Association between bovine milk infrared temperature and bacteriological results using the PathoProof™ Mastitis Complete-16 Kit

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Abstract

Infrared temperature (**IRT**) of the milk through the milking unit short milk tube and its association with bacteriological results using the PathoProof™ Mastitis Complete-16 Kit (**PtoPrf-16**) was evaluated. In addition, the sensibility and specificity of CHROMagar™ Mastitis Kit (**CHROM**) were studied using the PtoPrf-16 results. Individual mammary quarters (n=137) with subclinical mastitis (determined by the California Mastitis Test) from 9 dairy herds in Puerto Rico were evaluated. Milk samples (10 mL in duplicates) were collected and stored in ice for subsequent evaluation. During the a.m. milking, IR images were collected 2 min post milking unit attachment in 30s intervals. Ambient temperature (**AT)** and relative humidity (**RH**) were recorded using HOBO-U23Prov2Data Loggers. Somatic cell count (**SCC**) determination and milk plate culturing were done using a DeLaval Cell Counter and CHROM, respectively. Duplicated milk samples were sent to the Lancaster DHIA Lab for molecular identification of mastitic pathogens using the PtoPrf-16. A PROC GLIMMIX was used to determine differences between IRT and SCC by bacteriological results (PtoPrf-16) and contingency tables with a Chi-Square test to determine specificity and sensitivity of CRHOM plates relative to molecular results. The SCC was converted to somatic cell score (**SCS**) and bacteriological results grouped in Gram-positive, Gram-negative, and No-detection for analysis evaluation. The mean SCS differed between Gram-positive and no-detection (P=0.022) but not between the remaining comparisons (P=0.05). The mean IRT of the short milking tubes was higher in the Gram-positive group relative to no-

detection (P=0.052) with mean IRT±SEM of 33.48±0.20 and 32.86±0.29, respectively. No differences in IRT were observed between the remaining comparisons (P>0.05). Using the PtoPrf-16 as a comparative test tool, the CHROM presented an overall sensitivity and specificity of 70.5% and 52.17%, respectively. According to these findings, the use of IRT could be used as a discriminatory tool between subclinical mastitis quarters and justify more studies to better characterize these differences.

Resumen

La termografía infrarroja (**TIR**) de la leche a través de los tubos cortos de la unidad de ordeño y su asociación con los resultados bacteriológicos del PathoProof™ Mastitis Complete-16 kit (**PtoPrf-16**) fueron evaluados. En adición, se estudió la sensibilidad y especificidad del CHROMagar™ Mastitis Kit (**CHROM**) utilizando el PtoPrf-16. Se evaluaron cuartos mamarios individuales (n=137) con mastitis subclínica (utilizando la Prueba de Mastitis Californiana) de 9 vaquerías en Puerto Rico. Muestras de leche (10 mL en duplicados) fueron colectadas y guardadas en hielo para análisis subsiguientes. Durante el ordeño de la madrugada, imágenes IR fueron tomadas a los 2 min de haberse colocado la unidad de ordeño y en intervalos de 30s. Temperatura ambiental (**TA**) y humedad relativa (**HR**) fueron tomadas utilizando dos HOBO-U23Prov2Data Loggers. Se determinó el conteo de células somáticas (**CCS**) y las bacterias de los cuartos utilizando un DeLaval Cell Counter y CHROM, respectivamente. Los duplicados de las muestras de leche se enviaron a la Asociación de Mejoramiento de Hatos Lecheros (Manheim, PA) para identificar patógenos causantes de la mastitis utilizando la prueba molecular PtoPrf-16. Se utilizó un PROC GLIMMIX para determinar diferencias entre TIR y SCC por resultado bacteriológico (PtoPrf-16) y pruebas de contingencias con la prueba de Chi-Cuadrado para determinar la especificidad y sensibilidad de los platos CHROM al comparar sus resultados con los del PtoPrf-16. El CCS fue convertido a puntuación de células somáticas (**PCS**) y los resultados bacteriológicos se agruparon en Gram-positivo, Gram-negativo y no-detección para análisis. La media de PCS fue diferente entre Gram-positivos y no-detección (P=0.022) pero no entre las comparaciones

restantes (P=0.05). El promedio de TIR de los tubos cortos fue mayor en el grupo de Gram-positivo relativo a no-detección (P=0.052), con medias TIR ± SEM de 33.48±0.20 y 32.86±0.29, respectivamente. No se encontraron diferencias en TIR entre las comparaciones faltantes (P>0.05). Utilizando el PtoPrf-16 como herramienta comparativa, el CHROM presentó una sensibilidad y especificidad general de 70.5% y 52.17%, respectivamente. De acuerdo a estos resultados, la TIR podría utilizarse como herramienta discriminatoria entre cuartos mamarios con mastitis subclínica y justificar más estudios que caractericen mejor estas diferencias.

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List of acronyms

ALA: Alpha-linolenic acid

AT: Environmental Temperature

CMT: California Mastitis Test

CNS: *Coagulase negative staphylococci*

DCC: DeLaval Cell Counter

DHA: Docosahexaenoic acid

EC: Electric conductivity

IRT: Infrared thermography

LP: Lactose persistence

LPS: Lipopolysaccharide

LTA: Lipoteichoic acid

ND: No-detection

ORIL: Dairy Industry Regulatory Office of Puerto Rico

PAMP: Pathogen associated molecular pathogens

PtoPrf-16: PathoProof Mastitis 16 kit

qPCR: Real Time Polymerase Chain Reaction

RH: Relative Humidity

SCC: Somatic Cell Count

SCS: Somatic Cell Score

TLR: Toll like receptors

Chapter 1.

Introduction

Industrialization of milk production has persuaded many dairy farmers to focus genetic selection on traits associated with higher milk yields neglecting health traits (Koeck et al., 2017). Currently, mastitis is one of the most important diseases in the dairy industry, instigating economic losses associated with lower milk yields, poor milk quality, treatment costs, culling of animals, among others (Halasa et al., 2009) Mastitis is not only causing economic losses in the dairy industry, it is also affecting cattle's well-being (Seegers et al., 2003). The most economically important type of mastitis is its subclinical form; therefore, new methods are necessary for its detection. Early detection and treatment of mastitis increase cure rates, therefore improving milk yields and quality while safeguarding animal wellbeing (Lago et al., 2011). Detection of mastitis with the use of infrared thermography cameras could represent a feasible and a low-cost tool to detect subclinical mastitis. Previous researches have used this method to identify mastitis by imaging the skin of the mammary gland reporting favorable results (Colak et al., 2008; Hovinen et al., 2009). Other researches have used this technology in milk samples and milking components as a means to predict the somatic cell counts (**SCC;** Almeida, 2014). These authors reported that IRT of the milk had limited capabilities in estimating SCC; however, it correlated well when the SCC was grouped by bacterial Gram-family ($r= 0.84$; $P=0.008$). Nevertheless, the aforementioned study used a limited number of observations and a considerable number of milk samples with elevated SCC had no bacterial grow (55%; using bacterial culturing).

