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Real-time polymerase chain reaction for detection and enumeration of *Staphylococcus aureus* and *Streptococccus agalactiae* using different milk samplings

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ABSTRACT - The objective of the present study was to evaluate the qPCR for detection and enumeration of *Staphylococcus aureus* and *Streptococcus agalactiae* using different milk samplings in comparison to the conventional microbiology. Four dairy herds with a history of subclinical mastitis caused by *S. aureus* and *S. agalactiae* were selected. Sampling approach included milk samples from bulk tank (BT), cow level (composite samples, CO), and mammary quarter level (MQ) from 785 lactating cows. Three consecutive monthly milk samplings were carried out, totaling 3347 MQ milk samples, 912 CO, and 12 from BT. All collected milk samples were subjected to conventional microbiology and qPCR for detection and enumeration of *S. aureus* and *S. agalactiae.* The qPCR showed 71.5% of diagnostic sensitivity for *S. aureus* isolated from MQ milk samples, 71.8% for CO, and 50% for BT milk samples compared with conventional microbiology methodology. Taken together, the diagnostic sensitivity for *S. agalactiae* isolated from MQ milk samples was 90.2, 87.7 for CO, and 90.9% for BT milk samples. In general, the qPCR methodology enabled the detection of *S. aureus* and *S. agalactiae*, regardless of the type of milk sampling. The direct use of milk samples to estimate the counting of *S. aureus* by qPCR demonstrated lower sensitivity than the counting of *S. agalactiae*, which can be explained by the pathogen infection dynamics and differences in milk sample type.

Keywords: bovine mastitis, identification, molecular microbiology, qPCR

1. Introduction

Bovine mastitis is the main disease of dairy cattle in the world (Gonçalves et al., 2018a; Gonçalves et al., 2020) and results in great economic losses to the farmer (Bes et al., 2000; Gonçalves et al., 2018b; Gonçalves et al., 2021) and significant losses to the dairy industry (Santos et al., 2003). Mastitis usually occurs in response to an intramammary infection, mainly caused by bacteria, with a predominance of species of the genera *Staphylococcus* spp. and *Streptococcus* spp. (Bradley, 2002; Ruegg, 2017).

The diagnosis of mastitis-causing agents is very important to monitor herd udder health, prevent new cases, and control the disease (Bradley, 2002; Koskinen et al., 2010). Currently, conventional microbiology (CM) is the standard methodology for identifying microorganisms that cause mastitis (Ruegg, 2017). However, the identification of mastitis-causing pathogens by means of conventional

microbiology can take from two to seven days for the complete diagnosis at the species level, since it involves plating milk samples on solid medium culture plates, incubation, and assessment of any microbial growth, and then subsequent application of biochemical tests (Barreiro et al., 2012).

Quantitative real-time PCR (qPCR) has been used as alternative methodology for the identification of pathogens that cause bovine mastitis (Gillespie and Oliver, 2005; Taponen et al., 2009; Koskinen et al., 2010; Elias et al., 2012), as qPCR can reduce the limitations of conventional microbiology to diagnose bovine intramammary infection. Several reports have shown that qPCR has been utilized as a rapid and reliable methodology, complementary to conventional microbiology, for the identification of mastitis-causing bacteria (Gillespie and Oliver, 2005; Koskinen et al., 2010; Katholm et al., 2012).

Quantitative real-time PCR has a high diagnostic sensitivity for the identification of major pathogens (e.g., *S. aureus* and *S. agalactiae*) isolated from mammary quarter (MQ) samples (Gillespie and Oliver, 2005; Koskinen et al., 2010). However, the conventional microbiology method combined with qPCR evaluation for detection and enumeration of *S. aureus* and *S. agalactiae* using different milk samples (bulk tank, BT; cow level, CO; and mammary quarter level, MQ) has not yet been determined. Therefore, the objective of the present study was to evaluate qPCR for detection and enumeration of *S. aureus* and *S. agalactiae* using different milk samplings in comparison to conventional microbiology.

