abordaje sobre los diferentes métodos de detección de mastitis subclínica en leche desde convencionales o tradicionales hasta métodos alternativos de mayor precisión.

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INTRODUCTION

Milk is defined as the secretion of the mammary gland of dairy animals obtained by one or more milking without any addition or extraction, intended for consumption in the form of liquid milk or for further processing (FAO, 2011). Milk produced by cows, buffaloes, sheep, goats and camels is used in various parts of the world for human consumption. For much of the world's population, cow's milk represents most of the milk used for consumption, given that it is produced at approximately 86% compared to other dairy species used for the same purpose (FAO, 2020).

In the human diet, milk is one of the most important products to consume, mainly because of its nutritional value, since it provides minerals such as calcium, magnesium, selenium, vitamins, proteins and calories according to the portion consumed. However, this food is highly susceptible to contamination by various pathogenic agents, which alter its quality, often imperceptibly, generating health risks for consumers and in addition, important economic losses in the dairy sector (FAO, 2020).

World milk production in 2020 was estimated at 906 million tons, for South America a total of 82 million tons was reported and 22,592 tons, particularly for Colombia (FAO, 2021). This positions the country as the fourth largest dairy producer in Latin America, in addition to ranking 21st worldwide with daily production margins estimated at approximately 22 million liters (DANE, 2019), which represents a contribution of the dairy sector to the national GDP of 2.3% and to the agricultural sector of 24.3%, among other aspects, due to the generation of employment in the agricultural sector of 17% (Morales and Ospina, 2017).

In this context, regarding diseases that severely affect bovine dairy production, such as mastitis, especially subclinical mastitis, it is essential to know the methods for early detection in order to establish control and prevention measures from the production unit and minimize the impact in terms of treatment costs and milk discard, among others. The objective of this paper was to carry out a bibliographic review of conventional and non-conventional techniques for the diagnosis of subclinical mastitis.

Composition of raw milk

The nutritional composition of milk is a complex mixture of different substances, present in suspension or emulsion and others in solution form, such as water, fat, protein, lactose, vitamins, and minerals, which are called dry extract or total solids. This composition varies according to genetic, nutritional, and herd management conditions. The following is the average composition of bovine milk with its respective ranges of variation: 87.3% water (85.5-88.7%), 3.9% fat (2.4-5.5%), 8.8% non-fat solids (7.9-10.0%), protein 3.3% (3/4 parts casein), lactose 4.6%, minerals 0.8% (Ca, P, citrate, Mg, K, Na, Zn, Cl, Fe, Cu, sulfate, bicarbonate), acids 0.2% (citrate, acetate, lactate, oxalate), vitamins (A, C, D, thiamine, riboflavin) (Arroyave and Naranjo, 2007).

Water: the water content of the milk of different mammalian species can vary from 86 to 90.5%; however, it normally represents 87% of the total milk content. This variation is due to the alteration of any of its other components: proteins, lactose, and mainly, fat. Because of its high-water content, milk allows the distribution of its components to be relatively uniform, and thus any amount of milk, regardless of how small, contains almost all the available nutrients (Badui, 2006).

Fat: Lipids are among the most important constituents of milk and milk products, conferring unique flavor characteristics, nutritional content, and physical properties. Milk fat is a good source of energy and an excellent transport medium for fat-soluble vitamins A, D, E, and K. Carotene, a precursor of vitamin A, gives milk its "cream" color (German and Dillard, 2006).