Objectives

- Determine if infrared thermography could be used as a discriminatory tool between Gram-negative and Gram-positive mastitic quarters and healthy ones using molecular testing for bacterial identification.
- Determine the specificity and sensitivity of CHROMagar™ plates as an in field mastitis pathogen detection tool using the PathoProof™ Mastitis Complete 16 kit as a comparative test.

Literature Review

Milk and its contribution to civilization

Humans learned how to domesticate animals and crops, allowing them to evolve from nomads to settled people (Bellwood and Oxenham, 2008). However, the main problem that humans encountered was the susceptibility of their crops to seasonal changes (Vigne, 2008). Since then, milk became a key strategy to overcome this issue originating in the Middle East 10,500 years ago. People started to ferment milk to reduce lactose concentration. This allowed them to overcome the health issues associated with milk consumption usually experienced after the age of 7, when the production of the enzyme lactase was drastically reduced (Curry, 2013).

It was not until around the Neolithic era (10,200 BC) that a genetic mutation, an exchange of the base cytosine for thymine in the Lactase Persistence (**LP**) allele, spread from generation to generation resulting in the production of lactase regardless of the age in many parts of the world, like northern and central Europe (Curry, 2013). At present, it is possible that descendants from the firsts generations of LP can digest lactose found in fluid milk as well as other dairy products of nutritional significance such as cheese, yogurt, protein shakes, puddings and others.

Human's breast milk's long chains of polyunsaturated fatty acids play an important role in the nutrition of neonates as it provide fatty acids such as docosahexaenoic acid (**DHA**, 22:6n-3). Presence of DHA in diets is indispensable for the development of retina, visual cortex and brain tissue of humans (Uauy and Dangour, 2006). Furthermore, alpha-linolenic acid (**ALA**, 18:3n-3) is a precursor of DHA via a sequence of chemical reactions

involving desaturation, elongation and β-oxidation reactions (Moore et al., 1995) and it is present in bovine milk. Hence, consumption of dairy milk favored survival and enhanced brain development of human progenies since the beginning of lactose tolerance.

The Dairy Industry of Puerto Rico

In Puerto Rico, the dairy industry currently produces a surplus of fluid milk, generates around 23% of the net agricultural income of the country, protects around 52,287 acres of land destined for forage production or grazing, and generates around 25,000 employs (Dairy Industry Regulatory Office; **ORIL**, 2014). However, in the last few years the dairy industry of Puerto Rico has been drastically affected by the reduced demand of fluid milk. At the farmer level, the situation is more precarious when milk production efficiency is continually affected by high prices of grain and low quality of tropical forages. This scenario becomes even more challenging when taking into consideration the different diseases that dairy cattle are constantly exposed to and the aggravated effect of heat stress associated with the island's geographical location. Among the various disease that arise, mastitis is the most common and costly in dairy cattle worldwide (Seegers et al., 2003). Although more than 75% of the dairies in Puerto Rico average ≤400,000 SCC/mL, the island currently have one of the more lenient milk quality regulations with upper allowable limits for SCC and bacterial counts of 750,000 SCC/mL and 100,000 CFU/mL, respectively (ORIL, 2014). On the contrary, countries like the European Union, Australia and New Zealand, and some milk processors in the U.S.A. have lower limits (e.g., 400,000 SCC/mL; Hogeveen and Federation, 2005).

Mastitis

Mastitis is a word that comes from the greek “mastos”, meaning breast, with the suffix “-itis” to indicate inflammation. It is the term used to refer to any effector that causes inflammation of the mammary gland. Although mastitis can occur from physical injuries and biological agents such as fungi, yeasts and viruses; it is mainly caused by bacteria (Holm et al., 2017). Inflammation during bacterial mastitis occurs as a consequence of the antigens released by bacteria during their replication and death, activating the host’s immune system. The severity of the inflammation depends on the type of pathogen invading the udder (Lavon et al., 2011). Immunological responses associated with bacterial infections reduce milk yields (Wilson et al., 2004) and can also decrease reproductive performance (Lavon et al., 2011). It is also known that the reproductive performance is more predisposed when mastitis is caused by a Gram-positive bacteria relative to Gram-negative bacteria (Lavon et al., 2011).

With milk as one of the most important foods in the human diet (Haug et al., 2007; Pereira, 2014), dairy scientists have been raising aware about the importance of maintaining a healthy herd and its relation with production efficiency and milk quality. Wilson et al. (2004), reported that cows with mastitis produce lower quantity and quality of milk relative to healthy ones. In addition, herds with high prevalence of mastitis face great challenges fulfilling quality regulations for fluid milk, especially for SCC and bacterial counts, increasing the chances of sending milk contaminated with antibiotics to the bulk tank (Ruegg and Tabone, 2017), resulting in significant economic losses for the dairy producer.

Mastitis can be classified as clinical or subclinical. Subclinical mastitis do not display any visible signs associated with inflammation; for diagnosis, the milk has to be tested to determine its SCC (NMC, 2004). Field-tests such as the California Mastitis Test (**CMT**) and the DeLaval Cell Counter (**DCC**) are used to determine the SCC of suspected subclinical mastitic quarter. Laboratory tests, such as as the Fossomatic, are more frequently used for managing large number of samples, such as in the DHI. Depending on the host's health status and the etiological agent, subclinical mastitis could become clinical and vice versa. Clinical mastitis is characterized by visible abnormalities like coagulated milk or blood alongside inflammation, quarter redness and hardness or elevated temperature (Hovinen et al., 2009). The term chronical mastitis refers to an infection of long duration that could presents clinical symptoms intermittently (NMC, 2004). Regardless of the mastitis classification, early detection represents the most efficient and cost-effective approach to reduce its impact on milk quality, animal welfare and overuse of antibiotics. Moreover, it is still common to observe dairy farmers treating mastitic quarters only when the mastitis turns clinical (Hoe and Ruegg, 2017).

Economic impact of mastitis

Mastitis is the most common and costly diseases in dairy herds worldwide (Seegers et al., 2003). Considering the costs of milk losses, labor, medications, veterinary services and culling of mastitic cows, the estimated average yearly loss is $5,481 per every one hundred dairy cows (Halasa et al., 2009). According to ORIL's annual report, during the fiscal year 2014 in Puerto Rico the losses associated with discarded milk due to exceeding the regulatory upper limit of SCC and/or presence of antibiotics in bulk tank milk reached $

53,325 (ORIL, 2014). Considering the impact of mastitis on milk yield would provide a more accurate estimate of its economic impact. Inflammation associated with mastitis reduces the ability of milk secreting cells (alveolar epithelium) to produce milk (Shuster et al., 1991). In addition, milk clots can block the lactiferous ducts, aggravating inflammation and drastically reduce milk yield (Harding, 1995). A meta-analysis about the economic impact of mastitis in dairy herds found that cows with average milk SCC from 400,000 to 800,000 cells/mL produced 10% less milk relative to cows free of mastitis (<200,000 SCC/mL; Kvapilik et al., 2014). Considering the latest ORIL report (2014), dairy farmers produced 155.6 M liters of milk, averaging 421,523 SCC/mL. Using a 5% milk loss, according to Kvapilik et al. (2014), this would represent 12,531,249 L of milk that were not produced in Puerto Rico due to elevated SCC during fiscal year 2014. Using the pondered price paid in 2014 ($0.78/L), the estimated milk loss due to the reduced milk yield associated with high SCC may have reached $10,288,816.

