

Detection and Enumeration of *Streptococcus agalactiae* from Bovine Milk Samples by Real-Time Polymerase Chain Reaction

Nara Ladeira de Carvalho¹ · Juliano Leonel Gonçalves¹ · Bruno Garcia Botaro¹ · Luis Felipe de Prada e Silva¹ · Marcos Veiga dos Santos¹

Received: 24 February 2015 / Accepted: 27 April 2015 / Published online: 2 July 2015
© Springer Science+Business Media New York 2015

Abstract The aim of this study was to evaluate the use of real-time polymerase chain reaction (qPCR) combined with DNA extraction directly from composite milk and bulk tank samples for detection and enumeration of *Streptococcus agalactiae* (SAG) causing subclinical mastitis. Dilutions of sterile reconstituted skim milk inoculated with SAG ATCC 13813 were used to establish a standard curve (cfu/mL) for the qPCR assay targeting SAG. The analytical sensitivity and repeatability of the qPCR assay were determined. Bulk tank (BTM; $n = 38$) and composite milk samples (CM; $n = 26$) collected from lactating cows with positive isolation of SAG were submitted to the qPCR protocol and SAG plate counting, with results from both methods compared. Amplification of DNA was not possible in two out of 64 samples, indicating that qPCR was able to detect SAG in 96 and 97 % of BTM and CM samples, respectively. The inter-assay coefficient of variation was

<5 %, showing that the technique had adequate repeatability. The qPCR protocol can be a high-throughput and rapid diagnostic assay to accurately detect SAG from BTM and CM samples compared with conventional microbiological culture method. However, the evaluated qPCR protocol is not accurate for enumerating SAG in milk samples, probably due to quantification of DNA of non-viable cells.

Introduction

Mastitis is an important animal health and welfare issue that negatively impacts production efficiency and milk quality [21]. The most important contagious mastitis pathogens include *Streptococcus agalactiae* (SAG), *Staphylococcus aureus*, *Corynebacterium bovis*, and *Mycoplasma* spp. [30]. *S. agalactiae* (Lancefield group B) is an obligate contagious parasite of the mammary gland, which can survive for a long time in the gland [23]. This agent was a major cause of mastitis in the pre-antibiotic era, mostly causing subclinical mastitis which tended to be chronic [9].

During the last few decades, specific programs for prevention and control of mastitis have resulted in reduction of intramammary infection (IMI) caused by contagious pathogens. Historically, SAG was the most common contagious mastitis pathogen, but successful control efforts have reduced its prevalence. Makovec and Ruegg [30] reported that SAG decreased from 8.1 % (1994) to 3.0 % (2001) in Wisconsin over a 7-year period. However, the control of this contagious pathogen depends on several factors such as the size of the herd and production system adopted, seasonal trends, age of cow or stage of lactation, mammary gland immunology, and basic management

✉ Marcos Veiga dos Santos
mveiga@usp.br

Nara Ladeira de Carvalho
naralcarvalho@gmail.com

Juliano Leonel Gonçalves
julianolg@usp.br

Bruno Garcia Botaro
bbotaro@usp.br

Luis Felipe de Prada e Silva
lfpsilva@usp.br

¹ Department of Animal Sciences, School of Veterinary Medicine and Animal Sciences, University of São Paulo (USP), Avenue Duque de Caxias Norte, 225, Pirassununga, SP 13635-900, Brazil

practices for prevention and control of mastitis (blanket dry cow therapy, post-milking teat disinfectants, and effective pre-milking hygiene).

On the other hand, SAG remains one of the most important bovine mastitis pathogens in developing countries as well as those in Latin America. In South America, the herd prevalence has been reported to be 42 % in Colombia [22] and 11 % in Uruguay [12]. In Brazil, SAG has been diagnosed from different regions of the country in percentages varying from 3.2 to 33 % [38], probably due to heterogeneity of farm systems in Brazil, which include a high percentage of small producers who may be unaware of management protocols for the control of SAG mastitis.