The fat fraction of milk is in the form of microscopic globules 4.4 µm in diameter. Both total lipid and fatty acid contents can vary considerably in response to changes in diet, breed of animal, and lactation status by 3 to 6%, although typically the fat content can be between 3.5% and 4.7% (Badui, 2006; NOM, 2003). In addition, fat contains mainly triglycerides (about 98%), diacylglycerol (2%), cholesterol (less than 0.5%), phospholipids (about 1%) and free fatty acids (0.1%). On the other hand, saturated fatty acids make up 70% of the total weight of fat, with palmitic acid being the most common, accounting for 30% of milk fat by weight, followed by myristic and stearic acid, which make up 11 to 12% by

weight. Of the saturated fatty acids, 10.9% are short-chain fatty acids. The content of butyric and caproic acid averages 4.4%, and they represent only 2.4% of the total fatty acids (García-Garibay *et al.*, 2012).

Proteins: The primary function of milk proteins is to provide sufficient supply of indispensable amino acids and organic nitrogen for the synthesis and repair of tissues and other biologically important proteins. Cow's milk is considered an excellent source of proteins of high biological value, since it contains the ten indispensable amino acids. The protein fraction of milk regularly corresponds to 3-4% and two main categories can be distinguished, defined by their chemical composition and physical properties: casein, which constitutes 70% of milk proteins, contains phosphorus and coagulates or precipitates at a pH of 4.6; and whey proteins, which represent the remaining 30%, do not contain phosphorus but sulfur and remain in solution in milk at a pH of 4.6 (Badui, 2006).

Caseins: they consist of fractions a, b, k, and g caseins, which are distinguished from each other by their amino acid composition and functional properties. Caseins are suspended in milk through micelles, formed by macromolecular complexes of phosphoproteins and glycoproteins in colloidal suspension. The nutritional role of casein is the supply of amino acids, calcium and inorganic phosphorus (Zhang *et al.*, 2021).

Whey proteins: also known as seroproteins, they are considered soluble proteins and are mainly classified into albumins and globulins, including α -lactalbumins, b-lactoglobulins, immunoglobulins, protease-peptones and other non-specific minority nitrogenous compounds such as lactoferrin and lysozyme. Seroproteins are considered to be high biological value proteins with a broad amino acid profile that includes sulfur amino acids such as cysteine and methionine, branched-chain amino acids, and lysine and tryptophan, thus compensating for casein deficiencies (Zapata *et al.*, 2017).

Lactose: is the main carbohydrate in milk and contains approximately 4.5%. It is 85% less sweet than sucrose or common sugar and contributes, together with salts, to the overall flavor of milk, the amounts of lactose and salts being inversely proportional. Lactose is easily transformed into lactic acid by the action of bacteria.

For humans, lactose is the only source of galactose, an important constituent of nerve tissues (Aranceta and Serra, 2004).

Minerals: milk provides essential mineral elements for the human body and is the most important source of bioavailable calcium in the diet. Its good absorption is due to the presence of lactose and vitamin D and to its union with phosphopeptides derived from casein hydrolysis, in addition to the fact that the adequate calcium:phosphorus ratio (greater than the unit) favors its absorption in the human intestine. For this reason, cow's milk is considered to be the best source of calcium both for bone growth in young people and for the maintenance of bone integrity in adults (Aranceta and Serra, 2004).

Fat-soluble vitamins: both milk and dairy products are considered an important food source of vitamin A; this vitamin is involved in functions related to vision, gene expression, embryonic development, growth, reproduction, and immunocompetence. Both vitamin A and its precursors called carotenoids, mainly b-carotene, are present in different amounts in the fat fraction of milk (Miller *et al.*, 2007). Vitamin D is involved in the absorption of calcium and phosphorus in the intestine and is essential for the proper maintenance of the skeleton throughout life. It is found in very low concentrations in milk and dairy products to which this vitamin has not been added (Schmid and Walther, 2013). Vitamin E, also called tocopherol, is considered an antioxidant that protects cell membranes from free radical damage. It also participates in the immune response. Some studies even consider it a protective factor against some types of cancer and cardiovascular diseases. This vitamin is present in milk in low concentrations, as is vitamin K (Haug *et al.*, 2007).