2. Material and Methods

2.1. Herd selection and milk sampling

Thirteen herds with a history of clinical mastitis caused by *S. aureus* and *S. agalactiae* were pre-selected. Four herds in the Pirassununga/SP region (an average of 66 lactating cows) were chosen for having both *S. aureus* and *S. agalactiae* pathogens isolated from bulk milk samples. Afterwards, during three consecutive months, 12 BT samples, 912 CO level samples, and 3,347 MQ level samples were collected. Ninety-one MQ were considered non-functional. Research on animals was conducted according to the institutional committee on animal use (3004/2013).

All lactating cows were milked according to their herd milking routine, and clinical mastitis was diagnosed based on the detection of abnormal milk prior to sampling. Milk samples from each MQ as well as composite milk samples (a pool from the four MQ) were aseptically collected in separate sterile tubes. After milking, 40 mL of agitated BT milk were collected in sterile tubes with a sterile collecting spoon and stored at −20 °C until further analysis.

2.2. Identification of *S. aureus* and *S. agalactiae* by microbiological culture

The composite and MQ samples were subjected to microbiological culture to identify *S. aureus* and *S. agalactiae* according to the methodology described by the National Mastitis Council (NMC, 2017). With a platinum loop, 10 µL of the sample was plated on a solid blood agar medium and incubated at 37 °C. Bacterial growth patterns were observed at 24-h intervals and classified based on bacterial colony morphology (form, elevation, and margin) and hemolysis detection. After that, a Gram stain was carried out, and bacteria were examined for the presence of catalase.

The isolates were recognized as *S. aureus* based on their Gram-positive cocci shape and positive coagulase, which was confirmed by a yellow color on mannitol salt agar. Bacterial isolates were determined to be *S. agalactiae* based on their Gram cocci shape, negative reaction to catalase, positive CAMP test result, negative esculin hydrolysis, and PYR test results (NMC, 2017).

2.3. Real-time quantitative polymerase chain reaction (qPCR)

DNA extraction of pathogens (*S. aureus* and *S. agalactiae*) was performed directly from milk using a kit (Qiagen DNA isolation kit, Minneapolis, USA) per the manufacturer's instructions. After that, the extracted DNA was subjected to the purification step using a 1:1 ratio of UltraPure TM Phenol: Chloroform: Isoamyl Alcohol (25; 24; 1, v/v) (Invitrogen®, Carsbad, CA, USA), followed by

homogenization in a vortex for 15 s. The DNA microtube was centrifuged for 3 min at 14,000 rpm at 20 °C, and the supernatant was transferred to another microtube. For precipitation, absolute alcohol was added in the proportion of 2:1 purified DNA and sodium acetate in the proportion of 1:10 of the original volume of the DNA sample, followed by incubation at −20 °C for 24 h. After this period, centrifugation was carried out at 14,000 rpm for 35 min at 4 °C, and the supernatant was discarded. Subsequently, 500 µL of 70% alcohol was added, followed by homogenization for 15 min. The supernatant was discarded by inversion of the microtube, and the pellet was resuspended in 50 µL TE solution (Tris HCl pH 8.0 100 mM / EDTA pH 8.0 40 mM) provided by the extraction kit. At the end, the extracted, purified, and precipitated DNA samples were evaluated for concentration and purity using a NanoDrop 2000® (Thermo Fisher Scientific, Wilmington, EUA) and stored at −80 °C until qPCR analyses were performed.

The DNA was amplified using the 7500® qPCR system (Applied Biosystems, Foster, USA). Briefly, to detect *S. aureus*, the reaction was composed of 10 µL of SYBR Green PCR Master Mix® (Applied Biosystems, Foster, USA), 3.6 µL of each primer at a concentration of 0.2 µM, 1.8 µL of an ultrapure water, and 1 µL of DNA. To detect *S. agalactiae*, 10 µL of SYBR Green PCR Master Mix®, 1.0 µL of each primer at a concentration of 10 µM, 7.0 µL of ultrapure water, and 1 µL of DNA were used. DNA at 20 ng/µL of concentration was added in the final reaction with a total volume of 20 µL/sample. The primers for the amplification reactions of *S. aureus* and *S. agalactiae* were designed by PrimerExpress® software (Applied Biosystems, Foster, USA) (Table 1). For amplification, 50 cycles were used at 95 °C for 15 s and at 60 °C for 1 min, with an initial cycle of 95 °C for 10 min in a qPCR.