Although antimicrobial susceptibility for SAG remains very high [21], the self-cure rate is low [11, 21] if the IMI is not diagnosed or treated. The virulence of this pathogen is related to differences in their ability to adhere to the mammary epithelium [8]. Bacterial cells are shed in milk from infected quarters, and transmission to uninfected quarters and cows usually occurs during the milking period. Therefore, herds infected with SAG can also experience very high total bacteria count in bulk tank milk, because cows infected with SAG can shed up to 100 million bacteria/mL. Infected herds frequently have standard plate counts ranging from 20,000 to 100,000 cfu/mL [16]. Somatic cell counts (SCC) of >1,000,000 are not uncommon for SAG infections [21].

Routine microbiological methods applied to microorganism identification have remained nearly unchanged over the last century. These methods still employ classical approaches based on sample streaking, colony growth using various culture media, and morphological and biochemical characterization of the isolated bacterial species. On average, routine milk microbiological procedures take from 5 to 8 day to be completed and require the use of various biochemical tests and the evaluation of trained technicians [2]. Currently, biochemical tests and serological grouping are employed by most diagnostic laboratories to identify *Streptococcus* spp. [31]. Furthermore, commercial biochemical systems have improved turnaround time, standardization, and reproducibility but still incorrectly identify up to 8 % of isolates [17].

Considering the negative consequences of mastitis caused by SAG on the milk quality and the spread from cow to cow, this study aimed to evaluate the applicability of real-time polymerase chain reaction (qPCR) combined with DNA extraction directly from composite milk and bulk tank samples for detection and enumeration of SAG causing subclinical mastitis in comparison with conventional routine biochemical identification testing.

Materials and Methods

Preparation of Standards for the qPCR

Protocol and Reference Methods

To prepare standard curves to enumerate SAG, sterile skimmed-milk powder was used as the dilution medium. *S. agalactiae* ATCC 13813 (American Type Culture Collection, Manassas, VA, USA) kept at $-80\text{ }^{\circ}\text{C} \pm 1$ in brain-heart infusion broth (BHI; Merck, Darmstadt, Germany) with 20 % glycerol, at a concentration of 10^8 – 10^{10} cfu/mL, was used to artificially contaminate the dilution medium. One hundred microliters of the SAG strain solution were pipetted into 6 mL of BHI broth and incubated at $37\text{ }^{\circ}\text{C}$ for 15 h. Turbidity confirmed bacterial multiplication in the broth; 6 mL was then pipetted into 54 mL of sterile reconstituted skim milk. After mixing, 10 serial 1:10 dilutions of sterile reconstituted skim milk artificially inoculated with SAG were made. From each dilution, three aliquots were taken for SAG quantification by plate count and qPCR protocol.

Viable cell counts of SAG ATCC 13813 at each dilution were performed by plating 100 μL onto a modified Edward's agar with 5 % sheep blood (Oxoid, Basingstoke, UK) as recommended by Jayarao et al. [18]. Plates on which no SAG ATCC 13813 growth (0 cfu/mL) was detected were used to establish the analytical sensitivity of the qPCR protocol. Enumeration of SAG ATCC 13813 by flow cytometry was carried out using the BactoScan flow cytometer (Foss Electric, Hillerød, Denmark) to create reference values for the serial dilutions of SAG ATCC 13813 in milk, both for the standard plate count (based on enumeration of viable organisms in the sample) and for the qPCR method (based on the total counts of the target DNA).

Detection and Quantification by qPCR

Primers

The primers for SAG qPCR reactions (F: 5'-TTTGGTG TTTACTAGACTG-3' and R: 5'-TGTGTTAATTAC TCTTATGCG-3'; [32]) were specifically designed to SAG diagnostics. Meiri-Bendek et al. [32] developed it by 16S rRNA subunit sequence that was aligned [using GCG Pileup and Pretty programs (GCG Wisconsin package)] across 11 streptococcal strains (Genbank accession numbers: AB023574, AF135453, AF088900, AF015928, AB002517, AF076028, AB023576, AF003932, AF009494, AF104675, AJ243965). Using the Primer-BLAST software (National Center for Biotechnology Information, National

Library of Medicine, Bethesda, MD, USA; http://www.ncbi.nlm.nih.gov/tools/primer-blast/index.cgi?LINK_LOC=BlastHome), the primers for SAG were checked for annealing specificity among the primer set SAG and the genomic sequences found for SAG and other streptococcal species.