Mastitis not only reduces the milk production potential of dairy cows, but also their reproductive performance. Pro-inflammatory molecules associated with mastitis can alter the estrous cycle of ruminants, affecting their reproductive performance. Examples of these are cytokines, like tumor necrosis factor α (**TNF-α**), which suppress the formation of luteinizing hormone receptors (Darbon et al., 1989), or may cause an abnormal synthesis of Prostaglandin-$F_{2\alpha}$ associated with mastitis development during the luteal phase of the estrous cycle (Hockett et al., 2000). In fact, Schrick et al., (2001) found that cows with mastitis (subclinical or clinical) had higher days to first service and higher services required per conception, compared to uninfected cows.

Immunological responses associated with mastitis-causing bacteria

Activation of the mammary gland immune response occurs when an antigen, frequently of bacterial origin, surpasses the anatomical defenses of the mammary gland, the skin and teat end orifice. The teat end possesses a keratin lining that contains antimicrobial agents (Constable et al., 2017). If a pathogen passes through the teat end orifice and colonize the teat cistern, it could cause an immunological response (Capuco et al., 1992). At this point, three possible scenarios favoring mastitis development could occur, 1) immunocompromised host unable to eliminate the invading pathogen, 2) inappropriate milking practices favoring propulsion of bacterial cells deeper into the gland cistern, and 3) bacterial virulence overcoming an immune-competent host. The alternative outcome would be the opposite of the previous scenarios resulting in a healthy quarter.

Pathogens possess pathogen-associated molecular patterns (**PAMP**) that can be recognized by the mammalian host immune cells, including macrophages. Initiation of the innate immune response occurs when PAMPs binds to a specific pattern recognition receptor (**PRR**) expressed by macrophages or dendritic cells (Murray et al., 2005). Pattern recognitions are included in Toll-Like Receptors (**TLRs**) which are in charge of recognizing bacterial structures and activate the cytokine production (Murray et al., 2005).

After recognition, macrophages attach to bacteria through the TLRs. Subsequently, a phagocytic vacuole is formed around the microorganism, with part of the plasma membrane, and fuses with macrophages or neutrophils to inactivate and digest the microorganism (Paul, 2013). Neutrophils and macrophages phagocyte bacteria in the following order: attachment, internalization and digestion and cytokines are released by macrophages to attract leukocytes from the blood vessel to the mammary tissue (Murray et

al., 2005; Wellnitz and Bruckmaier, 2012). Cytokines are responsible for capillary expansion to increase blood flow and stimulate mast cells to release histamine and increase permeability of the endothelium for leukocytes mobilization to the insult area.

Leukocytes enter the infection site by passing from the bloodstream to the endothelium, sub-epithelial matrix, basement membrane and mammary epithelium into milk by slowly rolling, attaching and going through the capillary wall by a process known as diapedesis (Paape et al., 2003; Murray et al., 2005). Of all leukocytes, neutrophils are present in larger quantity in an infected quarter and are responsible of phagocytizing and killing pathogens with the use of chemical defenses like hydrogen peroxide, hydrolytic enzymes and lactoferrin (Paape et al., 2003).

Bacterial phagocitation and digestion by neutrophils and macrophages can be oxygen depended or oxygen independent (Murray et al., 2005). Oxygen dependent killing occurs by oxidative burst that later on forms hydrogen peroxide and combines with other compounds and kill the microorganism (Murray et al., 2005). Some bacteria are able to replicate and survive the attacks of the innate immune response and activate the systemic inflammation response (Murray et al., 2005).

Dendritic cells and macrophages produce pyrogenic factors such as TNF-α, Interleukin-1, and Interleukin-6 to activate the acute response in the liver (Bochsler and Slauson., 2002). Furthermore, macrophages produce prostaglandins and leukotrienes to enhance local inflammatory reaction (Bannerman et al., 2004). The inflammatory reaction increases blood flow by vasodilatation and consequently, increases local temperature producing fever which is unfavorable for bacteria growth and replication (Murray et al., 2005). If bacteria survive the attack by neutrophils, lymphocytes enter the site of infection.

Lymphocytes produce antibodies and are able to recognize antigenic structures and enhance immune system memory for future infections with the same bacteria (Sompayrac, 2011).

Mastitis is also characterized according to the type of bacteria isolated from mastitic quarters. Bacteria are categorized as Gram-positive or Gram-negative depending on cell wall composition (Murray et al., 2005). A major constituent of the cell wall of Gram-negative bacteria are the lipopolysaccharides (**LPS**) while in Gram-positive bacteria the principal component of the wall is known as lipoteichoic acid (**LTA;** Murray et al., 2005). Variations between Gram family mastitis symptoms could be attributed to differences in TLR recognizing antigens. For instance, LPS is identified by TLR-4 (Fu et al., 2013) and LTA is identified by TLR-2 (Strandberg et al., 2005). Previous research has reported differences in mammary gland immunological responses according to the isolated Gram family responsible for the infection (Wellnitz et al., 2011).

Mammary gland immunologic responses associated to a specific group of bacterial infections can be studied utilizing the LPS challenge or LTA challenge. A study at the University of Bern in Switzerland, reported increments in rectal temperatures during an LPS challenge in one udder quarter (Wellnitz et al., 2011). In this study, LPS concentration was directly proportional to the concentrations of SCC, pro-inflammatory molecules such as TNF-α and tissue damage markers such as lactate dehydrogenase during the inflammatory response. However, the same degree of association between the aforementioned responses were not observed when the mammary gland was infused with LTA (Wellnitz et al., 2011).

Methods for mastitis identification

Clinical mastitis is identified easily by observing appearance of the milk and udder during the pre-milking procedures. However, methods to estimate SCC are required to identify subclinical mastitis. The California Mastitis Test (**CMT**), is one of the most common field tests used to identify mastitic quarters (Holtgrew-Bohling, 2012). Although the test does not require extensive training its is relatively subjective. The CMT consists of a detergent solution, with bromocresol purple, that exposes the DNA of somatic cells in the sampled milk. The viscosity of the sample correlates with the amount of DNA exposed, making it possible to detect subclinical mastitis. However the amount of somatic cells in the milk sample can only be estimated inside a considerably large range (Whyte et al., 2005) and discrepancies can occur depending on the subject performing the test.