Water-soluble vitamins: both milk and its derivatives contain the vast majority of soluble vitamins in varying amounts, although the content of vitamin B2 (riboflavin) and niacin stand out; milk provides lesser amounts of vitamin B1 (thiamine), vitamin B6 (pyridoxine) and folic acid (Haug *et al.*, 2007).

Bovine Mastitis

The importance of milk in human life has already been

discussed, nevertheless, the main disease that afflicts cattle and has a direct impact on milk production is mastitis, a disease that produces inflammation of the mammary gland, due to traumatic, allergic or infectious causes, the latter being the most common caused by pathogenic microorganisms, mainly bacteria and, to a lesser extent, yeasts, fungi and algae (Ruegg *et al.*, 2015).

Traditionally, mastitis-causing pathogens are classified into contagious, environmental and minor pathogens, according to their mode of transmission and reservoir (Ruegg, 2012). Contagious microorganisms are those that are transmitted from cow to cow, therefore, the main reservoir is the animal itself (Fox and Gay, 1993). In this group are *Staphylococcus aureus*, Coagulase-Negative *Staphylococcus* (CoNS), *Streptococcus agalactiae*, *Mycoplasma spp* and *Corynebacterium bovis* (Fox and Gay, 1993; Barkema *et al.*, 2009). In the case of environmental pathogens, the reservoir is constituted by the habitat of the cow. Gram-negative bacteria such as *E. coli*, *Klebsiella pneumoniae* are the most common environmental pathogens, but can also be caused by gram-positive pathogens such as *S. uberis* and *S. dysgalactiae* (Smith and Hogan, 1993). Other pathogens include *Pseudomonas aeruginosa*, *Pasteurella multocida*, *Prototheca spp*, *Trueperella pyogenes*, *Mycobacterium spp*, *Nocardia spp*, and some yeasts (Williamson and di Mena, 2007; Tarazona-Manrique *et al.*, 2019).

The main route of entry for contagious and environmental pathogens is through the teat orifice, either during milking or between milking (Bradley, 2002). After the bacterial invasion, depending on the invading pathogen, they infect different locations in the mammary gland and cause different symptoms and duration of the infection (Svennesen *et al.*, 2019).

In response to the invasion of these pathogens, innate and acquired immune response mechanisms are activated (Medzhitov, 2007). Toxins and virulence factors induce neutrophil migration and secretion of proinflammatory cytokines; subsequently, antigens from invading bacteria are processed in macrophages and B lymphocytes and appear on membranes in association with major histocompatibility complex (MHC) type I

or II, so they can be recognized by different types of lymphocytes (Ezzat *et al.*, 2014). Thus, they are the lymphocytes that, once activated and proliferated, fight the infection that develops in the mammary gland (Kehrli and Harp, 2001).

According to the signs of inflammation, bovine mastitis is classified as clinical mastitis (where alterations occur in milk, mammary gland, or even systemically) and subclinical (no noticeable signs in milk, mammary gland, or systemically) (Erskine, 2020). Regardless of the form of presentation, the importance of the study, diagnosis, and treatment of mastitis lies, among other things, in the fact that this disease produces severe economic losses within the livestock sector both in the country and in the world (Andrade-Becerra *et al.*, 2014).

Subclinical mastitis

In subclinical infection, there are no visible changes in the appearance of the milk, nor are there any manifestations of the disease in the cow, but milk production decreases, and its composition is altered. Its detection is based on somatic cell counts (Blowey and Edmonson, 2010), where values higher than 200,000 cells mL⁻¹ are considered positive for intramammary infection. The occurrence of subclinical mastitis has commonly been attributed to contagious pathogens when undetected by the producer, the critical point is the progression to a state of critical point, and as a consequence, in many cases, leading to the discard of the animal (Dohoo *et al.*, 2011).