Table 1 - Primer oligonucleotides used for qPCR reactions

Gene	Primer	Bp	Tm (°C)
SAG[1]	F: 5′-TTTGGTGTTTACACTAGACTG-3′ R: 5′-TGTGTTAATTACTCTTATGCG-3′	120 bp	77-78
SAU[2]	F: 5′-CCTGAAGCAAGTGCATTTACGA-3′ R: 5′-CTTTAGCCAAGCCTTGACGAACT-3′	166 bp	75-76

qPCR - quantitative real-time polymerase chain reaction; Bp - base pairs; Tm - melting temperature.
[1] Meiri-Bendek et al. (2002).
[2] Graber et al. (2007).

2.4. *S. aureus* and *S. agalactiae* count: conventional methodology

Conventional counting of *S. aureus* from BT, CO, and MQ samples, expressed in colony forming units (CFU/mL), was performed from 1 mL of milk on 3M™ Petrifilm™ Staph Express Disk (3M, Minneapolis, MN, USA). Samples were diluted in sterile saline solution under four decimal dilutions of 10 to 10^{-3} CFU/mL before inoculation in Petrifilm, and then the plates were incubated for 24 h at 35 °C. Colonies that showed a dark blue color were identified as *S. aureus*, confirmed with a DNAse disk by the presence of a pink halo after 2 h of incubation at 37 °C.

Conventional counting of *S. agalactiae* from BT, CO, and MQ samples, expressed in CFU/mL, was determined from 1 mL of milk on Edwards Modified medium (Himedia, India). Samples with positive isolation were diluted in four decimal dilutions in sterile saline solution from 10 to 10^{-3} CFU/mL before inoculation, identified, and counted as those that showed blue color, β-hemolysis, and absence of esculin fermentation.

2.5. *S. aureus* and *S. agalactiae* count: qPCR

A standard qPCR curve for both pathogens was performed using a serial dilution of 10 to 10^{-10} (CFU/mL) *Staphylococcus aureus* ATCC 25915 (Curve-SAU) and *Streptococcus agalactiae* ATCC 13813 (Curve-SAG) (American Type Culture Collection, Manassas, VA, USA). Each dilution contained 1 mL of BHI broth with standard strain of *S. aureus* or *S. agalactiae* in 9 mL of sterile, autoclaved, pasteurized, homogenized whole milk with low somatic cell count (SCC) and total bacterial count. Through the

standard counting curve of *S. aureus* and *S. agalactiae*, the ringing temperature (melting -Tm) and efficiency of the specific primer for *S. aureus* (primer_SAU) and *S. agalactiae* (primer_SAG) were obtained as well as the cycle threshold (Ct) values. Samples that presented the Tm corresponding to the primer used were considered positive for the presence of the bacteria in the milk sample. Samples that did not amplify at any time during the 50 cycles used and that presented Tm equal to 61 °C, or that presented Tm different from that of the primer used, were considered negative, without *S. aureus* and *S. agalactiae* detection.

2.6. Determination of diagnostic sensitivity, agreement, and equivalence

The diagnostic sensitivity was determined by the MedCalc® software (MedCalc Software, 1993) as the proportion of true positive results that were correctly identified by the analyzed test. The diagnostic sensitivity of qPCR was analyzed from the quantification curve of *S. aureus* and *S. agalactiae*. All samples were analyzed in duplicate by qPCR regardless of the counting result of *S. aureus* on 3M™ Petrifilm™ Staph Express Disk and of *S. agalactiae* on Edwards modified medium (Himedia, India). The detection limit in qPCR was considered the lowest detectable concentration of *S. aureus* ATCC 25915 and *S. agalactiae* ATCC 13813 when performing a serial milk sample.