Primers to confirm the DNA in the samples after extraction procedure were used targeting the bovine mitochondrial cytochrome *b* encoding region (BMCB; accession number V00654; F: 5'-GCAATACACTACACATCCGACACAA-3' and R: 5'-GCGTGTATGTATCGGATGATTCAG-3'), were specifically designed for qPCR (PrimerExpress, Applied Biosystems, Foster, CA, USA), and yielded a 98-bp amplicon. This single-copy, low-mutation-rate gene [15] was chosen as a positive control.

qPCR Simplex Reactions

Every simplex reaction (targeting either SAG or BMCB in different reaction vials) was performed in duplicate (StepOne, Applied Biosystems, Foster, CA, USA). Reactions were prepared as recommended by the manufacturer, with the following modifications: each amplification reaction was composed of 10 μ L of SYBR Green I PCR Master Mix (Applied Biosystems), 0.5 μ L of the primer set (SAG or BMCB) concentration in 20 pmol, 8.5 μ L of DNA-free water (Qiagen Inc, Valencia, CA, USA), and 0.5 μ L of nucleic acids extracted directly from the milk samples by a commercially available protocol (QIAamp Spin Procedure, QIAamp DNA Blood Mini Kit, Qiagen Inc, Minneapolis, MN, USA) with modifications.

For extraction of nucleic acids directly from the milk samples, 200 μ L of milk was added to lysis buffer (200 μ L, provided in the QIAamp kit) into one standard 1.5-mL microcentrifuge tube and homogenized. The mixture was incubated for 10 min at 95 °C. Proteinase K (20 μ L, provided in the QIAamp kit) was added to the mixture and homogenized. Mixture was incubated for 10 min at 70 °C and then absolute ethanol (200 μ L) was added. After vortexing, the mixture was transferred to a silica-gel membrane column (provided in the QIAamp kit) and centrifuged for 60 s at 6000 \times *g* to bond the DNA to the membrane. Washing buffer (500 μ L, provided in the QIAamp kit) was added to the column and then centrifuged (60 s at 6000 \times *g*). A second wash procedure was carried out with the appropriate washing buffer provided in the QIAamp kit for this step (500 μ L), and the column was then centrifuged for 3 min at 20,000 \times *g*. A second centrifugation step (1 min at 20,000 \times *g*) was performed to dry the pellet. The silica column was coupled to a fresh, standard 1.5-mL microcentrifuge tube, and 200 μ L of the provided elution buffer was added to the retained DNA in the membrane for incubation (5 min at 20 °C). The eluted

DNA was collected after being centrifuged twice (60 s at 6000 \times *g*) and kept at -20 °C until further analysis. One aliquot of viable cells of SAG ATCC 13813 was submitted to the same protocol extraction described previously. Five serial dilutions of the DNA extracted from SAG ATCC 13813 were performed in DNA-free water. Then, the relationship between DNA concentrations diluted and the respective C_t values was observed by qPCR (Fig. 1).

The reactions were prepared in 48-well reaction plates (MicroAmp, Applied Biosystems). The thermal cycler program consisted of five cycles at 95 °C for 15 s, 55 °C for 30 s, 72 °C for 30 s, 40 cycles at 95 °C for 15 s, 51 °C for 30 s, and 72 °C for 30 s, with an initial cycle of 95 °C for 10 min. In each cycle, the accumulation of PCR products was detected by monitoring the increase of fluorescence of the fluorophore binding to the double-stranded amplicons generated. Subsequently, a melting curve was constructed in the range of 60–95 °C for each targeted genome with its respective primer set. Average melting temperatures (T_m) were 78.4 °C for SAG and 77.8 °C for BMCB. The final result of each reaction was expressed as threshold cycles (C_t). Reactions were qualitatively considered positive for both detection and quantification of SAG (primer annealing to its target) when the coefficient of variation (CV) between the C_t values of duplicates was <3 % [6] (T_m SAG = 78.4 °C \pm SD) and when amplification of the bovine somatic cell positive control (T_m BMCB = 77.8 °C \pm SD) was achieved. In contrast, samples amplified at C_t >44, with a CV between the C_t values of >3 % and amplification of the positive control sample (T_m BMCB = 77.8 °C \pm SD) were qualitatively classified as negative.