Clinical mastitis

This disease is characterized by the presence of visible alterations in the milk (formation of lumps, changes in color, presence of clots, etc.), changes in the mammary gland (inflammation, pain, heat, tumor, and redness) and, in certain cases, it can reach a systemic involvement with anorexia, fever, and shock (Radostits *et al.*, 2006; Erskine, 2020). According to severity, it is classified as mild (abnormalities in milk), moderate (abnormalities in milk and mammary quarter, based on 6 parameters of alteration of the quarter), and severe (abnormality in milk, signs of systemic disease with or without alterations in the mammary quarters). Regarding the frequency of presentation of clinical mastitis cases according to severity, the majority of cases are mild, followed by

moderate, and, to a lesser extent, severe (Roberson, 2012).

Considering that subclinical mastitis is approximately 40 times more common than clinical mastitis and that, additionally, it does not present visible changes in milk, there is a need to explore and develop new technological tools that, in a sustainable way, contribute to the reduction of losses in dairy herds through the early detection of bovine mastitis and the isolation of sick cows, preventing the spread to healthy cows.

Characteristics of the pathogens causing bovine mastitis

The characteristics and consequences of pathogens on cow health are described below. Gram-positive pathogens such as *Staphylococcus aureus* and *Streptococcus agalactiae*, cause infections of prolonged duration in the subclinical phase, with high somatic cell counts ($>800,000$ cells mL^{-1}), with an affinity for the mammary parenchyma and mucosa of the mammary cistern and ducts. Other gram-positive pathogens such as coagulase-negative *Staphylococcus*, *Streptococcus* spp and *Corynebacterium bovis* cause infections of short to moderate duration with somatic cell counts up to $500,000$ cells mL^{-1} , with an affinity for the teat canal and mucosal surfaces. Gram-negative pathogens such as *Escherichia coli*, *Klebsiella* spp, and *Serratia* spp, cause infections with counts ranging from $500,000$ to $1,000,000$ cells mL^{-1} , subclinical infections of short to the prolonged course, and clinically 20 to 30% develop systemic signs. Nevertheless, the expectation of spontaneous bacteriological cure is moderate to high (Ruegg *et al.*, 2015).

Other rare pathogens such as *Mycoplasma* spp can cause increases in somatic cell counts up to $500,000$ cells mL^{-1} , with prolonged duration periods in the subclinical phase, in addition to the involvement of other organs and low probability of spontaneous bacteriological cure, the main impact of mastitis caused by such an agent (Ruegg *et al.*, 2015).

Effect of mastitis on the compositional and nutritional quality of raw milk

As mentioned above, milk is a product with diverse compositional and nutritional characteristics, being one of the most consumed products worldwide. However,

bovine mastitis, mainly of subclinical course, has a negative impact on the quality of milk, causing alterations in it and, as a consequence, the impossibility of its use in the dairy industry.

In addition to the increase in somatic cell count, a parameter that, from the regulatory aspect, is considered in many countries to evaluate milk quality (European Commission, 2020), components such as proteins change dramatically. Casein, the main milk protein, decreases, and whey proteins of lower quality increase, affecting the flavor. Proteins are degraded by the presence of enzymes such as plasmin (Kibebew, 2017; Ismail and Nielsen, 2010). On the one hand, the concentrations of sodium and chloride are increased, in an attempt to maintain osmotic balance. The change in these elements or ions allows monitoring of the evolution of mastitis because they cause an increase in the electrical conductivity of the milk (Fox *et al.*, 2015). On the other hand, it decreases potassium and calcium, since the latter is associated with casein (Calderón-Rangel *et al.*, 2014). Lactose, the milk sugar, is also decreased due to three causes: altered synthesis due to cell damage, loss of lactose in urine, and use of lactose as a substrate by mastitis-causing pathogens (Costa *et al.*, 2019).

Mastitis detection

As mentioned, clinical mastitis presents obvious signs or symptoms in cattle health, so its detection should not be a problem. Nevertheless, subclinical mastitis can only be diagnosed by a series of tests, which will be described below. To make it simpler, the tests will be divided into conventional methods and alternative or non-conventional diagnostic methods.

