Application of udder surface temperature by infrared thermography for diagnosis of subclinical mastitis in Holstein cows located in tropical highlands

Juan Velasco-Bolaños,^{1,2*} Cristian C. Ceballes-Serrano,³ Daniel Velásquez-Mejía,¹ Juan Carlos Riaño-Rojas,³ Carlos E. Giraldo,⁴ Jorge U. Carmona,⁴ and Alejandro Ceballos-Márquez^{1,2}

¹Grupo de Investigación en Calidad de Leche y Epidemiología Veterinaria (CLEV), Departamento de Producción Agropecuaria, Universidad de Caldas, Calle 65 No 26-10, 170004 Manizales, Colombia

²Grupo de Investigación en Biología de la Producción Pecuaria, Departamento de Producción Agropecuaria, Universidad de Caldas, Calle 65 No 26-10, 170004 Manizales, Colombia

³Computational Applications Group, Departamento de Matemática y Estadística, Universidad Nacional de Colombia, Carrera 27 No 64-60, 170004 Manizales, Colombia

⁴Grupo de Investigación Terapia Regenerativa, Departamento de Salud Animal, Universidad de Caldas, Calle 65 No 26-10, 170004 Manizales, Colombia

ABSTRACT

Several reports have indicated that udder surface temperature (UST) can be a useful indicator of subclinical mastitis (SCM). The objective was to evaluate UST by infrared thermography (IRT) as a diagnostic tool for SCM and intramammary infection (IMI), and to assess the influence of environmental conditions in the potential diagnosis of this disease in dairy cows located at high-altitude tropical regions. A total of 105 cows (397 quarters) from 3 dairy farms with mechanical and manual milking methods were enrolled in the study. Subclinical mastitis was diagnosed when quarter samples had a somatic cell count (SCC) $\geq 200 \times 10^3$ cells/mL, microbial growth (MG) was defined when a major pathogen (≥ 1 cfu/plate) or *Corynebacterium* spp. (≥ 10 cfu/plate) was isolated, and IMI was defined as the presence of MG and SCC $\geq 100 \times 10^3$ cells/mL. Infrared images were taken with a thermal camera placed 1 m away from the udder, and shots of the rear and left and right lateral view were made during the morning milking, before any manipulation of the udder and employing dark cardboard on the contralateral side to avoid artifacts in the background. A multilevel mixed effects linear regression model clustered within cows and herd was performed to evaluate the associations with UST. Clinical performance was evaluated using the Youden index to establish the optimum UST thresholds, which were set at 32.6°C for any case definition when milking was by hand, at 33.7°C for MG, and at 34°C for SCM and IMI in machine-milked

quarters. Sensitivity (Se), specificity (Sp), area under curve (AUC), and positive likelihood ratio (+LR) were also assessed. Test agreement was assessed by kappa coefficient (κ). The UST of healthy quarters ranged between (95% CI) 32.4 and 32.6°C, lower than SCM quarters (n = 88) at 32.9°C (95% CI: 32.7–33.1°C), MG quarters (n = 56) at 33.5°C (95% CI: 33.3–33.7°C), and IMI quarters (n = 50) at 33.5°C (95% CI: 33.2–33.7°C). The UST was also related to the milking method: higher temperatures were observed for hand milking (n = 90) compared with machine milking (n = 185). No relation between environmental conditions such as wind speed, atmospheric temperature, relative humidity, and temperature-humidity index and UST were observed during this study. For hand milking, the optimal UST threshold was 32.6°C; for SCM, Se = 0.53, Sp = 0.89, AUC = 0.71, κ = 0.4; for MG, Se = 0.83, Sp = 0.93, AUC = 0.88, κ = 0.77; and for IMI, Se = 0.82, Sp = 0.92, AUC = 0.87, κ = 0.74. The machine milking threshold for SCM resulted in Se = 0.42, Sp = 0.97, AUC = 0.70, κ = 0.47; for MG, Se = 0.82, Sp = 0.89, AUC = 0.85, κ = 0.60; and for IMI, Se = 0.82, Sp = 0.98, AUC = 0.90, κ = 0.79. These findings suggest that UST determined by IRT is higher in machine-milked cows and in quarters with MG and IMI than in healthy quarters; therefore, UST by IRT is a reliable, clinically useful method for MG and IMI diagnosis.

Key words: dairy cow, intramammary infection, somatic cell count, thermal camera

INTRODUCTION

Mastitis is one of the most common disease of dairy cattle worldwide. It affects milk yield and quality, which leads to a lower economic return and affects farm sustainability. Many etiological agents may cause mastitis,

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*Corresponding author: juan.velasco@ucaldas.edu.co

such as *Staphylococcus aureus*, *Streptococcus agalactiae*, and *Streptococcus uberis*, among others (Gonçalves et al., 2018). In response to udder infection, a neutrophilic migration occurs from the blood to the milk, with a final increase of SCC in the milk (Harmon, 1994).

Mastitis may be classified as subclinical (SCM) or clinical, according to whether clinical signs are observed or not. The mammary gland with clinical mastitis can show signs, such as edema, tumefaction, pain, warmth, redness, and physical changes in the milk (Moreira et al., 2018). By contrast, the subclinical form is difficult to detect without any test. Currently, several on-farm tests, such as the Wisconsin Mastitis Test and the California Mastitis Test, can help in diagnosing. However, the interpretation of these tests is mainly subjective and requires trained technicians to avoid variation in results (Dingwell et al., 2003; Sanford et al., 2006; Riggio et al., 2013; Ruegg, 2017; Moroni et al., 2018). The SCC is described as a useful SCM detection method, but it uses different thresholds that represent different sensitivity (Se) and specificity (Sp) levels, and the results can be affected by several physiological factors, such as milk production level, breed, season, lactation number, and stage of lactation (Viguier et al., 2009; Jashari et al., 2016). Therefore, the definition of IMI differs from that of SCM, because IMI combines the SCC with the isolation of a pathogenic microorganism from the milk (Pyörälä, 2003).