Another indirect method of estimating SCC the milk electric conductivity (**EC;** Sharma, 1974). This method detects mastitis according to changes in the presence of ions in milk (NaCl). During inflammation, the permeability of the alveoli capillary net increases. As a result, Na^+ and Cl^- increase in milk due to their passage into the alveolar lumen through the tight junctions while lactose and K^+ exit the lumen in order to compensate osmolality. Determination of EC is more accurate when testing milk samples after the milk stripping relative to milk samples collected prior to this (Norberg et al., 2004), Currently, EC technology has been applied to on-line milking systems like Afimilk (Lien et al., 2015). With this system, measurements of EC are recorded daily and an average is calculated for each cow. If a recorded measurement deviates from their average the system alerts the farmer for a possible case of mastitis.

Another mastitis detection field test is the DeLaval Cell Counter (**DCC,** DeLaval©, Tumba, Sweden). The DCC uses a disposable cassette which absorbs 60 µL of milk by capillarity and mixes it with propionic iodine, a fluorescent marker for somatic cell's nuclei. With the use of a built-in digital camera it counts the nuclei of each somatic cell, making the measurement of SCC highly accurate (Ogola et al., 2007). Similarly, the Fossomatic™ FC (FOSS, Hilleroed, Denmark) uses propidium iodide to stain the nuclei of somatic cells (Oosterbroek and van den Berg, 2003). Yet, the Fossomatic uses a technique known as flow cytometry (measurement of particles in a fluid with the use of laser) allowing this method to analyze large amounts of samples in a relatively short period (e.g., 600 milk samples per hour).

Infrared thermography as a mastitis detection tool

The inflammatory process of mastitis is associated with a temperature increment at the insulted area due to an increase in blood flow (Kehrli and Harp, 2001). The IRT of the milk could be used as a non-invasive tool to detect the mammary gland's temperature. This tool is used to measure convective heat fluxes with thermal sensors and a two-dimensional transducer for precise measurements (Carlomagno and Cardone, 2010). Moreover, IRT has a high sensitivity which makes it a useful and reliable tool for measuring convection temperatures (Carlomagno and Cardone, 2010).

The use of IRT has been previously studied as a novel detection tool for subclinical mastitis, presenting positive correlations between milk IRT and CMT results (Colak et al., 2008). However, it is important to emphasize that external factors such as environmental temperature (**AT**), solar radiation and daylight variations during the milking process can

influence the udder surface temperature (Berry et al., 2003). Another study in Puerto Rico evaluated the use of milk's IRT as a subclinical mastitis tool (Almeida et al., 2014). These researchers evaluated the use of IRT to indirectly measure the milk's temperature through the short milking tubes and estimate the SCC. They reported that, in addition to what was reported by Berry et al., (2003), the IRT of the milk was affected by rectal temperature and activity level of the cows, and the time of milking. Interestingly, the study reported a positive correlation between IRT and bacterial Gram family ($r=0.84$, $p=0.0086$; Almeida, 2014). Since these findings were not a predetermined objective, a limited amount of observations were used.

Methods for bacterial identification in milk

Once a mastitis infection is detected, identifying the etiological agent is a crucial step. Incorrect treatment of mastitis decreases farm profitability and could favor bacterial development of antibiotic resistance (Suriyasathaporn et al., 2012). In order to appropriately select an effective treatment regime, the etiological organism as well as data regarding reproduction and production status, treatments and outcomes, should be considered. In extreme cases, such as in clinical mastitis, in which the udder may be swollen and milk ducts blocked, systemic antibiotics may be necessary because these factors reduce the efficiency of intra-mammary antibiotic treatments against bacteria (Gruet et al., 2001). Failure to effectively resolve the infection in a short period represents a longer withdrawal time of milk leading to greater economical loses.

Lago et al. (2011) found economic benefits, including less discharged milk, lower days in treatment and better cure rates, when cows were assigned to a cultured-based

mastitis treatment. Contrarily, the indiscriminate use of antibiotics have been directly related to emergence of antibiotic resistant bacteria (Suriyasathaporn et al., 2012). A study by Tenhagen et al. (2006) demonstrated that the degree of ampicillin resistance of *S. aureus* increased as the cow's days in milk increased. Another study conducted in Sweden found that 7.1% of *S. aureus* and 12.5% of *Coagulase Negative Staphylococci* (**CNS**) were resistant to penicillin (Bengtsson et al., 2009). Since penicillin is commonly used to treat Gram-positive mastitis infections, both of these studies effectively highlighted the importance of identifying the mastitis etiological agent before choosing an antibiotic.

Traditional Bacteriological Tests

Protocols must be followed in order to obtain an aseptic milk sample for bacterial identification. The National Mastitis Council established procedures intended to reduce contamination of milk during sampling collection (NMC, 2004). Once the milk samples are obtained, different bacteriological tests can be performed to determine the etiological agent. Agar plate cultures are the most commonly used method to identify bacteria and the number of colonies per mL (expressed as colony forming units/mL; CFU/mL), known as standard plate count (Sanders, 2012).

Agar is made from polysaccharide agarose isolated from cell walls of red algae. The medium is prepared by reconstituting the dehydrated agar with boiling water (99.9° C), stirring, cooling and pouring it on petri dishes (approximately 15 mL in each petri dish). Antibiotics can be added to the agar mix to selectively growth bacteria. Some of the most used agars are: Blood agar (allows identification of homology type in all bacteria), MacConkey agar (for Gram-negative bacteria) and Triptyc Soy agar (for various types of

bacteria). The favorable outcome of using a broad-spectrum agar when culturing can be the discovery of new bacteria. Since isolation of bacteria is possible after culturing, their metabolic information like carbohydrate consumption, growth inhibition and/or antibiotic resistance can be studied (Sutton and Lee, 2000). Furthermore, culturing is an exceptional tool for the pathogenesis study of a disease (Houpikian and Raoult, 2002). Moreover, after culturing pathogens, antigens can be utilized for vaccine development (Sutton and Lee, 2000).

If viable bacteria are present on the cultured sample, morphology of bacterial colonies can be observed after incubating the sample for 12 to 72 h at 38° C. Each colony means that one bacterium reproduced itself exponentially to the point where it is visible. If colonies are not observed in the surface of the agar plate after incubation, it could mean that the sample is free of bacteria or that the bacteria were not able to grow under the given circumstances (Sanders, 2012).

For milk cultures, the spread plate technique is recommended, which is the aseptically spreading of a milk sample (100 µl) on the surface of the agar plate, This technique allows growth of aerobic microorganisms. However, the inability to grow anaerobic microorganisms when using this technique and the possible contamination of the agar while its being prepared or incubated are some disadvantages of this bacterial identification method.

CHROMagar™ Mastitis: differential and selective test for mastitic bacterial identification

A chromogenic technology (CHROMagar™, Paris, France) was developed in 1979 for the isolation and differentiation of mastitic pathogens using chromogenic technology. The chromogenic media found in CHROMagar plates uses colorless molecules with substrate content that can be targeted by specific enzymatic activity of a specific bacterium. Once the enzyme targets the chromogenic conjugate in the agar and the chromophore is released, it portraits a specific color to the colony. Using a color legend, the bacterial colonies can be identified. The CHROMagar Mastitis kit (**CHROM**) consists of two different media: one for identification of Gram-positive and another for the identification of Gram-negative bacteria.