Sampling

To evaluate the proposed qPCR protocol for SAG detection and quantification, 38 bulk tank milk (BTM; SCC

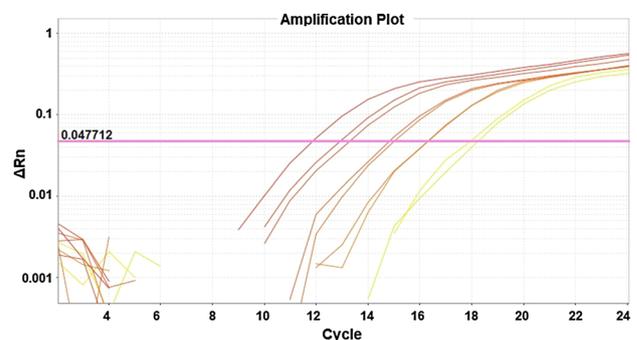


Fig. 1 Real-time PCR amplification plot obtained from five serial dilution of SAG ATCC 13813 DNA in DNA-free water. As observed in Fig. 1, the greater C_t value the lower DNA concentration. DNA concentrations in \log_{10} were 2.77, 2.47, 2.17, 1.69, and 1.39 with respective C_t 11.83, 13.05, 14.86, 16.25, and 18.04

>600,000 cells/mL \pm SD) and 26 composite milk (CM; SCC >200,000 cells/mL \pm SD) samples were selected from 13 commercial dairy herds and lactating Holstein cows in the midwest region of São Paulo State, Brazil, in which subclinical SAG mastitis cases have been previously reported. Herds had an average of more than 60 lactating cows at the time of this study. Herds were milked in herringbone parlors. Pre- and post-milking disinfection were routinely performed on all farms, and no segregation of high-SCC cows was performed on either farm. Cows with clinical mastitis or under treatment for any local or systemic disease were not selected for sampling. Milk samples were collected aseptically for microbiological culture and enumeration of SAG [35] and used for detection and quantitation of SAG by qPCR. This research, protocol number 2608/2012, agrees with Ethical Principles in Animal Research adopted by “Ethical Committee in the use of animals” of the School of Veterinary Medicine and Animal Science of University of São Paulo.

Microbiological Procedures

Microbiological culture of SAG was performed as recommended by the National Mastitis Council [35]. Briefly, to isolate SAG from CM samples through microbiological culture, a 10 μ L aliquot of milk was spread onto a plate of trypticase soy agar with 5 % of defibrinated bovine blood (TSA, Becton, Dickinson and Company, Sparks, MD, USA). The plates were aerobically incubated at 37 ± 1 °C for up to 72 h and were evaluated for bacterial growth after 24, 48, and 72 h. Bacterial colonies were evaluated according to morphology (shape, size, number, and color), hemolytic ability (presence and type), and possible contamination [35]. The isolates were considered SAG when particular characteristics were observed: small (1–2 mm diameter), usually grayish, smooth, translucent, convex colony. Most presented a clear zone of lysed cells (beta-hemolysis). A few were nonhemolytic or surrounded by a zone of greenish, or discolored erythrocytes (alpha-hemolysis) on TSA agar.

For bulk tank milk, 100 μ L of milk was spread on modified Edward’s agar with 5 % blood (Oxoid, Basingstoke, UK). Plates were aerobically incubated at 37 ± 1 °C for 24 h, and SAG was detected and quantified as recommended by Jayarao et al. [18]. SAG colonies on Edward’s agar presented bluish coloration and lack of fermentation. After incubation, all SAG isolates were checked by microscopy and catalase, hippurate, esculin, and CAMP tests. All isolates that were catalase negative, hippurate positive, esculin negative, CAMP-positive gram-positive cocci were considered to be SAG.