To assess the agreement between the methodologies, data obtained by qPCR were divided into two groups, presence or absence of *S. aureus* and *S. agalactiae*, regardless of the Ct amplification average, using the Analyze-it program version 3.90.1 (Analyze-it Software Ltd., Leeds, West Yorkshire, United Kingdom). Kappa's non-parametric test was used to determine the intensity of agreement between the results (Bland and Altman, 2010). For the interpretation of Kappa values, values were determined among: $\kappa < 0.00$ (no agreement), $0.00 < \kappa < 0.19$ (poor agreement), $0.20 < \kappa < 0.39$ (reasonable agreement), $0.40 < \kappa < 0.59$ (moderate agreement), $0.60 < \kappa < 0.79$ (substantial agreement), and $0.80 < \kappa < 1.00$ (almost perfect agreement) (Landis and Koch, 1977). The limits of agreement for the *S. aureus* and *S. agalactiae* count between the methodologies were defined with a confidence interval of 95% of the differences. To assess the equivalence regarding the *S. aureus* and *S. agalactiae* count between the methodologies, the non-parametric test of Bland-Altman differences was used (Bland and Altman, 2010), assuming that the null hypothesis has no significant difference ($P>0.05$).

2.7. Statistical analysis

All numerical results of the BT, CO, and MQ milk samples of *S. aureus* and *S. agalactiae*, as well as those of SCC were subjected to base 10 logarithmic transformation ($\log_{10}$) to reduce the skewness and improve the normality of SCC values. The estimation of the *S. aureus* and *S. agalactiae* count using Ct was performed by linear regression using PROC REG using the SAS® software (Statistical Analysis System, version 9.0) according to the model:

$$Y\log_{10} = b_{slope} \times Ct_{S.\ aureus\ or\ S.\ agalactiae} + a_{intercept}$$

in which $Y\log_{10}$ in CFU/mL = estimated CFU/mL value transformed to Log_{10} for each type of pathogen; Ct = cycle threshold values of *S. aureus* or *S. agalactiae*, obtained by qPCR; b_{slope} = estimated slope for the variable Ct; and $a_{intercept}$ = intercept. For all analyzes, the significance was considered as $P \leq 0.05$.

3. Results

3.1. Isolation frequency of *S. aureus* and *S. agalactiae* by microbiological culture and qPCR

All 12 BT milk samples were positive for *S. aureus* and *S. agalactiae* by conventional microbiology. The presence of 8.3% of *S. aureus* and 6.3% of *S. agalactiae* isolates in CO milk samples (n = 912) was observed by conventional microbiology, whereas 9.9% of *S. aureus* and 16.4% of *S. agalactiae* were detected by qPCR analyses. Out of 3347 MQ milk samples, 4% of *S. aureus* and 4.8% of *S. agalactiae* were identified by conventional microbiology, while 2.9% of *S. aureus* and 4.4% of *S. agalactiae* were detected by qPCR analyses (Table 2).

Table 2 - Frequency of pathogen isolation by microbiological culture and qPCR

Sample type	*S. aureus*		*S. agalactiae*		Total
	MC	qPCR	MC	qPCR	
Composite	76 (8.3%)	90 (9.9%)	58 (6.3%)	150 (16.4%)	912
Mammary quarters	134 (4.0%)	98 (2.9%)	161 (4.8%)	147 (4.4%)	3347

qPCR - quantitative real-time polymerase chain reaction; MC - microbiological culture.

3.2. Conventional counting and counting curves for *S. aureus* and *S. agalactiae* using qPCR

There was a decrease in the DNA concentration extracted from the milk sample according to the dilution increase, due to the reduction in the *S. aureus* and *S. agalactiae* count, resulting in a peak melting curve reduction. The standard curve efficency was 59.2% with a coefficient of determination (R^2) equal to 0.95 when tested for *S. aureus* (Ct ranging from 26.9 to 35.9, corresponding to the first and the last dilution) and 78.1% with $R^2 = 0.96$ when tested for *S. agalactiae* (Ct ranging from 24.9 to 35.9).