Infrared thermography (IRT) is a passive, remote, and noninvasive technique that allows one to obtain the surface temperature of a body through the infrared radiation of the electromagnetic spectrum that it emits, wherein each pixel corresponds to a temperature intensity, where warmest areas appear white or red and coolest areas are blue or black (Eddy et al., 2001; Polat et al., 2010; Usamentiaga et al., 2014). Briefly, this diagnostic tool is based on the principles of infrared radiation, which has a longer wavelength than visible light and a shorter wavelength than microwaves. Its wavelengths range between 0.7 and 1,000 μm , and it is invisible to the human eye. All objects whose temperature is above absolute zero (-273.15°C) emit infrared radiation proportional to their temperature, according to the Stefan-Boltzmann law via conduction convection and radiation; even cold objects emit infrared radiation, which can be absorbed, emitted, reflected, or transmitted. In that order, based on the amount of heat generated, thermal cameras acquire and process radiation and display thermal images, called thermograms (Eddy et al., 2001).

It has been described that IRT can be used for determining some physiological parameters such as breathing, stress, or estrus in cattle (Stewart et al., 2007, 2017; Talukder et al., 2014), skin and hoof in-

juries (Nikkhah et al., 2005; Poikalainen et al., 2012), and subclinical diseases in calves (Schaefer et al., 2004; Lowe et al., 2016). Previous research shows that IRT can be useful to determine variations in the udder surface temperature (UST) related to milking, environmental conditions, and exercise (Berry et al., 2003), but also, IRT has had some encouraging results for the identification of mastitis, either subclinical or clinical, due to the ability of IRT to perceive changes in the UST in response to different mammary gland infections (Colak et al., 2008; Hovinen et al., 2008; Polat et al., 2010; Zaninelli et al., 2018).

The aim of this study was to evaluate the UST by IRT as a diagnostic tool for SCM and IMI, and to assess the influence of environmental conditions in the potential diagnosis of these conditions in Holstein dairy cows in herds located at high-altitude tropical regions.

MATERIALS AND METHODS

The committee on animal experimentation of the Universidad de Caldas, Manizales, Colombia, approved the protocols for image acquisition and sample collection. The cows employed in this study were used under authorization of their owners after signing an informed consent form. The personnel in charge of thermography did not have access to the results of milk analyses and vice versa; both were processed separately, and only the personnel in charge of the statistical analyses had access to the merged database.

Farms and Animals

A quarter-level analytical cross-sectional study was conducted between March and June of 2017. A total of 105 Holstein cows (397 quarters and 23 nonfunctional or blind quarters) in their first to ninth lactation were selected from 3 dairy herds in Villamaría county ($5^\circ 02' 44''\text{N}$ $75^\circ 30' 55''\text{W}$), Caldas, Colombia. Herds were located at 2,100 m above sea level. Cows were managed under rotational grazing systems and received supplementation with concentrates according to milk yield. Predominant pasture was Kikuyu grass (*Pennisetum clandestinum*). Mineral supplements and water were available ad libitum.

Animals were milked twice a day, during the morning between 0200 and 0700 h, and in the afternoon between 1230 and 1700 h. Milk yield average was 17.8 L/cow per day (95% CI: 16.7–18.6 L/cow per d), and cows were in late lactation, 205 DIM (95% CI: 185–226 DIM). Milking was performed on the left or right side of the cow indiscriminately, depending on the side of the tent selected by the cow to enter (Figure 1). Additional data and environmental conditions are shown in Table 1.

Table 1. Number of cows, quarters, and cases, and average and SE for milk yield, DIM, and environmental conditions at morning milking by farm; ranges in parentheses indicate 95% CI

Item	Farm			Overall
	A	B	C	
Herd information				
Milkings/cow per day	2	2	2	2
Herd size	44	21	40	105
Quarters included	90	60	125	275
Right front	26	12	34	72
Left front	24	15	25	64
Right rear	21	17	33	71
Left rear	19	16	33	68
Milking method	Hand	Machine	Machine	—
Milking location	Paddock	Paddock	Paddock	—
Milk yield (L/cow per day)	20.1 (18.6–21.6)	17.8 (14.3–21.3)	16.0 (15.0–17.1)	17.8 (16.7–18.6)
DIM	252 (211–293)	245 (172–316)	165 (146–184)	205 (185–226)
Environment				
Wind speed (m/s)	0.10 (0.03–0.18)	0.01 (0.00–0.01)	0.06 (0.02–0.10)	0.06 (0.03–0.09)
Atmospheric temperature (°C)	19.1 (18.3–20.0)	15.2 (15.0–15.4)	15.6 (15.3–15.9)	16.7 (16.3–17.0)
Relative humidity (%)	70.7 (67.6–73.9)	89.6 (88.8–90.4)	91.2 (90.6–91.8)	84.2 (82.6–85.7)
Temperature-humidity index (%)	64.5 (63.4–65.6)	59.3 (59.0–59.6)	59.9 (59.4–60.4)	61.3 (60.8–61.8)
Number of cases (quarters)				
Subclinical mastitis	45	14	29	88
Microbial growth ¹	29	10	17	56
IMI	28	10	12	50

¹Microbial growth case definition includes major pathogens (≥ 1 cfu) and *Corynebacterium* spp. (>10 cfu).

Inclusion Criteria

At the beginning of the study, all cows were clinically evaluated to determine their health status. Only healthy lactating cows that had experienced no cases of clinical mastitis in the current lactation and that had received no treatment with antibiotics for any other disease in the last 30 d were included. Cows with concomitant infectious diseases or showing inflammation, tumefaction, redness, or any alteration in the udder tissue, as well as visible changes in the milk such as the presence of flakes, clots, blood, or serous milk, were not included.