Conventional methods

Somatic cell count (SCC): Somatic cell count is one of the conventional methods used to detect the presence of mastitis in herds and to assess the sanitary quality of milk. In raw milk, a high somatic cell count value determines not only that the cows have mastitis, but also, information on biochemical changes in the milk, up to production losses (Riveros-Galán and Obando-Chávez, 2021).

Somatic cell counts below $200,000$ cells mL^{-1} are considered physiologically normal, while those above

300,000 cells mL⁻¹ generally indicate the presence of inflammation. For somatic cell counts in different countries of the European community, the established norms are between 400,000 to 750,000 cells mL⁻¹ as the maximum value, while in Colombia, the maximum accepted count is 800,000 cells mL⁻¹ (Gómez, 2015).

California Mastitis Test (CMT): it is the most widely used field test in dairy cattle for the diagnosis of subclinical mastitis, it does not count numerical results but categorical results. It consists of adding a detergent to milk, linear alkylbenzene sulphonate, causing the release of DNA from leukocytes present in the udder, and this is converted, in combination with protein agents in the milk, into a gelatinous complex. The categorization of the results is given in several ways, such that it is negative when the reagent and the mixed milk is still watery. When the cell count is higher, the mixture of reagent and milk almost solidifies (Saran and Chaffer, 2000).

The reagent used in the CMT test is characterized because it has a surfactant among its components with the ability to decrease the surface tension of the leukocytes present in mastitis milk. When the surface tension decreases, a burst of leukocytes is immediately produced, which in contact with the reagent, forms the gelatinous complex on the paddle used for the test (Echeverry *et al.*, 2010; Moroni *et al.*, 2018). Thus, when there are more cells, a higher concentration of DNA is released and, therefore, the higher the degree of the gelatinous complex, which allows determining the inflammatory response based on the viscosity of the gel formed by mixing the same amount of affected milk with the CMT reagent, the paddle with four compartments evaluates each quarter separately (Moroni *et al.*, 2018; Aguilar and Álvarez, 2019). The CMT was developed in 1957 with the purpose of rapidly detecting abnormalities in milk, the personnel who perform the test only require basic training and coincides with the fact that as the Somatic Cell Count or Leukocyte Count increases, so does the CMT score, making it a reliable field test (Sanford *et al.*, 2006).

Wisconsin Mastitis Test (WMT): This test can be used to sample milk from individual cows and milk from cooling tanks. It is characterized by the estimation of somatic

cell content. The procedure uses a reagent very similar to that of the CMT, the difference is that the results are measured quantitatively depending on the viscosity, not qualitatively (Philpot and Nickerson, 2000; Bedolla, 2007; NMC, 2016).

Whiteside test: Similar to the California test, this test is based on the increase of leukocytes, where a gelling reaction occurs when mixing mastitic milk with a 4% NaOH solution on a glass plate homogenized with a glass rod for 20 seconds. The result is measured according to milk precipitation as negative, trace, and positive (Hasan and Ahasan, 2013).

Alternative methods

Electrical conductivity: one of the pioneers works carried out for the detection of subclinical mastitis, using non-conventional technology, was that by Nielen *et al.* (1995a), the authors developed a model where they acquired, online and automatically, every 5 seconds, data of variables such as electrical conductivity per mammary quarter, milk temperature and milk production per cow since they concluded that the combination of these parameters would help to improve the detection results in terms of sensitivity (percentage of successfully classified cows with subclinical mastitis) and specificity (percentage of successfully classified healthy cows) (Shoshani and Berman, 1992).