Statistical Analysis

Heterogeneity of variance was removed from all quantitative data (enumeration of SAG) by \log_{10} transformation. Data were anti- \log_{10} -transformed for presentation of results. The normality of residuals was assessed using the Kolmogorov–Smirnov test with the PROC Univariate command of SAS (version 9.2, SAS Institute Inc., Cary, NC, USA).

Standard Curves

To estimate SAG (ATCC 13813) enumeration (cfu/mL) using qPCR, the linear correlation (PROC CORR, version 9.2, SAS Institute) between the SAG C_t value of a given sample and its plate count (cfu/mL) determined by the reference method was constructed. Once linearity was observed between these values ($P < 0.05$), we established the equation ($Y_1 = a_1 + b_1X_1$) to estimate the SAG counts (cfu/mL) of a given sample based on the SAG C_t value (CtSAG) determined by qPCR (PROC REG, version 9.2, SAS Institute), where Y_1 = estimated \log_{10} cfu/mL; a_1 = intercept; X_1 = \log_{10} CtSAG resulting from SAG qPCR of the sample; and b_1 = slope for X_1 .

Sensitivity and Repeatability of the qPCR Protocol

The parameters for assessing the analytical sensitivity and repeatability of the assay were done as described previously Office International des Épizooties [10]. The analytical sensitivity of the qPCR method was evaluated using aliquots taken from the 1:10 serial dilutions of SAG ATCC 13813 used to construct the pathogen standard curve. From the aliquot that yielded cfu/mL = 0 to that classified as slightly below “too numerous to count” (TNTC) by the modified Edward’s agar with 5 % sheep blood (Oxoid, Basingstoke, UK), all aliquots were subjected to SAG qPCR. The detection limit for SAG qPCR consisted of the value of the sample classified immediately after the sample with cfu/mL = 0, as determined by the reference method. In contrast, the quantification limit was the highest count (cfu/mL) of SAG ATCC 13813 in milk immediately before that of the sample with a TNTC value (cfu/mL).

Repeatability was assessed by analysis of the variability of CtSAG obtained from six samples subjected to the entire qPCR protocol on two different days (day 1 and 2) in duplicate (day 1, A1’, and A’’; day 2, B’, and B’’); the intra-assay variation (CV A; CV B) was obtained by calculating the CV of duplicates; the inter-assay variation (CV 1, 2) was determined by calculating the CV of results obtained from samples analyzed on different days.

Diagnostic Agreement

Nonparametric kappa test [1] was used to determine agreement between the microbiological reference procedure and the proposed SAG qPCR protocol. Data from SAG qPCR were classified as having either the presence or absence of the pathogen, regardless of the average C_t between duplicates, provided that quality control qPCR parameters between duplicates were kept. The κ coefficient was determined (Epidat version 3.1, Epidemiological Analysis Software for Tabulated Data, Pan American Health Organization/World Health Organization, Santiago de Compostela, Spain) considering two methods of observation (microbiological culture for isolation of SAG vs. SAG qPCR), two categories (presence or absence of SAG), the number of samples analyzed by both methods, and a 95 % confidence level. The κ parameters were used to interpret the coefficient of agreement [1] in cases of significant agreement ($P < 0.001$) between methods and to be able to reject the null hypothesis ($\kappa = 0$).

Agreement Between Methods of Measurement

The agreement between SAG quantification by qPCR and by the reference methods was assessed using Bland–Altman nonparametric test of differences [3] (Analyze-it, Analyze-it Software Ltd., Leeds, UK) coupled Microsoft Excel (Microsoft Corp., Redmond, WA, USA).

Results

Microbiological Cultures, DNA Extraction Directly from Milk, and qPCR Amplification

Streptococcus agalactiae was isolated by microbiological culture from all BTM and CM samples. From CM samples, qPCR detected 96.15 % ($n = 25$) SAG-positive samples. *S. agalactiae* was detected by qPCR in 97.63 % of BTM samples ($n = 37$). Two samples had no DNA amplification, one from CM and the other from BTM. The qPCR methodology showed threshold cycle (C_t) values for each type of milk sample evaluated, which were used to estimate SAG plate counts using the standard curve.