Figure 1. Diagram of temporary milking tent. Dimensions: 4 × 3 × 2 m.

Cow Preparation for IRT and Milk Sample Collection

The udder surface of each cow was inspected for any external artifact that could affect the surface temperature, such as dirt, mud, grass, or manure that could add cold spots in the thermogram. No IRT were taken during fog or rain, because this generates a wet layer on the skin and hair of the cows that blocks the infrared radiation.

A quarter-level milk sample for bacteriological and SCC measurement was collected from every cow just before it was milked in the morning and after the IRT image was captured. For sample collection the first strips of milk were discarded, and then teat ends were disinfected using gauze soaked in alcohol before the samples were taken. A 50-mL cisternal milk sample was collected in plastic sterile tubes. Later, in the laboratory, the sample was split into 2 aliquots: one was used for microbiological diagnosis, and the other had a bronopol tablet added and was shipped under refrigeration to another laboratory for SCC measurement using an automated cell counter (Fossomatic, Foss). The SCC results were transformed into the natural logarithm (LnSCC , $\times 10^3$ cells/mL) to normalize data distribution.

IRT Protocol

Infrared images were taken only during the morning milking before the clinical examination, and before the shot, any manipulation of the udder was avoided.

A FLIR T450sc thermal camera (FLIR Systems Inc.) on a tripod was employed, located 1 m away from the objective. Also, any reflective objects in the visual field and direct sunlight were avoided. The udders were photographed under a temporary homemade tent, consisting of a plastic roof and 6 pillars without walls (dimensions $4 \times 3 \times 2$ m; Figure 1), while cows were fed with concentrate. The images were taken from the rear and lateral view. The rear image, employed for the rear quarters, was taken before the legs were tied, to obtain a better amplitude; the tail was moved to one side, so it would not interfere with the image (Figure 2, left-hand panel). The lateral view (right and left) was used only for the front quarters, and IRT was captured after tying the legs and using a dark piece of cardboard on the contralateral side, covered with a dark cloth, to avoid artifacts in the background (Figure 2, right-hand panel). Photographs were taken in the total absence of fog, to avoid mistakes in the UST measures.

To measure the UST, it was necessary to set the emissivity, relative humidity, and reflected temperature in the camera, to compensate for the effects on the udder caused by different radiation sources (Testo SE and Co., 2017).

Determination of Udder Temperature

Because the camera takes an image of the entire udder (field of view), and each pixel represents a different temperature, it is necessary to determine an average temperature for the udder. Thus, the infrared images captured by the camera were exported to the computer

using FLIR Tools 5 software (FLIR Systems Inc.), after registering the emissivity, reflected temperature, distance, atmospheric temperature, and relative humidity. Later, using the ResearchIR 4.20 software (FLIR Systems Inc.), each image was upload to MatLab 2014a (MathWorks). In this software, using the tool *roipoly*, a manual polygonal segmentation of the region of interest was performed, resulting in a mask for the image (Figure 3); then the mask is multiplied by the original rainbow-patterned IRT, and, over the resulting region, the average, median, mode, maximum, minimum, and standard deviation of the UST were calculated by the software. Photographs of udders that show cold spots due to the presence of mud, grass, or excessive hair were removed from the database and not analyzed.

During the process, relative humidity (%), atmospheric temperature ($^{\circ}\text{C}$), and wind speed (m/s) were measured using a thermo-hygro-anemometer (Extech 45160 3-in-1, Extech Instruments). Temperature-humidity index (**THI**) was calculated using the equation $\text{THI} = (0.8 \times \text{atmospheric temperature}) + [(\% \text{ relative humidity} \div 100) \times (\text{atmospheric temperature} - 14.4)] + 46.4$, described by Mader et al. (2006).

Milk Analysis

Microbiological diagnosis was performed according to the National Mastitis Council (Hogan et al., 1999). Milk was streaked with a sterile cotton swab into blood esculin (5% of sheep blood and 0.1% of esculin) and Edwards modified medium. Afterward, plates were incubated at 37.5°C and visually inspected at 24 and 48

Figure 2. Infrared thermography of the udder surface temperature. Left-hand panel: rear view; right-hand panel: lateral view.

h for bacterial growth. Morphology and gram-positive bacteria were identified as follows. The catalase test was used to differentiate staphylococci from streptococci. The coagulase test was performed on catalase-positive cocci to confirm *Staph. aureus*. Catalase-negative cocci were differentiated by their reaction to the hydrolysis of esculin under UV light. The Christie–Atkins–Munch–Petersen test and esculin reaction were used to differentiate *Strep. agalactiae* and *Streptococcus dysgalactiae*, and esculin reaction and culture in enterococcosel agar were used for the identification of *Strep. uberis*. Morphology and Gram staining were used to identify *Corynebacterium* spp. Those cultures that showed more than 2 bacterial species were considered contaminated and not informative of IMI. Mixed infections were those with growth of 2 bacterial species. “No significant growth” was established when a milk sample yielded fewer than 3 colonies and no major pathogens were isolated. *Staphylococcus aureus*, *Strep. agalactiae*, *Strep. dysgalactiae*, and *Strep. uberis* were considered as major pathogens (Barkema et al., 1997; Riffon et al., 2001).

Case Definition

Three case definitions were employed. A case of SCM was diagnosed when a quarter sample had SCC ≥ 200

$\times 10^3$ cells/mL (Schukken et al., 2003; Dohoo et al., 2011). A microbial growth case (MG) was defined when a microbiological culture was positive for a major pathogen (≥ 1 cfu/plate) or *Corynebacterium* spp. (≥ 10 cfu/plate; Gonçalves et al., 2018). Finally, IMI was defined as the presence of MG and SCC $\geq 100 \times 10^3$ cells/mL (Hamann, 2003; Pyörälä, 2003). Quarters without a case of SCM, MG, or IMI were analyzed as a control group.