The system, which used neural networks (Nielen *et al.*, 1995a), was able to flag a cow or udder quarters when an abnormality was detected through measured parameters, mainly electrical conductivity. They identified that a high somatic cell count coincided with high milk electrical conductivity values. Cows with SCC < 200,000 cells mL⁻¹ were considered healthy cows and cows diagnosed with subclinical mastitis were those with SCC > 500,000 cells mL⁻¹. Healthy cows diagnosed with clinical mastitis or those in the range of 200,000 to 500,000 cells mL⁻¹ were not taken into account for the study. The results showed that, of the total number of milking, 19.2% were positive for subclinical mastitis, while 80.2% were negative, i.e., healthy milking. The model had a sensitivity of 53% and a specificity of 97% (Nielen *et al.*, 1995b). They state that the power of an online system lies in the evaluation of subclinical mastitis from data taken over a long period of time (from 1991 to

1992) and not by data from a few samples. In another study (Paudyal *et al.*, 2020), they obtained sensitivity and specificity of 89.9% and 86.8%, respectively, using the same diagnostic technique.

Infrared thermography: any object, material, or body emits radiation, in the form of heat, depending on its temperature. In the field of veterinary medicine, infrared thermography is sensitive enough to perceive changes in skin surface temperature (SST) and relate it to the severity of mammary gland infection. One study measured SST using infrared thermography and one milk sample per quarter in 94 cows using the California Mastitis Test (CMT) and found a strong correlation between SST and CMT ($r=0.92$), suggesting that thermography is a sensitive technique, as well as being non-invasive for detecting different degrees of mastitis. One of the drawbacks of the technique is that it requires the adaptation of a dark room to perform the measurement using infrared cameras, as well as its calibration, in addition to being expensive and lacking specificity with respect to etiology (Colak *et al.*, 2008). Regarding sensitivity and specificity, other authors report values of 95.6 and 93.6%, respectively (Colak *et al.*, 2010). It should also be taken into account that there are other factors that can affect the temperature of the udder skin, such as humidity in the environment, physiological state, and production level of the bovine and aspects related to feeding and milking, which directly influence the measurement (Colak *et al.*, 2008).

Piezoelectric sensors: piezoelectric materials are those that have the ability to produce an electrical potential difference when subjected to mechanical deformation and vice versa. From the point of view of sensors with biological applications, piezoelectric can be used to fabricate biosensors (Pohanka, 2018). The operating principle can be explained as follows: an alternating voltage on the faces of the piezoelectric surface produces oscillations in the crystal. A mass (biological sample) on the surface of the piezoelectric, will change the frequency of oscillations in the material. The change in the frequency of oscillations is proportional, among other things, to the mass in contact with the piezoelectric surface; (García-Martínez *et al.*, 2011; Zhang *et al.*, 2011; Pohanka, 2017).

The application of these devices to evaluate milk quality is related to the monitoring of acetone, lactose, N-acetyl-

β -d-glucosaminidase (NAGase), l-lactate dehydrogenase (LDH), and progesterone (Brandt *et al.*, 2010). In this regard, several works have been developed to determine lactose concentrations. In these, devices based on the use of enzymes as lactose sensors have been used (Eshkenazi *et al.*, 2000). Other studies have focused on the measurement of urea in milk. In this case, they use a pressure biosensor that measures CO_2 from urea hydrolysis. The biosensor is capable of detecting urea concentrations between 2 and 7 mM and can be implemented in online measurement systems (Jenkins and Delwiche, 2002).

Flow cytometry: is a quick technique recognized by the International Dairy Federation (IDF) (Remón-Díaz *et al.*, 2019) that uses laser light for counting cells and other particles in suspension. The technique consists of passing a laser light beam through the sample in solution. The particles, in suspension, interact with the light beam producing two types of signals, one related to light scattering and the other with light emission coming from the fluorochromes present in the cells or particles in suspension. Through the processing and the technique, it is possible to know the characteristics of the cell, such as size, as well as to determine whether or not antigens are present in different parts of the cell, which makes the test specific and sensitive (Barrera *et al.*, 2004).