This technology offers various advantages relative to traditional culturing plates as it allows for the enumeration, differentiation and identification of bacteria in a single plate. The kit uses the same equipment as the traditional culturing plates and do not require additional testing (e.g., fermentation of specific sugars), with the exception of *E. coli*, some *Enterococcus* and *Proteus* (CHROMagar, 2017). Also, according to the manufacturer, the kit identifies mastitis pathogens according to the typical appearance of the colonies according to the color-legend described in Table 1.

Table 1. Typical appearance of colonies in the CHROMagar Gram-positive plates and Gram-negative plates.

CHROMagar Mastitis Gram-positive		**CHROMagar Mastitis Gram-negative**	
Pathogen	**Colony color**	**Pathogen**	**Colony Color**
S. agalactiae	Blue-green	*E. coli*	Red
S. uberis	Metallic Blue	*Klebsiella, Enterobacter, Citrobacter*	Metallic Blue
S. aureus	Mauve with mauve halo	*Proteus*	Brown Halo
Other microorganisms	Various	*Pseudomonas*	Translucent
-	-	*C. albicans*	White, Opaque, Small

Some disadvantages of the CHROM are the limited amount of Gram-positive bacteria that it can identify and the color deviations observed in some colonies. For instance, metallic blue colored colonies may indicate the presence of *S. uberis* when in reality represents an *Enterococcus* strain. Therefore, additional tests, like fermentation of carbohydrates, are required to confirm metallic blue colonies. Also, an Indole test may be required for identification of *E. coli* and a TDA (tryptophane deaminase) test for *Proteus* (CHROMagar™, 2017). To our knowledge, evaluation of the specificity and sensitivity of the CRHOM has not been previously studied or published. Culturing bacteria with CHROM is an easy and low cost method. If identification of the colonies is not possible, classification of Gram family is depending on the plate (Gram-positive and Gram-negative) on which the bacteria grew. The cost per analyzed milk sample is around $3.00 (taking in consideration the kit cost and culturing miscellaneous).

Molecular Testing for the identification of mastitic pathogens

Molecular testing is the study of single genes or short lengths of DNA to identify the presence of mutations, variations or presence of organisms like bacteria (Houpikian and Raoult, 2002). One of the available methods for genetic testing is the Real-time polymerase chain reaction (qPCR), known for its accurate quantification of a particular copy of DNA present in a sample (Solcà et al., 2014). The qPCR involves three steps: amplification reactions, a DNA-targeted system for optical detection and instrument software. Amplification reactions consist of multiple heating and cooling cycles of the samples for nucleotide denaturation, annealing and DNA amplification (Rocha et al., 2016). Once the samples are mixed with all necessary reagents, is then placed in a reaction block that is

typically a 96-well plate. Once the DNA amplification is obtained, the optical detection system is used to detect the presence of fluorescent molecules attached to the targeted DNA. An instrument software is used for interpretation of the results (Dorak, 2007).

PathoProof™ Mastitis complete 16 Real Time PCR kit

The PathoProof™ Mastitis kit, by Thermo Scientific (**PtoPrf-16**), was specifically developed to identify mastitic pathogens responsible for 95% of all mastitis cases (Asmussen et al., 2014). A fast qPCR machine and the Norden-Lab Studio software for result interpretations (Norden Logic Oy, Helsinki, Finland) are required. The kit includes all the reagents for extraction of DNA. The PtoPrf-16 targets the following mastitis causing pathogens: (Table 2).

Table 2. Mastitic pathogens targeted by the PathoProof Mastitis Complete 16 kit.

Corynebacterium bovis	*Prototheca spp.*
Enterococcus spp. (E. faecalis and E. faecium)	*Serratia marcescens*
Escherichia coli	*Staphylococcus aureus*
Klebsiella oxytoca and/or pneumoniae	*Staphylococcal β-lactamase gene*
Mycoplasma bovis	*Staphylococcal spp.*
Mycoplasma spp.	*Streptococcus agalactiae*
Streptococcus dysgalactiae	*Streptococcus uberis*
Trueperella pyogenes and/or Peptoniphilus indolicus	*Yeast*

Supré et al. (2013), examined 5,093 bovine milk samples with clinical mastitis and with microbiological culture identified those with bacteria growth and no-growth (no colony growth when plated). Minor pathogens and no-growth milk samples were selected for molecular testing using the PtoPrf-16. The 50.7% of the samples in which the microbiological culture found no-growth had bacterial DNA identified by the PtoPrf-16.

Another study evaluated milk samples with subclinical and clinical mastitis that had no bacterial growth when cultured using microbiological culturing methods (Taponen 2009). The study found that almost half of the clinical mastitis cases that were conventionally cultured and presented no-growth had pathogens present in the milk sample and often in extensive quantities. Their findings could be attributed to the death of bacteria due to antimicrobials in collected samples or to the presence of bacteria unable to grow on conventional media but detectable with the PtoPrf-16. Both studies (Taponen et al., 2009; Supré et al., 2013) suggest that PCR is a useful tool for detection and identification of mastitic pathogens due to its accuracy.

Additional advantages of using the PtoPrf-16 is that results can be obtained in up to 6 hours which represents an advantage when seeking an accurate and quick treatment for mastitis. According to the manufacturer (Thermo Scientific, Finland), additional advantages of using the PtoPrf-16 kit for identification of mastitic pathogens include: reduced risk of cross over contamination due to working with closed reaction vessels and identification of microorganisms even when dead, specially important during management of samples, allowing the freezing and thawing of milk. The PtoPrf-16 simultaneously identifies 15 of the most common microorganisms that cause mastitis as well as the β-lactamase gene which confers penicillin resistance to bacteria such as *S. aureus* and CNS. In addition, allows for semi-quantification of bacterial DNA in the sample as low-, medium- or high-relative frequency; making possible to identify the predominant pathogen in the sample.

Materials and Methods

Dairy herds and environmental parameters

The research included 137 quarters from ten Dairy Farms in Puerto Rico including the following counties: Aguadilla, Lajas, Hatillo, Hormigueros, Moca and Quebradillas. Dairy farms were chosen according to the following criteria: milking time from 0200 to 0600 and bulk tank milk SCC higher than 400,000 cells/mL. Relative humidity (**RH**) and AT were collected during the milking process using two U23-01 HOBO Data Logger (Onset®; Massachusetts, USA). Data was collected in one-minute intervals. Thermometers where distributed accordingly to the location of cows during milking, right and left side of the milking parlor.