Statistical Analysis

The association of UST and environmental and microbiological variables was explored through a multilevel mixed effects linear regression model clustered within cows and herd. All the bacteria found and the variables shown in Table 1 (categorical variables: milking method, milking location; continuous variables: herd size, milk yield, DIM, wind speed, atmospheric temperature, relative humidity, and THI) were evaluated as model predictors. Three models were assessed according to each case definition; thus, an unconditional analysis was conducted, and variables with $P \leq 0.15$ were included in the initial model. The final model was built with backward manual elimination procedure with significance level ≤ 0.05 as cutoff to be retained

Figure 3. Region of interest of the left rear (left-hand panel) and right front (right-hand panel) quarters of the udder. The light patch corresponds to the manual segmentation (mask).

in the model. The model was evaluated via analysis of the residuals at the lowest level (quarter). Milking method was one of the factors influencing the outcome; hence, analyses including all cow quarters milked by hand were performed separately from machine-milking quarters.

Sensitivity was calculated as the probability of a positive test result given that the disease is present, and Sp as the probability of obtaining a negative test result given that the disease is absent (Dohoo et al., 2009; Ying et al., 2020). For several cutoff points, the Youden index (**YI**) was calculated as the maximum expression for $Se + Sp - 1$, and the value of the cutoff point that achieved the maximum YI was considered the optimal threshold (Fluss et al., 2005; Kandeel et al., 2019). For the same cutoff points mentioned, the positive likelihood ratio (**+LR**) was obtained by the computation of $Se/(1 - Sp)$; values from 5 to 10 represent a moderate effect on post-test probability, and $+LR > 10$ were considered to be good (or large), increasing the post-test probability of diagnosis (Hayden and Brown, 1999; Grimes and Schulz, 2005). Also, the area under the curve (**AUC**) was obtained for several cutoff points. According to the YI, the UST threshold was selected, and receiver operating characteristic (**ROC**) curves were calculated for each case definition threshold and were compared using a nonparametric test for equality for ROC AUC (DeLong et al., 1988). Finally, the Cohen's kappa coefficient (κ) was calculated to determine the agreement beyond chance among the optimum cutoff points for each case definition and the IRT. The SCM case definition (microbiological analysis and SCC measurement) was considered as a reference method. A κ value between 0 and 0.2 was

considered slight, 0.2 to 0.4 fair, 0.4 to 0.6 moderate, 0.6 to 0.8 substantial, and >0.8 excellent agreement (Landis and Koch, 1977). The positive and negative predictive values are not reported, due to their relation with prevalence in the population included in the study (Dohoo et al., 2009).

Somatic cell count values were log-transformed during analyses and back-transformed to be presented as regular units (cells/mL). All the analyses were carried out using a statistical software epidemiological functions (*xtmixed*, *ttest*, *diagt*, *roccomp*, *rocfitt*, *kap*, and *kapci*; Stata 14.2, Stata Corp.), and statistical significance was declared at $P < 0.05$.

RESULTS

A total of 275 (69.3%) quarters were included and analyzed in the study; 122 (30.7%) were not analyzed in this study because cows or udders were in contact with mud during rainy season and udders were not entirely clean. Dirt or mud add cold spots to the IRT and alter the results obtained with this technology.

Microbiological diagnosis showed that CNS were the most frequent bacteria ($n = 53$, 19.3%), followed by *Staph. aureus* ($n = 24$, 8.7%), *Strep. uberis* ($n = 22$, 8.0%), and *Corynebacterium* spp. ($n = 20$, 7.3%; Table 2). Major pathogens were isolated in 45 samples (16.4%). We detected no difference among the SCM cases by quarters: right front 15.3% (95% CI: 6.8–24.8%), left front 20.3% (95% CI: 10.2–30.4%), right rear 21.1% (95% CI: 11.4–30.9%), left rear 13.2% (95% CI: 5.0–21.5%; $P = 0.55$). The average of LnSCC was 4.6×10^3 cells/mL (95% CI: 4.4 – 4.8×10^3 cells/mL; equivalent to 102,500 cells/mL).

Table 2. Microbiological diagnosis of 275 quarter milk samples grouped by the 3 case definitions, and SCC mean (with 95% CI)¹

Microbiologic diagnosis	Isolates, n (%)	SCC, mean (95% CI)	Cases, ² n		
			SCM	MG	IMI
Major pathogens					
<i>Staphylococcus aureus</i>	24 (8.7)	756 (462–1,234) ^a	23	24	23
<i>Streptococcus agalactiae</i>	1 (0.4)	567 ³	1	1	1
<i>Streptococcus uberis</i>	22 (8.0)	1,094 (544–2,199) ^a	20	22	20
Other pathogens					
<i>Corynebacterium</i> spp.	20 (7.3)	90 (50–164) ^b	6	11	8
<10 cfu	9 (3.3)	41 (21–79) ^d	0	0	8
≥10 cfu	11 (4.0)	174 (78–386) ^b	6	11	0
CNS	53 (19.3)	134 (81–219) ^b	22	0	0
<i>Streptococcus</i> spp.	10 (3.6)	801 (223–2,884) ^{ac}	7	0	0
Mixed infections	125 (45.5)	67 (37–122) ^{ac}	11	8	8
No growth or no significant growth	158 (57.5)	52 (42–64) ^d	20	0	0

^{a–d}Bacterial species with different superscript letters differ in LnSCC ($P < 0.05$).

¹SCC mean and 95% CI values are presented back-transformed from the natural logarithm of SCC (LnSCC).

²MG = microbial growth; SCM = subclinical mastitis.

³The CI could not be calculated for *Strep. agalactiae* because there was only 1 sample.