In the area of veterinary medicine, this technique has been used for the detection and identification of bacteria present in milk samples from cattle with mastitis (Langerhuus *et al.*, 2013; Gunasekera *et al.*, 2003). In the reference paper (Ruiz-García and Sandoval-Monzón, 2018), it is concluded that the correlation between flow cytometry and the somatic cell count is low with a regression or correlation coefficient of 26%.

Ultrasound: milk and its components can be evaluated in a simple and fast way using ultrasound or other waves of the electromagnetic spectrum (Brandt *et al.*, 2010). This has encouraged research in ultrasonography to evaluate mastitis, in fact, the reference study has performed in goats (Fasulkov *et al.*, 2015). Although it wasn't performed in cattle, the study shows that this ultrasonographic technique allows to evaluate changes in the length of the breast canals and in the thickness of its walls, as well as in the diameter of the ducts through which milk is expelled. It also allows for the visualization

of large hyperechoic areas, which is an indicator of inflammation (Fasulkov *et al.*, 2015; Santo *et al.*, 2015). With this technique, the progress of the disease can be monitored, and it has been evidenced that, after three days of medical treatment, ultrasonographic images show a normal, anechoic breast. Other investigations have used ultrasonography to study structures with differences in echogenicity, suggesting the presence of mastitis, edema, hematomas, atrophies and fibrosis, and intraluminal obstructions (Porcionato *et al.*, 2009, Rambabu *et al.*, 2009). Another ultrasonography study focused on evaluating the correlation between mammary gland biometry and possible alterations such as mastitis and milk production. The gland biometry consisted of measuring udder circumference, width, and quarter height. They concluded that there is no correlation between milk production, mammary gland biometric data, and ultrasonographic changes (Santos *et al.*, 2016). As can be seen, ultrasound (ultrasonography) is used to determine, through images, alterations in the mammary gland of cattle and thus diagnose mastitis, however, its use as a non-invasive technique, analyzing the milk instead of the breast, lacks study and application.

Infrared spectroscopy: An alternative method for analyzing fats, proteins, and lactose in milk is mid-field infrared spectroscopy. It is a highly accurate and repeatable method both in the laboratory and in the field. However, due to the limited penetration depth, this spectroscopy is considered unsuitable for analyzing milk in online systems (Brandt *et al.*, 2010). On the other hand, near-infrared spectroscopy can be used to analyze milk. In this case, the sensor is cheaper, and this method requires a little sample, even without preparation, making it more attractive for use in online systems (Brandt *et al.*, 2010). Some wavelength regions where information related to fats, proteins and lactose is found are known, which are between 1100 and 2500 nm (Brandt *et al.*, 2010) and between 600 and 1050 nm. Tsenkova *et al.* (2001) showed that the infrared spectrum of milk changes with high somatic cell count, due to alterations in proteins and changes in electrolytes contained in milk. Finally, spectroscopy in the visible region can be used to detect changes in milk coloration. Wiedemann and Wendl (2004) evaluated milk in the range between 400 and 520 nm. These results were superior compared to colorimetric measurements since

the technique ignores the influence of fat content on milk color. The authors were able to correctly classify 85% of healthy quarters with less than 100,000 cells mL⁻¹ and 71% of infected quarters with more than 500,000 cells mL⁻¹ and a specificity of 95% (Wiedemann and Wendl, 2004).

Accuracy of diagnostic tests

The diagnostic tests CMT, somatic cell count, and electrical conductivity have been shown to have good sensitivity and good specificity for detecting subclinical mastitis in dairy herds (Dego, 2020). Likewise, CMT and somatic cell count have been characterized as correlated and useful in the diagnosis of bovine dairy (Suárez *et al.*, 2014). In goat dairy the same situation does not occur due to physiological differences, the standard values for somatic cell count are not shown to be accurate for a good diagnosis of mastitis (Haenlein, 2002).