Evaluation of mammary quarters and milk sample collection

During the udder preparation, teats were disinfected with a pre-dip and stripped three to four times to evaluate milk appearance; this to assure that quarters included in the study did not have clinical mastitis. Quarters with normal milk appearance, no skin redness or inflammation were tested for subclinical mastitis using a CMT. Quarters with CMT scores ≥ 1 were selected for the study. Duplicated milk sample (10mL) were collected in sterile tubes (Falcon™ 15mL Conical Centrifuge Tubes) following the Microbiological Procedures for the Diagnosis of Bovine Udder Infection and Determination of Milk Quality (NMC, 2004). Samples were kept on ice until laboratory arrival and later stored under temperatures of approximately 4.0° C for SCC determination and milk plate culturing using a DCC (DeLaval Tumba, Sweden) and CHROM in no more than 24 h with respective to milk

collections, respectively. Duplicated milk samples were kept frozen until molecular identification of mastitic pathogens using the PtoPrf-16.

Infrared Thermography Imaging

The temperature of the milk from selected quarters was estimated using a FLIR E-8 IR camera (FLIR Systems Inc, Oregon, U.S.A.). The images were obtained from the short milking tubes of each selected quarter while being milked (Image 1). These images were taken in 30s intervals after two minutes from the milking unit attachment (to allow time for conduction of milk's heat through the short milking tubes). Thermography images were analyzed to determine the higher IRT registered from the images of the short milking tubes using the FLIR Tools-plus Software (FLIR® Systems, Inc, Wilsonville, OR). The Max IRT was used for data analysis.

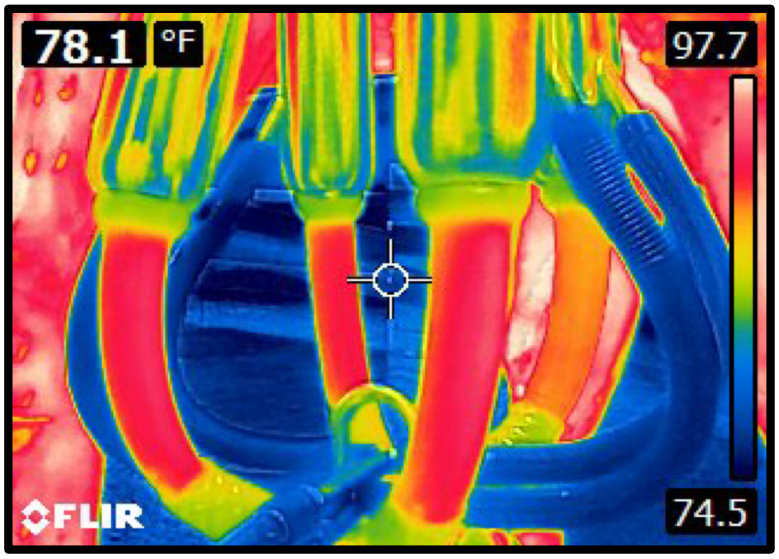

Image 1. Infrared thermography imaging of the short milking tube used to estimate milk temperature during the milking process.

Bacteriological Analysis of milk samples

Immediately upon arrival to the Animal Molecular Biology Laboratory, one of the duplicated milk samples was freeze at -80° C for subsequent delivery to the Dairy Herd Improvement Association laboratory (DHIA; Manheim, PA) for molecular identification of mastitis pathogens using the PtoPrf-16. The other milk sample (10 mL) was used to inoculate Petri dishes with chromogenic media (CHROM). Subsequently, the same milk sample was used for SCC analysis using a DeLaval Cell Counter (DCC; DeLaval International USA, Tumba, Sweden). The CHROM was used for bacterial isolation and differentiation of Gram-negative and Gram-positive bacteria. Preparation of Gram-positive plates consisted in the mixture of the agar dehydrate in the proportion of 42.4 g/L of boiling (100° C) purified water. A supplement is required in a proportion of 8 mL/L to provide growth factors to the medium (included in the kit). The mixture is stirred until creating a homogeneous solution and then 10-15 mL are poured per petri dish. For the Gram-negative plates, the same process is required with a proportion of 33.2 g/L of the dehydrate powder. A supplement is not needed to complete the reaction of this agar. Once the plates are solidified, the inoculation can be made by spreading 0.1 mL of a milk sample over each of the CHROMagar mastitis plates, ultimately incubating it upside down for 48 hours at 37° C (CHROMagar™, 2017). The inoculation process was realized following the recommendations of the NMC (2004). Colony growth in CHROM plates were visible after 48 h, allowing Gram categorization. If both Gram families were present in a milk sample, the predominant bacterium was selected according to relative molecular expression in the PtoPrf-16 report.

Statistical analysis

For data analysis, the mastitis pathogens identified with the PtoPrf-16 were categorized in Gram-positive,, Gram-negative, and no-detection (when no pathogen was detected). The SCC was converted to SCS using the formula: log2(SCC/100,000)+3 (Shook, 1993). To compare the IRT of the milk through the short milking tubes, according to the PtoPrf-16 results, a PROC GLIMIX analysis was used in SAS University Edition (SAS Institute Inc., NC, US). Independent variables in the model included, herd, PtoPrf-16 results, and cow ID as random variable. Dependent variables included IRT and SCS. Differences between IRT among PtoPrf-16 results and categories of SCS were compared using LSMEANS, with a Tukey adjustment. A PROC CORR in SAS was used to determine the correlation coefficients between quantitative variables such as SCC, IRT of the milk, AT and RH. To determine the sensibility and specificity of the CHROM, using the PtoPrf-16 as the comparative tool, contingency tables were performed using the PROC FREQ in SAS.

Sensitivity for CHROM Gram-positive detection was defined as the number of cases in which the CHROM detected any Gram-positive bacteria when the PtoPrf-16 did too. The same was used for sensitivity of Gram-negative bacteria. The specificity was defined as the number of cases in which no bacterial colonies matched the manufacturer legend in the CHROM and when the PtoPrf-16 did not detect any of the targeted mastitic pathogens.

Results and Discussion

Infrared temperature of the short milking tubes, somatic cell count and environmental parameters

Neither RH nor AT interacted with herd to affect the IRT of the short milking tube (P>0.05). In addition, AT and RH did not affect IRT (P=0.103 and P=0.065, respectively), which could mean that the use of IR images collected during milking times from 0200 to 0600 was effective in reducing the effects of diurnal temperature variations on the IRT of the short milking tubes, as previously reported by Almeida (2014). Moreover, Colak et al., (2008) reported that time relative to milking could be a variable that could affect IRT.

The PtoPrf-16 results did not interacted with herd to affect milk IRT (P=0.854). However, herd affected the IRT of the short milking tube (P=0.017). This could be attributed to differences in management, such as walking distance from pens to the milking parlor (*i.e.*, cow activity), which have been previously reported to affect milk temperature (Almeida, 2014).

Differences in IRT of the short milking tube according to PtoPrf-16 results where observed (P=0.052; Figure 1). This difference could be attributed to the inflammatory effect of mastitis and it subsequent influence on milk temperature (reference de arriba). No significant differences were found between Gram-negative and Gram-positive (P=0.680) or between Gram-negative and no-detection (P=0.492). This might be due to the limited amount of observations on the Gram-negative group, which could be associated to the type

of mastitis investigated (Gram-positive mastitis is commonly of subclinical nature while Gram-negative is of clinical nature) .