Table 3. Final mixed effects linear regression model of surface udder temperature (°C) by infrared thermography, clustered by cows and herds

Predictor ¹	Coefficient	SE	P-value	95% CI	
Case definition, SCM: SCC > 200 × 10 ³ cells/mL					
Milking method	1.06	0.14	0.00	0.78	1.34
Major pathogens	1.16	0.12	0.00	0.93	1.40
Intercept	31.72	0.12	0.00	31.49	31.95
Random effects parameters					
Herd (variance)	0.00	0.00		0.00	6.65
Cow (variance)	0.35	0.07		0.23	0.53
Residual (variance)	0.35	0.04		0.28	0.44
Case definition, MG: major pathogens (1 cfu/mL) and <i>Corynebacterium</i> spp. (>10 cfu/mL)					
Milking method	0.98	0.14	0.00	0.71	1.25
MG cases	1.07	0.11	0.00	0.86	1.29
Intercept	31.75	0.11	0.00	31.53	31.97
Random effects parameters					
Herd (variance)	0.00	0.00		0.00	1,524.22
Cow (variance)	0.31	0.07		0.20	0.48
Residual (variance)	0.36	0.04		0.29	0.45
Case definition, IMI: SCC >100 × 10 ³ cells/mL and MG					
Milking method	0.99	0.14	0.00	0.71	1.27
IMI cases	1.10	0.12	0.00	0.87	1.33
Intercept	31.76	0.12	0.00	31.53	31.99
Random effects parameters					
Herd (variance)	0.00	0.00		0.00	66,097.91
Cow (variance)	0.35	0.07		0.23	0.52
Residual (variance)	0.36	0.04		0.29	0.44

¹SCM = subclinical mastitis; MG = microbial growth.

Subclinical Mastitis, Microbial Growth, and IMI

Subclinical mastitis was diagnosed in 45 cases in farm A (manual milking) and 43 cases in farms B and C (machine milking), for a total of 88 cases according to the definition in the Materials and Methods section. Average SCC for quarters with SCM was 874.6×10^3 cells/mL (95% CI: 696.8 – $1,097.7 \times 10^3$ cells/mL), against 37.5×10^3 cells/mL (95% CI: 32.8 – 42.9×10^3 cells/mL) in healthy quarters. Microbial growth cases were observed in 56 quarters; of those, 29 occurred in hand-milked quarters and 27 in machine-milked quarters. The SCC in MG quarters was 647.5×10^3 cells/mL (95% CI: 434.7 – 964.1×10^3 cells/mL), against 64.1×10^3 cells/mL (95% CI: 52.5 – 78.4×10^3 cells/mL) in quarters without MG. Finally, IMI was declared in 50 cases: 28 on farm A, and 22 on farms B and C. Average SCC in cows with IMI was 915.4×10^3 cells/mL (95% CI: 661.3 – $1,267.3 \times 10^3$ cells/mL), against 63.2×10^3 cells/mL (95% CI: 51.9 – 76.8×10^3 cells/mL) in healthy quarters. In addition, SCC according to the microorganism isolated is presented in Table 2.

IRT and Mixed Effects Linear Regression Model

The average UST of cow quarters enrolled in the study was 32.6°C (95% CI: 32.5–32.8°C), whereas cow quarters with SCM had 32.9°C (95% CI: 32.7–33.1°C), MG cases 33.5°C (95% CI: 33.3–33.7°C), and IMI UST

was 33.5°C (95% CI: 33.2–33.7°C). Additionally, UST of quarters without a case declaration ranged between (95% CI) 32.4 to 32.6°C ($P < 0.001$). The final mixed effects linear regression models, one for each case declaration, included as predictor the milking method and the case declaration in MG and IMI; however, SCM was the only case declaration that was not retained in the final model, being replaced by the presence of major pathogens. The coefficients of the final models are shown in Table 3. No environmental variables were found related to UST.

Receiver Operating Characteristics

The optimum cutoff point selected for the UST in hand-milked quarters was 32.6°C for all case definitions. At this UST threshold, SCM had Se = 0.53, Sp = 0.89, AUC = 0.71, YI = 0.42, and +LR = 4.6; MG values were Se = 0.83, Sp = 0.93, AUC = 0.88, YI = 0.76, and +LR = 12.6; and IMI had Se = 0.82, Sp = 0.92, AUC = 0.87, YI = 0.74, and +LR = 10.2 (Table 4). In addition, optimum thresholds for UST in machine milking were different according to the case definition. The SCM and IMI threshold was 34.0°C, and MG was set at 33.7°C. In this respect, SCM values were Se = 0.42, Sp = 0.97, AUC = 0.70, YI = 0.39, and +LR = 14.9; MG had Se = 0.82, Sp = 0.89, AUC = 0.85, YI = 0.70, and +LR = 7.15; and IMI had Se = 0.82, Sp = 0.98, AUC = 0.90, YI = 0.79, and +LR = 33.3. This information

Table 4. Sensitivity, specificity, area under the curve (AUC), Youden index (YI), and positive likelihood ratio (+LR) at different udder surface temperature cutoff points (°C) by infrared thermography, grouped by 3 case definitions