The equation obtained for estimating the *S. aureus* count according to the Ct obtained by qPCR was $Y_{SAU} = (-0.3866 \times Ct_{SAU} + 29.668)$ ($R^2 = 0.9299$), and $Y_{SAG} = (-0.6526 \times Ct_{SAG} + 24.005)$ ($R^2 = 0.9848$) for counting *S. agalactiae*. Through this regression equation, it was possible to estimate the *S. aureus* and *S. agalactiae* counts, expressed in CFU/mL, by the Ct of the qPCR methodology in milk samples from commercial herds (BT, CO, and MQ samples). Overall, the Ct value increased according to the reduction of the DNA concentration of the evaluated pathogens.

3.3. Sensitivity of the qPCR methodology

The diagnostic sensitivity for identification via qPCR in MQ milk samples was 71.5% for *S. aureus* and 90.2% for *S. agalactiae*. In CO milk samples, the diagnostic sensitivity for identification of *S. aureus* was 71.8% and 87.7% for *S. agalactiae*. In BT milk samples, the diagnostic sensitivity was 50% for identification of *S. aureus* but 90.9% for *S. agalactiae* (Table 3).

Table 3 - Diagnostic sensitivity of qPCR for identifying *S. aureus* and *S. agalactiae* isolated from bulk tank (BT) milk samples, cow level (composite samples, CO), and mammary quarter level milk samples (MQ)

S. aureus					*S. agalactiae*				
		Microbiological culture					Microbiological culture		
	MQ[1]	Presence	Absence	Total		MQ[2]	Presence	Absence	Total
qPCR	Presence	93	0	93	qPCR	Presence	138	0	138
	Absence	37	0	37		Absence	15	0	15
	Total	130	0	130		Total	153	0	153
		Microbiological culture					Microbiological culture		
	CO[1]	Presence	Absence	Total		CO[2]	Presence	Absence	Total
qPCR	Presence	56	16	72	qPCR	Presence	50	65	115
	Absence	22	20	42		Absence	7	10	17
	Total	78	36	114		Total	57	75	132
		Microbiological culture					Microbiological culture		
	BT[1]	Presence	Absence	Total		BT[2]	Presence	Absence	Total
qPCR	Presence	6	0	6	qPCR	Presence	10	2	12
	Absence	6	0	6		Absence	1	0	1
	Total	12	0	12		Total	11	2	13

qPCR - quantitative real-time polymerase chain reaction.
[1] Sensitivity: MQ = 71.5% (IC 95%: 62.9-79.1%); CO = 71.8% (IC 95%: 60.5-81.4%); BT = 50% (IC 95%: 21.2-78.8%).
[2] Sensitivity: MQ = 90.2% (IC 95%: 84.3-94.4%); CO = 87.7% (IC 95%: 76.3-94.9%); BT = 90.9% (IC 95%: 58.7-98.5%).

3.4. Equivalence and agreement between conventional count versus qPCR

The mean differences in *S. aureus* and *S. agalactiae* counts observed by conventional microbiology and qPCR had no equivalence (P = 0.99; Table 4), indicating that both methodologies were different for counting *S. aureus* and *S. agalactiae* in milk samples. According to the Kappa test, samples with positive isolation of *S. aureus* and *S. agalactiae* demonstrated significant identification agreement between the conventional microbiology and qPCR methodologies (Table 4). However, when sample type differences were included in the model, fair agreement value for *S. agalactiae* counting (κ = 0.28 and 0.29) was observed when comparing both methodologies (conventional microbiology vs. qPCR), whereas no agreement values were observed for *S. aureus* counting when comparing both methodologies (Table 5).

Table 4 - Agreement and equivalence values of microbiological culture and qPCR methodologies for counting *S. aureus* and *S. agalactiae*

Pathogen	Count equivalence				Identification agreement		
	N	CI 95%	SEM	P-value	κ	CI (95%)	SEM
S. aureus	5	−0.5191 to 0.5207	0.1872	0.9968	0.71	−1.00 to 0.79	0.052
S. agalactiae	4	−0.6077 to 0.6113	0.1915	0.9931	0.64	−1.00 to 0.75	0.066

qPCR - quantitative real-time polymerase chain reaction; κ - Kappa; CI - confidence interval; SEM - standard error of the mean.