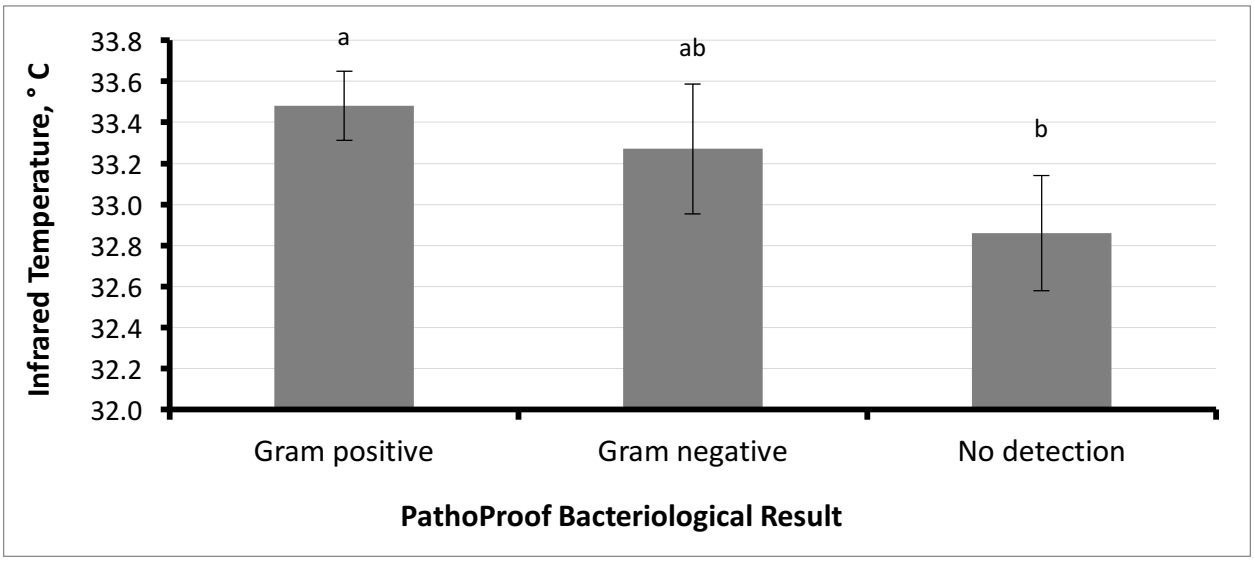

Figure 1. Mean infrared temperature of the milk ± SEM from subclinical mastitic quarters grouped by bacteriological results.

However, the authors originally hypothesized that milk IRT of quarters with Gram-negative mastitis could be higher relative to Gram-positive or healthy ones. According to Wellnitz et al. (2011), the toxins produced by Gram-negative bacteria induce a dose dependent immune response producing greater amounts of pro-inflammatory molecules such as TNF-α.

In general, immune responses associated to pathogen invasion could trigger immunological events associated with an increase in temperature at the mammary gland level (Strandberg et al., 2005; Wellnitz et al., 2011; Wellnitz and Bruckmaier, 2012). The immunological responses associated with this increment in temperature could be related

to the SCC of the mammary gland (Wellnitz et al., 2011). However, in the present study, milk SCS of mammary quarters in which no mastitic pathogens were detected, using the PtoPrf-16, were higher relative to quarters with Gram-positive mastitis (P=0.022; Table 3).

Table 3. Mean Somatic Cell Score ± SEM according to bacteriological result (n=123).

PtoPrf-16	n	Linear Score	SCC/mL
no-detection	31	7.224 ± 0.158^{a}	2,158,516 ± 185,100
Gram-positive	84	6.747 ± 0.147^{b}	1,803,678 ± 124,557
Gram-negative	8	4.932 ± 0.826^{ab}	839,750 ± 295,336

Means SCS with different letters were significantly different with a (Tukey-Kramer adjustment, P=0.022).

The higher SCS in no-detection samples could be attributed to the presence of pathogens that are not identified by the PtoPrf-16 and that can cause mastitis such as Pseudomonas, *Streptococcus parauberis, Streptococcus salivarius, Enterococcus saccharolyticus,* among others (Carrillo-Casas and Miranda-Morales, 2012). In fact, 26.7% of milk samples with >200,000 SCC had not bacterial identification using the PtoPrf-16 (Table 4). Since all the quarters included in the study had CMT scores ≥1, it is possible that remnant SCC from previous infections, or that other causes of inflammations, such as injury or chemical irritation, could have caused an increase in SCC (Stevenson, 1946). However, these statistical differences in SCS might not represent substantial changes when considering the SCC (Table 4). Additional studies should be considered to evaluate the influence of pro-inflammatory cells according to bacteriological results and their association with milk temperature.

Table 4: PtoPrf-16 bacteriological results categorized as healthy or mastitic using a cut off point of 200,000 SCC/mL.

PathoProof Mastitis Complete 16 kit				
SCC/mL	**Gram-negative**	**No-detection**	**Gram-positive**	**Total**
<200,000	3 42.86%	0 0.0%	4 57.14%	7
>200,000	5 4.31%	31 26.72%	80 68.97%	116
Total	8	31	84	123

There were no correlations between the IRT of the short milking tubes and somatic cells (SCC and SCS) or environmental parameters such as RH and AT (Table 5). The lack of association between environmental parameters can be explained by the selected milking time to measure the short milking tubes IRT, as mentioned above. In addition, the cow's physiological state, previous physical activities and other herd associated factors could also influence the IRT of the milk (Colak et al., 2008; Polat et al., 2010).

Table 5. Simple correlations between IRT, Somatic Cells and Environmental Parameters

	SCS	**CMT**	**SCC**	**RH**	**AT**
IRT	-0.13555 0.2053	-0.19348 0.0693	-0.19561 0.0662	-0.01025 0.9241	0.16841 0.1147
SCS		0.50908 <.0001	0.85122 <.0001	0.05584 0.5991	0.0796 0.4532
CMT			0.6041 <.0001	-0.11154 0.2925	-0.05122 0.6297
SCC				0.03492 0.7424	-0.0198 0.8522
RH					-0.63085 <.0001

The SCS did not affect IRT of the short milking tubes (P=0.692). Previously, Almeida (2014) found no significant correlation between IRT and SCS. Nevertheless, Polat et al., 2010 reported a positive correlations between the IRT of the udder skin surface and SCC (r=0.73). The discrepancies among these studies could be explained by the different methodologies used before collection of IR images. For instance, in the study of Polat et al., (2010), cows were moved to a room with controlled temperature for acclimatization before entering the milking parlor. Contrary to our study, every visited dairy farm had their own methodology for moving their cows to the milking parlor. In fact, another study showed that 2 h of exercise in dairy cattle increases udder skin temperature by approximately 1° C (Berry et al., 2003). Additionally, in the present study infrared images were taken from the short milking tubes relative to the udder skin surface as in Polat et al., (2010) study.