Cutoff point (°C)	Sensitivity (95% CI)	Specificity (95% CI)	AUC (95% CI)	YI	+LR
Hand-milking method					
Subclinical mastitis					
32.2	0.644 (0.488–0.781)	0.600 (0.443–0.743)	0.622 (0.521–0.723)	0.244	1.61
32.4	0.578 (0.422–0.723)	0.800 (0.654–0.904)	0.689 (0.595–0.783)	0.378	2.89
32.6 ¹	0.533 (0.379–0.683)	0.889 (0.759–0.963)	0.711 (0.620–0.797)	0.422	4.60
32.8	0.444 (0.296–0.60)	0.933 (0.817–0.986)	0.688 (0.607–0.771)	0.377	6.67
33.0	0.289 (0.164–0.443)	0.955 (0.845–0.994)	0.622 (0.548–0.696)	0.244	6.36
Microbial growth					
32.2	0.931 (0.772–0.992)	0.672 (0.54–0.787)	0.802 (0.726–0.877)	0.603	2.84
32.4	0.862 (0.683–0.961)	0.836 (0.719–0.918)	0.849 (0.770–0.928)	0.698	5.26
32.6 ¹	0.828 (0.642–0.934)	0.934 (0.841–0.982)	0.881 (0.804–0.958)	0.762	12.6
32.8	0.724 (0.528–0.879)	0.967 (0.887–0.996)	0.846 (0.760–0.931)	0.691	22.1
33.0	0.483 (0.294–0.675)	0.983 (0.911–1.00)	0.733 (0.639–0.827)	0.466	29.0
IMI					
32.2	0.929 (0.765–0.991)	0.661 (0.530–0.780)	0.795 (0.718–0.872)	0.59	2.74
32.4	0.857 (0.673–0.96)	0.823 (0.705–0.908)	0.840 (0.758–0.921)	0.68	4.83
32.6 ¹	0.821 (0.631–0.939)	0.919 (0.822–0.973)	0.870 (0.790–0.950)	0.74	10.20
32.8	0.714 (0.513–0.868)	0.952 (0.865–0.990)	0.833 (0.744–0.922)	0.666	14.80
33.0	0.464 (0.275–0.661)	0.967 (0.887–0.996)	0.716 (0.619–0.812)	0.431	14.20
Machine-milking method					
Subclinical mastitis					
33.6	0.535 (0.377–0.688)	0.838 (0.767–0.894)	0.687 (0.605–0.768)	0.373	3.30
33.8	0.488 (0.333–0.645)	0.901 (0.840–0.945)	0.695 (0.615–0.774)	0.389	4.95
34.0 ¹	0.419 (0.27–0.579)	0.972 (0.929–0.992)	0.696 (0.619–0.771)	0.391	14.9
34.2	0.256 (0.135–0.412)	0.972 (0.929–0.992)	0.614 (0.619–0.771)	0.228	9.08
34.4	0.190 (0.086–0.341)	0.986 (0.950–0.998)	0.588 (0.527–0.649)	0.176	13.5
Microbial growth					
33.6	0.815 (0.619–0.937)	0.848 (0.782–0.9)	0.832 (0.752–0.911)	0.663	5.36
33.7 ¹	0.815 (0.619–0.937)	0.886 (0.826–0.931)	0.851 (0.772–0.929)	0.701	7.15
33.8	0.778 (0.577–0.911)	0.911 (0.856–0.951)	0.845 (0.762–0.928)	0.689	8.78
33.9	0.704 (0.498–0.862)	0.962 (0.928–0.99)	0.833 (0.747–0.925)	0.666	22.2
34.0	0.704 (0.498–0.862)	0.981 (0.946–0.996)	0.843 (0.754–0.931)	0.685	37.1
IMI					
33.6	0.864 (0.651–0.971)	0.834 (0.768–0.888)	0.849 (0.770–0.928)	0.698	5.21
33.8	0.864 (0.651–0.971)	0.902 (0.845–0.943)	0.883 (0.806–0.960)	0.766	8.80
34.0 ¹	0.818 (0.597–0.948)	0.975 (0.938–0.993)	0.897 (0.813–0.980)	0.793	33.3
34.2	0.500 (0.282–0.718)	0.975 (0.938–0.993)	0.738 (0.630–0.845)	0.475	20.4
34.4	0.381 (0.181–0.616)	0.988 (0.956–0.999)	0.685 (0.578–0.791)	0.369	31.0

¹Cutoff point with the largest AUC and YI values in each category.

is expanded in Table 4. In addition, the Se-Sp plots of maximum Se and Sp obtained for each case definition are presented in Figure 4. Furthermore, the ROC analysis of selected thresholds showed that SCM AUC was significantly lower than those of MG and IMI ($P < 0.001$) for hand and machine milking; however, AUC of MG and IMI were similar for both milking methods ($P > 0.05$; Figure 5).

Agreement of the Tests

The observed agreements between SCM, MG, and IMI case definitions methods and the UST obtained by IRT with a cutoff point equal to 32.6°C for hand milking were 70.0, 90.0, and 88.9%, respectively. In that order, the math for Cohen's kappa coefficient showed moderate agreement between UST and SCM ($\kappa = 0.40$; 95% CI: 0.23–0.58) and substantial agreement in MG

($\kappa = 0.77$; 95% CI: 0.63–0.91) and IMI ($\kappa = 0.74$; 95% CI: 0.59–0.89) case definitions with UST. In the same way, in quarters milked by machine, the diagnosis of SCM and IMI using UST with a cutoff point of 34.0°C showed observed agreement of 84.3% and 95.7%, respectively. Hence, SCM ($\kappa = 0.47$; 95% CI: 0.31–0.62) showed moderate agreement, whereas IMI agreement was substantial ($\kappa = 0.79$; 95% CI: 0.66–0.93). The MG observed agreement was 87.6%, with moderate agreement between the tests ($\kappa = 0.60$; 95% CI: 0.45–0.75). All κ were higher than would be expected due to chance ($P < 0.001$).

DISCUSSION

As mentioned, several studies have evaluated IRT as a diagnostic tool for mastitis, either clinical, sub-clinical, or IMI, in dairy cattle. However, these studies

Figure 4. Sensitivity (dashed line) and specificity (dotted line) plot (with 95% CI; gray lines) of udder surface temperature (°C) as a predictor of subclinical mastitis according to 3 case definitions, when milking method was by hand (left-hand column) or machine (right-hand column).

were performed under weather-controlled conditions in milking parlors, which limits the practical value in field conditions (Colak et al., 2008; Polat et al., 2010). In line with this, we intend to perform a study in the usual environmental and topographical conditions in which our dairy cattle are routinely milked and managed.