Table 5 - Agreement values of microbiological culture and qPCR counting of *S. aureus* and *S. agalactiae* from bulk tank, composite, and mammary quarter milk samples

Sample type	Pathogen	κ	CI (95%)	SEM
Bulk tank	*S. aureus*	0.00	−1.00 to 0.00	0.000
Bulk tank	*S. agalactiae*	0.28	−1.00 to 0.29	0.005
Composite	*S. aureus*	0.00	−1.00 to 0.00	0.000
Composite	*S. agalactiae*	0.29	−1.00 to 0.32	0.017
Mammary quarters	*S. aureus*	0.00	−1.00 to −1.00	0.000
Mammary quarters	*S. agalactiae*	0.29	−1.00 to 0.36	0.043

qPCR - quantitative real-time polymerase chain reaction; κ - Kappa; CI - confidence interval; SEM - standard error of the mean.

4. Discussion

4.1. Diagnostic sensitivity between the identification methodologies of *S. aureus* and *S. agalactiae*

In the present study, the diagnostic sensitivity of milk samples containing *S. agalactiae* by qPCR was 90.2% for MQ, 87.7% for CO, and 90.9% for BT milk samples, which was similar to that reported by Gillespie and Oliver (2005), who demonstrated that the qPCR methodology showed high sensitivity (95.5%) in comparison with conventional microbiology and correctly identified 91.70% of *S. aureus* and 98.20% of *S. agalactiae.* However, when compared with *S. agalactiae* detection found in this current study and with those results reported by Gillespie and Oliver (2005), the qPCR approach demonstrated lower diagnostic sensitivity for detection of *S. aureus* in milk samples, which was directly related to the type of milk sample (71.5% for MQ, 71.8% for CO, and 50% for BT milk samples). According to Koskinen et al. (2010), both methodologies can present between 11 to 25% false negative results. In addition, while utilizing the microbiological culture method, the aforementioned researchers reported that different types of bacteria were recovered from 600 out of 780 milk samples (77%), whereas 89% of the samples had qPCR detection. Different from that found in the present study, Botaro et al. (2013) obtained a sensitivity of 100% for identification of *S. aureus* in milk samples. As DNA extraction of pathogens was performed directly from milk, we hypothesized that the selection of the optimal DNA

extraction strategy and high-fidelity DNA polymerase (e.g., Hot Start Taq Master Mix), which is less sensitive to PCR inhibitors naturally present in milk, could have improved the diagnostic sensitivity of the qPCR (Rossen et al., 1992; Cremonesi et al., 2006; Dibbern et al., 2015). According to Elias et al. (2012), milk contains components such as calcium (Ca^{2+}), proteinases, fats, and proteins that hinder the action of DNA polymerase on the DNA target during qPCR reaction, acting as a shield and avoiding the counting equivalence between qPCR and conventional microbiology.

In CO and MQ milk samples, the qPCR methodology showed high diagnostic sensitivity of *S. agalactiae* when compared with the results of conventional microbiology. The lower diagnostic sensitivity observed by conventional microbiology could be attributed to the smaller milk volume used to inoculate in agar (10 µL) compared with the 200-µL sample of DNA extraction protocol. In addition, a lower shedding of *S. agalactiae* in milk samples has already been reported, which could be linked to the false negative detection of conventional microbiology (Sears et al., 1990). Other point of view is the bacterial competition, milk samples containing more than one pathogen (e.g., greater likelihood in BT and CO milk samples) would limit the development of *S. agalactiae* due to the greatest growth challenge (Katholm et al., 2012). According to Reyher and Dohoo (2011), the sensitivity of the identification methodology may decrease when composite milk samples are used, mainly due to the dilution of the healthy milk samples over the infected ones under the final sample. Therefore, it would help to explain our results of *S. aureus* identification by qPCR methodology. The qPCR methodology is a tool for identifying and counting agents that cause bovine mastitis and can be an excellent strategy for those milk samples without bacterial growth by conventional microbiology (Taponen et al., 2009).