The mean IRT of the short milking tubes according to SCS categorizations are presented in Figure 2. No differences in IRT were found (P=0.167) when the SCS was categorized in SCS<4.0 (n=4), SCS>4.0≤6.0 (n=19), SCS>6.0≤8.0 (n=77) and SCS>8.0 (n=21). Although no differences were found, milk IRT through the short milking tubes from mammary quarters with SCS from 4 to 6 had 0.53° C higher temperature than those with SCS lower than 4 (33.89 vs 33.36 ° C, respectively). The limited amount of observations per SCS categorization could have accounted for this lack of statistical differences.

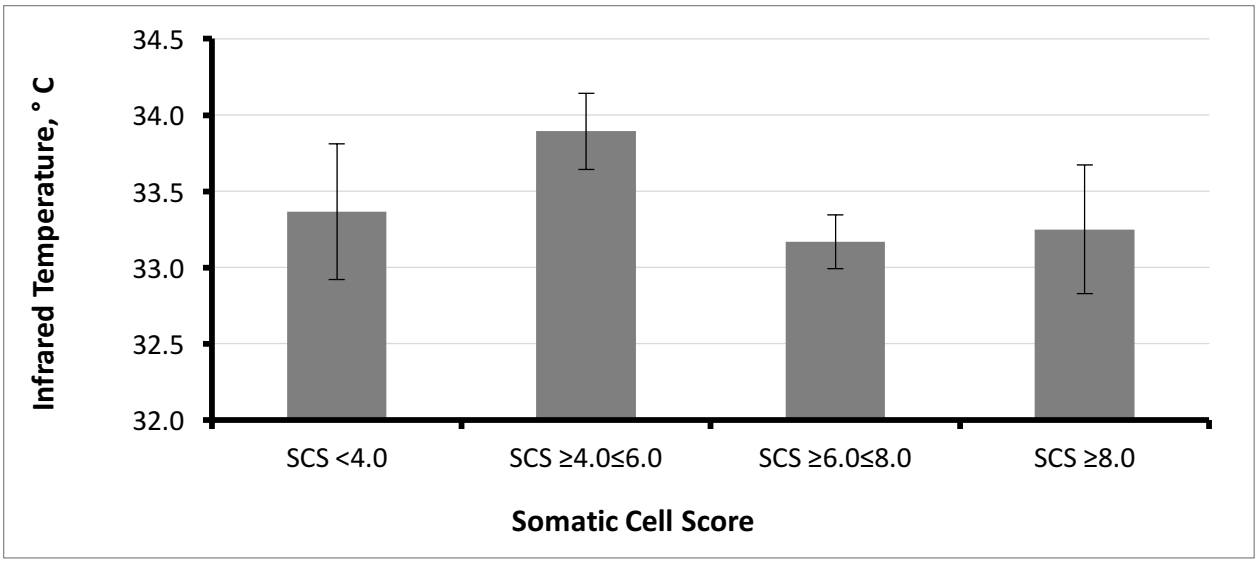

Figure 2. Infrared temperature (° C) according to somatic cell scores (SCS) categories: <4.0 (n=4), SCS>4.0≤6.0 (n=19), SCS>6.0≤8.0 (n=77) and SCS>8.0 (n=21; P=0.692).

Specificity and sensitivity of CHROMagar plates compared to the PtoPrf-16 kit

The CHROMagar results were compared with the PtoPrf-16 to calculate its specificity and sensitivity using contingency tables (Table 6). Colonies with colors differing from the CHROM colony identification legend were not further identified with differential tests. This discrepancy could have been caused by the growth of pathogens not included in CHROM targets. However, according to the manufacturer, light exposure may affect the chromogenic media and consequently change the resulting colony color (CHROMagar, 2017).

Table 6. Contingency table of CHROMagar vs. PathoProof-16 results.

PathoProof-16	**CHROMagar**			Total
	Gram-positive	Gram-negative	No-detection	
Gram-positive	46 (73.0%)	11	6	63
Gram-negative	2	1 (20.0%)	2	5
no-detection	10	1	12 (52.2%)	23
Total	58	13	20	91 100%

In this study, the CHROM had a combined specificity (Gram-negative and Gram-positive plates) of 52.17 % and a sensitivity of 73.02 % for Gram-positive plates and 20.0 % for Gram-negative plates, resulting in a combined sensitivity for mastitic pathogens of 70.5%, when using the PtoPrf-16 as a comparative test. Differences in bacteriological results among these tests could be attributed to several factors. As mentioned above, 26.7% of mastitic quarters (>200,000 SCC/mL) had no bacterial detection when using the PtoPrf-16.. Hence, the use of tests such as gene sequencing analysis to identify specific mastitic pathogens should be considered for future validation studies.

To our knowledge, the evaluation of the CHROM plates has not been published before. The main purpose of evaluating the capacities of detection of mastitic pathogen using the CHROM, according to molecular testing (PtoPrf-16), was to provide suggestions for local dairy farmers about strategies towards mastitis precision treatment and its benefits. Some of the benefits of using CHROM plates as an on-farm mastitis detection tool are the identification of Gram-positive mastitis cases, which in this study had a sensitivity

of 73.0 %. This tool could be of benefit to farmers that tend to treat mastitis cases indiscriminately. In fact, the predominant mastitic pathogens detected in the dairy farms included in the study were S*. aureus,* CNS and *C. bovis* (Table 7). However, farmers should be trained in strategies for aseptic milk sample collection, management, and culturing techniques in order to reduce false positive results.

Table 7. Mastitic pathogen in quarter milk samples and their frequencies, using PathoProof Mastitis Complete 16 kit.

Gram-positives	Frequency	Gram-negatives	Frequency
S. aureus	42	*Coliforms*	8
Coagulase Negative Staph.	24	-	-
C. bovis	17	-	-
S. dysgalacteae	4	-	-
Enterococcus	3	-	-
S. uberis	2	-	-

Conclusions

- The higher IRT of the short milking tubes observed in gram-positive mastitic quarters relative to no-detection could be attributed to an active mastitis infection (confirmed by the presence of bacteria in the mammary quarters), causing a greater degree of infiltration of pro-inflammatory components and a greater milk temperature.

- The lack of association between SCC and IRT of the short milking tube could be attributed to the stage of mastitis of experimental quarters, the limited lack of observations in each SCS classification and/or due to the fact that pro-inflammatory cells are not accounted in the SCC.

- The IRT of the short milking tube was not affected by AT or RH when collecting IRT images during milking from 0200 to 0600.

- For future experiments, identification of pro-inflammatory molecules such as IL-8 or TNFα in mastitic milks could provide a better understanding of the interaction between IRT, SCS and mastitis pathogens.

- The high incidence of contagious mastitic pathogens such as *S. aureus, SCN and C. bovis* isolated from the mammary gland quarters included in the study, insinuates that milking procedures should be improved and taken into constant consideration.

- The results obtained in this study indicate that the use of IRT of the short milking tube could represent a practical discriminatory tool for mastitis detection.

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