The most frequently detected bacteria were CNS; despite the variety of species of CNS, they were classified as one homogeneous group. In this study 41.5% ($n = 22$) of samples with CNS showed SCC over the SCM threshold (200×10^3 cells/mL), and 58.5% ($n = 31$) of CNS samples had SCC above the threshold value selected for this research. In fact, the most frequent CNS are evenly distributed between high and low SCC (Thorberg et al., 2009; Condas et al., 2017). By contrast, *Staph. aureus*-positive milk samples had SCC values over the threshold (SCM or IMI) in 95.8% of cases ($n = 23$), in accord with previous findings, where

a positive correlation between them has been described (Djabri et al., 2002; Jayarao et al., 2004; Barkema et al., 2015). Likewise, in 90.1% of the quarter milk samples where *Strep. uberis* was isolated, SCM and an IMI were diagnosed. Finally, the respective low and zero prevalences of *Strep. agalactiae* and *Strep. dysgalactiae* prevented the analysis of this group of bacteria separately, in accord with previous studies developed in Colombia. The zero prevalence of *Strep. dysgalactiae* is expected because this pathogen is rarely isolated from milk quarter samples in the country, with prevalence reports ranging from 0.6 to 5.2% (Ramírez et al., 2001, 2014; Velasco-Bolaños et al., 2019).

All IRT were taken during the predawn (0200 h) and morning hours (0700 h), avoiding the hours of maximum solar radiation and heat, because solar radiation along with low wind speed can increase an animal's heat load (Mader et al., 2006) and therefore affect UST. How-

ever, to improve udder temperature measurement, the high-resolution thermal camera included environmental parameters.

It has been described that IRT is highly dependent on working conditions such as wind speed, humidity, or environmental temperature; likewise, UST can be affected by external factors when animals are not in controlled environmental conditions (Berry et al., 2003; Polat et al., 2010; Porcionato et al., 2010). Unconditional analysis between several environmental factors showed that none of them were associated with UST; nevertheless, when mixed effects linear regression analysis was performed, the environmental factors were not present in the final model as a predictor of UST. Those results suggest that environmental factors in grazing systems are not related to UST if environmental factors are controlled when the IRT is taken. It is worth mentioning that animals included in this study were milked directly in the paddocks where cows were on pasture, using portable machine milking systems or by hand; they did not have to walk long distances to the milking parlor, and no resting period was needed because friction generated with the displacement was not a factor to be controlled, as was done in other studies (Berry et al., 2003; Colak et al., 2008; Polat et al., 2010). The maximum distance walked to the milking tent by the cows included in this study was less than 50 m; however, on some farms in tropical countries, including Colombia, cows must walk long distances from the paddocks to a waiting room and then into the milking parlor. According to other

authors, this exercise could affect UST and should be considered a confounder in other studies aiming at using UST for mastitis diagnosis (Berry et al., 2003).

The THI, which combines relative humidity and environmental temperature, was reported as a factor directly associated with surface corporal temperature of cattle, including UST (Peng et al., 2019). Nasr and El-Tarabany (2017) also describe that THI could increase related to the increase of SCC. During the entire period that the measurements of our study were taken, THI was below 72 points; this could be interpreted as thermoneutral conditions for the animals, where heat stress should not appear and cattle's body thermoregulation ability would be unaffected (Dash et al., 2016).

The average UST in this study was similar to those reported in cows from Estonia, Canada, and Brazil (Berry et al., 2003; Porcionato et al., 2009; Poikalainen et al., 2012; Silva et al., 2019); however, some researchers have found average temperatures of $>34^{\circ}\text{C}$ in Europe and Asia (Colak et al., 2008; Zaninelli et al., 2018; Peng et al., 2019).

Two factors affected UST in our study, one of which was the milking method. The UST in machine milking was close to 1.0°C (Table 3) warmer than that of udders milked by hand, regardless of the case definition. This is the first report finding such a difference, as all previous reports, according to our literature review, were done with machine milking. However, the specific mechanism involved in the observed relationship between milking method and UST response are not known, and it would

Figure 5. Left-hand panel: Receiver operating characteristics (ROC) analysis for udder surface temperature (UST, $^{\circ}\text{C}$) to assess the capacity of the test to predict subclinical mastitis (SCM, $\blacksquare$), microbial growth (MG, $\bullet$), or IMI ($\blacktriangle$) with hand milking. Data were obtained from 90 quarters from 44 cows. The optimal cutoff point for detecting SCM, MG, and IMI was 32.6°C , and areas under the curve (AUC) were 0.71, 0.88, and 0.87 for SCM, MG, and IMI, respectively. Right-hand panel: ROC analysis for UST to assess the capacity of the test to predict SCM ($\blacksquare$), MG ($\bullet$), and IMI ($\blacktriangle$) with machine milking. These data were obtained from 185 quarters from 61 cows. The optimal cutoff points for detecting SCM and IMI were 34.0°C and 33.7°C for MG, and the AUC was 0.70, 0.85, and 0.90 for SCM, MG, and IMI, respectively. The diagonal line represents the ROC curve for a test of no predictive ability.

be necessary to determine which specific factors in the milking method are responsible for these results. One hypothesis could be related to the udder distance from the ground, as higher udders with smaller teats are found commonly on machine-milking farms. Such udders are more influenced by body temperature radiation because they are also closer to the cow than those from farms where cows were milked by hand, where cows typically have large udders and long teats, closer to the ground, where they can easily release heat. Nonetheless, this parameter was not measured in this study.

The other aspect related to UST was MG and IMI: IMI leads to variation in skin vasomotor activity, with vasodilation and blood cell perfusion (mainly influx of leukocytes to the site of the infection) resulting in hyperemia, which leads to temperature fluctuation that can be detected by IRT (Hovinen et al., 2008; Viguié et al., 2009). This can also explain why healthy udders are cooler than those diagnosed as an MG or IMI case. The SCM case definition was not related to UST and was excluded from the final model. Instead, the presence of major pathogens remained in the model, and the coefficient indicates that these increase the UST by 1.16°C.