The primer for the gene encoding the bovine mitochondrial cytochrome b (BMCB) was used for confirmation of DNA extraction directly from milk samples. This procedure was done as positive control. Moreover, as C_t increased, bacterial and DNA concentrations decreased. A correlation coefficient of 79 % between C_t values and DNA extracted from SAG in the 1:10 dilutions was observed ($r = 0.792$; $P < 0.001$). The equation to estimate the DNA

concentration of SAG by qPCR was \log_{10} DNA concentration = $19.391 - 9.8178 \log_{10} C_t$.

Standard Curve for Counting SAG Using qPCR

Counts of SAG ATCC 13813 in 1:10 serial dilutions of SAG-inoculated skim milk ranged from 300 to 3.2×10^6 cfu/mL. Linearity between data from plate count and flow cytometry of SAG ATCC 13813 ($r = 0.99$, $P < 0.001$) and between data from SAG qPCR and flow cytometry ($r = -0.99$, $P < 0.001$) was observed; a standard curve to estimate SAG ATCC 13813 levels in milk by SAG qPCR was constructed (Fig. 1). The SAG counts using 16S rRNA gene amplification of SAG ATCC 13813 (\log_{10} CtSAG/mL) ranged from 2.47 to 5.51 \log_{10} cfu/mL and were linearly associated to plate count data ($r = 0.971$, $P < 0.001$), which ranged from 300 to 3.2×10^6 cfu/mL.

Sensitivity and Repeatability of the qPCR Protocol

The bacterial counts based on the analysis of serial dilutions of SAG ATCC 13813 in sterile reconstituted skim milk by qPCR were highly correlated ($r = 0.971$; $P < 0.001$), consistent with the results from plate counting in the range of 900– 2.0×10^5 cfu/mL. To assess intra- and inter-assay repeatability of the SAG qPCR protocol, variations of up to 5 % between C_t values (Table 1) were considered acceptable [17]. Intra-assay CV from milk samples with higher SAG numbers (2.0×10^5 cfu/mL) were 0.57 and 2.87 % on day 1 and 2, respectively. For DNA extraction and qPCR 16S rRNA gene amplification, intra-assay CV were 0.07 and 1.89 % (at 900 cfu/mL) on day 1 and 2, respectively. The CV of results of bacterial counts for protocols carried out on both days was 2.87 % for milk samples with 2.0×10^5 cfu/mL of SAG ATCC 13813 and 4.56 % for milk samples with 900 cfu/mL (Fig. 2).

Diagnostic Agreement Between Microbiological Culture and qPCR

Composite Milk

Data for enumeration of SAG in CM samples obtained from 25 dairy cows quantified by the plate-counting method (ranging from 1.74 to $4.87 \cong 2.76 \log_{10}$ cfu/mL) and estimated by SAG qPCR (ranging from 3.28 to $4.87 \cong 5.61 \log_{10}$ CtSAG/mL) without standard deviations between measurements are shown in Fig. 3a. *S. agalactiae* plate counts (cfu/mL) was not significantly correlated to qPCR (C_t) ($r = 0.46$; $P = 0.245$). The magnitude of observed differences (in \log_{10} cfu/mL) between results obtained by the plate-counting method and by SAG

Table 1 Coefficients of variation (CV) within and between trials of serially diluted milk samples with decreasing numbers of *Streptococcus agalactiae* subjected to real-time quantitative PCR targeting the 16S rRNA gene of *Streptococcus agalactiae* (SAG qPCR) on two different days