Signorini *et al.* (2008) conducted a study between May 1999 and August 2007 on "farm-level predictive values of mastitis from individual diagnostic test characteristics and sampling size". The diagnostic techniques analyzed were the California Mastitis Test (sensitivity 0.75 and specificity 0.54), electrical conductivity (sensitivity 0.61 and specificity 0.79), history of clinical mastitis (sensitivity 0.50 and specificity 0.54), and a hypothetical test with proposed sensitivity and specificity of 0.95 for both parameters. They used the statistical package InfoStat (National University of Córdoba) as a regression analysis where the sample size and the sensitivity and specificity of the diagnostic test were used as independent variables. Carpenter and Gardner (1996), analyzed a hypothetical case of the relationship between the sensitivity and specificity of diagnostic tests and the number of animals sampled on the predictive value at the farm level and the sensitivity at the farm level. Both authors agreed that there is an inverse relationship between diagnostic test sensitivity and sensitivity at the farm level for low and medium prevalence and an inverse relationship between specificity and sensitivity at the farm level when prevalence is high.

Dasohari *et al.* (2018) between February and August 2015 conducted a study on subclinical mastitis cases where 115 quarters of 30 cows were analyzed to compare the efficacy of different diagnostic tests such as

the California Mastitis Test (CMT), Somatic Cell Count (SCC), Whiteside Test (WST), among others. It was found that the most sensitive test was CMT (74.6%), followed by SCC (69.5%) and WST (59.3%). However, the best specificity was shown by WST (83.9%). CMT and SCC showed a specificity of 78.6%. The highest probability of having the disease if the test is positive was WST (79.6%) followed by CMT (78.6%) and SCC (77.4%). Overall, CMT is the most reliable field test after laboratory diagnostic test such as SCC.

Economic impact of bovine mastitis

Bovine mastitis is considered to be the disease that causes the greatest economic losses to dairy producers since its presence in the herds is reflected in excessive expenses for the producer and a decrease in income due to a decrease in production, which should generally be perceived within the farm (Hogeveen *et al.*, 2019).

The losses caused by this disease can be grouped as follows: Decreased production, milk discard, cost of medications, veterinary fees, extra work, and loss of genetic potential (Saran and Chaffer, 2000; Halasa *et al.*, 2007). It is estimated that a cow with subclinical mastitis decreases her production by 10.9% (Paudyal *et al.*, 2020).

Subclinical mastitis is more important and dangerous in dairy cattle because it is not possible to measure its dimension, it is underestimated since it produces chronic productivity losses with imperceptible alterations in the milk, which usually causes measures to be taken against the process when the suppression of productivity is already very large and the procedure to cure it is very expensive (Romero *et al.*, 2018).

Subclinical mastitis, whose frequency is 20 to 50 times higher than clinical mastitis, is nowadays the main problem of the whole pathological complex represented by mastitis. Careful analysis indicates that 80% of milk production losses are due to subclinical mastitis (Romero *et al.*, 2018).

The cost attributable to subclinical forms of mastitis amounts to the majority of the total cost, which is between \$100 and \$150 per cow year⁻¹, or 50 to 80% of the industry's total production losses from mastitis

(Burvenich *et al.*, 2004), while milk production losses due to subclinical mastitis and cow replacement costs associated with somatic cell counts were estimated at \$960 million (Wellenberg *et al.*, 2002).

CONCLUSIONS

Subclinical mastitis is a silent disease that has a major impact on the health of bovines on the one hand, and on humans, as well as the world economy on the other. These are some of the reasons why detection methods are important in the diagnosis of the disease. Conventional methods are used worldwide such as CMT, somatic cells count, and WMT because of their practicality and ease to use. However, alternative methods are gaining more attention due to the sensitivity and specificity of the techniques. Besides, state-of-the-art technology allows for early detection of subclinical mastitis and other diseases saving or minimizing the impact it can cause on humans, animals, and milk producers.

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