Our study compares UST for diagnosis of SCM, MG, and IMI. Based on our results for Sp, Se, AUC, YI, and +LR, we suggest that SCM is not well identified by UST, and that an important number of SCM quarters will not be diagnosed by the UST method; however, MG and IMI show encouraging results for UST as a diagnostic tool, based on those diagnostic test evaluations when the optimum threshold was used for either hand or machine milking under field conditions. According to Kandeel et al. (2019), diagnostic tests should have Se and Sp as high as possible, $AUC > 0.80$, and $+LR > 10$; in our study, the UST cutoff point selected to diagnose IMI fulfilled all those indices, suggesting that UST had good performance. In this regard, we can suggest, like other authors, that an evident relation exists between mammary gland health and UST as measured by IRT (Metzner et al., 2014; Zaninelli et al., 2018), and, according to our findings, UST could be a reliable method for diagnosis of IMI and, to a lesser degree, of MG.

Because Se, Sp, AUC, and +LR of IRT depend on the threshold used, practitioners could select the cutoff point to modify one of the mentioned parameters, to increase the probability of isolating healthy animals in large herds with high prevalence of SCM (Sp), the probability of identifying all the SCM in the herd (Se), or the probability of the post-test diagnosis (+LR; Hayden and Brown, 1999; Stokes et al., 2012). In this study, UST had high Se and Sp values for diagnosing MG or IMI, but the values obtained for SCM, defined

as an $SCC > 200 \times 10^3$ cells/mL, were not reliable, in either hand or machine milking. Additionally, differences were observed between the AUC ROC analyses according to the case definition, where SCM had lower values compared with UST as a tool for diagnosing MG and IMI, and these latter showed similar values of AUC.

The maximum AUC obtained for the threshold value for UST, for hand and machine milking, and the Se and Sp differed from those reported by Polat et al. (2010) in Brown Swiss dairy cows with SCM definition similar to our study (Se = 0.83, Sp = 1.00, AUC = 0.91). In addition, our findings for IMI were higher than those reported by Zaninelli et al. (2018), in which the AUC, Se, and Sp were, respectively, 0.80, 0.79, and 0.78. In addition, Polat et al. (2010) and Zaninelli et al. (2018) used an SCC threshold of 400×10^3 cells/mL to define an SCM case. For Zaninelli et al. (2018), this resulted in a detrimental effect over AUC, Se, and Sp, whereas Polat et al. (2010) showed better values for AUC and Se but a considerable decrease in Sp, as expected.

The kappa coefficient found in our study indicated substantial agreement between UST and MG and IMI for hand and machine milking, but only moderate agreement for the SCM case definition (in both hand and machine milking). The substantial agreement could be described as clinically useful according to Dohoo et al. (2009). However, as described in Torres et al. (2009), the kappa coefficient depends on Se, Sp, and distribution of the prevalence; thus, when an unbalanced low prevalence appears, kappa results can be low despite relatively good agreement (Thompson and Walter, 1988; Cicchetti and Feinstein, 1990). Accordingly, kappa can be underestimated for machine-milking farms due to the low prevalence of SCM, MG, and IMI case declarations (<23%).

According to several authors, IRT could be described as a noninvasive early-detection method with enough potential to diagnose mastitis (Colak et al., 2008; Hovinen et al., 2008; Polat et al., 2010; Zaninelli et al., 2018). Nevertheless, no standardized method currently exists to capture IRT, including distance from the objective, which ranges from 0.5 to 2 m (Porcionato et al., 2009; Nakagawa et al., 2016; Pampariene et al., 2016); the region of interest, which may or may not include the teat, the teat end (Barth, 2000; Porcionato et al., 2009), the whole udder (Hovinen et al., 2008; Polat et al., 2010; Poikalainen et al., 2012), and the lower, median, and upper fractions of the udder (Porcionato et al., 2009; Sathiyabarathi et al., 2016); and the view (front, rear, or lateral). Additionally, region of interest can be defined using rectangles, lines, or polygons (Metzner et al., 2014). In this study, from both IRT images an average UST value was found by using a

manual polygon segmentation of the udder, which was described as reliable by Metzner et al. (2014). Additionally, there is no consensus in the UST average or in the cutoff point, and currently there is lack of agreement over the UST value to be used for diagnosis (average, maximum, or median; Polat et al., 2010; Metzner et al., 2015; Zaninelli et al., 2018) and the moment of the IRT image capture, whether before or after sample collection. Porcionato et al. (2009) performed IRT after sampling and found a lack of association between UST and SCM; based on that result, it could be that the previous manipulation of the udder increases the UST evenly in all teats due to heat transfer from the technician's hand to the udder and the friction that occurs during milk sampling, which may mask the true surface temperature. All of this is without mentioning the variety of thermal cameras and software for thermal image analysis employed in existing research.

Finally, some concerns exist about the implementation of IRT for measuring UST, not due to test performance but because it is a nonstandardized test, although its relationship with mammary gland health status and usefulness have been demonstrated several times (Polat et al., 2010; Metzner et al., 2014; Zaninelli et al., 2018). However, currently useful common methods exist, such as the California Mastitis Test, which is a low-cost, fast, portable, and accurate technique for mastitis diagnosis (Polat et al., 2010; Kandeel et al., 2019).

CONCLUSIONS

The findings of this research suggest that UST established by IRT was not related to SCM cases as defined in this study. Additionally, UST was 1.07 and 1.10°C higher in quarters with MG and IMI, respectively, than in healthy quarters; therefore, UST is a reliable and clinically useful method for MG and IMI diagnosis on grazing systems in tropical pastures, with animals milked by either hand or machine. However, milking method is an important factor when UST is used. In addition, there was no effect of environmental conditions on the UST.

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