4.2. Analysis of agreement and equivalence between the counting methods of *S. aureus* and *S. agalactiae*

In the present study, regardless of the type of milk sample containing *S. aureus*, no agreement or equivalence of results was observed between both methodologies. In BT and MQ milk samples with *S. agalactiae* isolation, the methodologies (conventional microbiology and qPCR) showed equivalent results. However, neither methodology was equivalent for CO milk samples. When sample type differences were taken into account in the model, fair agreement values of *S. agalactiae* counting were also seen when comparing the two methodologies (conventional microbiology and qPCR). Our results differ from those described by Elias et al. (2012) who obtained a substantial agreement (κ = 0.6686 ± 0.0477; 95% CI = 0.5752 - 0.7620) between the conventional microbiology and qPCR, but only for BT milk samples. According to Katholm et al. (2012), qPCR for analyzing BT milk samples may be considered an important tool to evaluate milk quality based on the pathogen detection and quantification.

The lack of agreement between counting methodologies could be attributed to a variety of factors, including those related to pathogen characteristics and infection dynamics, DNA extraction protocol, sampling frequency, volume collected and used for analysis, sample conservation (Barrio et al., 2003), and the presence of DNA from non-viable cells (Nogva et al., 2003). The presence of qPCR inhibitory substances naturally present in milk, or the PCR efficiency, could explain the non-agreement between the two methodologies. However, we believe the discrepancy between the two methodologies is primarily due to the presence of DNA from non-viable cells detected by qPCR. An alternative approach to increasing the agreement between the counting methodologies and to only amplify the viable DNA (i.e., intact cells, growthing bacteria), would be the use of monoazidic ethidium bromide (MEB). When added to milk samples, MEB only binds to damaged DNA, binding to the double strand of DNA, making it impossible to amplify by qPCR (Nogva et al., 2003). In the present study, we did not use MEB to consider only viable cells in the performed analysis and, for that reason, it can be recognized as our limitation. Additionally, we found that the type of sampling (BT, CO, or MQ milk sample) may interfere with the agreement between the counting methodologies (conventional microbiology vs. qPCR). Note that MQ milk samples have been routinely used for identification by conventional microbiology, while BT or CO milk samples have been eventually subjected to qPCR for detection and confirmation of pathogens causing mastitis (Mahmmod et al., 2013).

5. Conclusions

S. aureus and *S. agalactiae*, which are frequently isolated as the causative agents of bovine subclinical mastitis, can be detected by qPCR regardless of the type of milk sample used. Mammary quarter milk samples have a higher qPCR detection rate of *S. aureus* and *S. agalactiae* in comparison with other sampling levels. The direct use of milk samples to estimate the counting of *S. aureus* by qPCR is not recommended, because no equivalence and agreement were observed in comparison to conventional microbiology. However, the counting of *S. agalactiae* from bulk tank milk samples can be used to infer the presence of this pathogen in composite and mammary quarter milk samples.

Conflict of Interest

The authors declare no conflict of interest.

Author Contributions

Conceptualization: J.L. Gonçalves, B.G. Alves, J.R. Barreiro, K. Anderson and M.V. Santos. Data curation: A.G. Dibbern, J.L. Gonçalves, B.G. Alves, J.R. Barreiro and K. Anderson. Formal analysis: A.G. Dibbern, J.L. Gonçalves and J.R. Barreiro. Funding acquisition: M.V. Santos. Investigation: J.L. Gonçalves and M.V. Santos. Methodology: A.G. Dibbern and J.R. Barreiro. Project administration: J.L. Gonçalves and M.V. Santos. Resources: M.V. Santos. Software: J.R. Barreiro. Supervision: K. Anderson and M.V. Santos. Validation: J.L. Gonçalves and K. Anderson. Visualization: J.L. Gonçalves, B.G. Alves, J.R. Barreiro and K. Anderson. Writing – original draft: A.G. Dibbern and J.L. Gonçalves. Writing – review & editing: J.L. Gonçalves, B.G. Alves, J.R. Barreiro, K. Anderson and M.V. Santos.

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