| Sample | Count ^a | Day 1 | | | Day 2 | | | CV 1, 2 (%) |
|--------|-----------------------|-------------------|--------------------|----------|-------------------|--------------------|----------|-------------|
| | | C _t A' | C _t A'' | CV A (%) | C _t B' | C _t B'' | CV B (%) | |
| 1 | 2.0 × 10 ⁵ | 23.75 | 23.56 | 0.57 | 24.42 | 24.85 | 1.23 | 2.87 |
| 2 | 3.3 × 10 ⁴ | 25.97 | 25.88 | 0.25 | 26.26 | 25.76 | 1.36 | 0.23 |
| 3 | 5.0 × 10 ³ | 29.04 | 28.77 | 0.66 | 28.46 | 29.10 | 1.57 | 0.31 |
| 4 | 3.2 × 10 ³ | 29.35 | 31.86 | 5.80 | 28.55 | 28.27 | 0.70 | 5.26 |
| 5 | 1.7 × 10 ³ | 31.18 | 31.64 | 1.04 | 31.02 | 30.14 | 2.03 | 1.89 |
| 6 | 9.0 × 10 ² | 22.10 | 22.70 | 1.89 | 21.01 | 20.99 | 0.07 | 4.56 |
| Média | | | | 1.70 | | | 1.16 | 2.52 |

Independent assays A' and A'' and B' and B'' were independently performed on day 1 and 2; C_t = threshold cycle in which SAG qPCR occurred; CV A and CV B = CV between C_t obtained from assays A' and A'' and B' and B''; CV 1, 2 = CV between C_t obtained from assays performed on day 1 and 2

^a Count (cfu/mL) of *Streptococcus agalactiae* ATCC 13813 determined by modified Edward's agar with 5 % sheep blood (Oxoid, Basingstoke, UK)

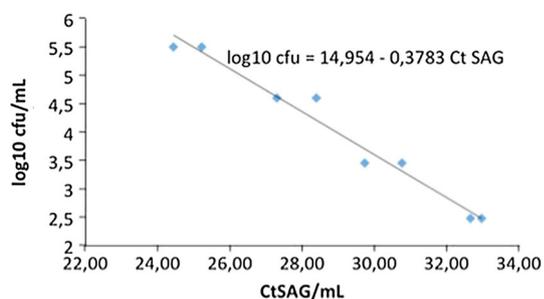


Fig. 2 Standard curve of SAG ATCC 13813 count (\log_{10} cfu/mL) as determined by plate counting (\log_{10} cfu/mL) of SAG-inoculated skim milk and by real-time quantitative PCR (SAG qPCR, CtSAG/mL). C_t = threshold cycle of the qPCR reaction

qPCR as a function of the average results obtained by both methodologies was expected to fall constantly across the identity line and to be approximately normally distributed (Fig. 3b); however, the large limits of agreement (-5.49 to -0.11) determined poor agreement ($P < 0.0001$) between methods.

Bulk Tank Milk

Data for enumeration of SAG in milk samples obtained from 37 BTM based on the plate-counting method (ranging from 2 to $5.30 \cong 3.36 \log_{10}$ cfu/mL) and estimated by SAG qPCR (ranging from 2.11 to $7.64 \cong 4.26 \log_{10}$ CtSAG/mL) without standard deviations between measurements are shown in Fig. 4a. SAG counts from plate counting (cfu/mL) were correlated to qPCR (C_t) ($r = 0.29$; $P = 0.009$). The magnitude of observed differences (in \log_{10} cfu/mL) between results obtained by the plate-counting method and by SAG qPCR as a function of the average results obtained by both methods was expected to fall constantly across the identity line and to be

approximately normally distributed (Fig. 4b); however, the large limits of agreement (-2.9 to 1.17) determined poor agreement ($P < 0.0001$) between methods.

Discussion

The aim of the present study was to evaluate the equivalence between two methodologies (plate counting vs. qPCR) used to enumerate and detect SAG from milk samples. For that, we created a standard curve, using SAG ATCC 13813 to estimate bacterial counts using threshold cycle determined using qPCR. By combining DNA extraction directly from milk with qPCR, it was possible to detect SAG faster than with the plate-counting method. On the other hand, despite the qPCR method being widely used in quantification of bacteria in foods like milk and dairy products [37, 43], enumeration of SAG by qPCR obtained in the present study from BTM and CM was not equivalent to plate-counting method. The non-equivalence may have occurred because of the presence of dead or non-viable SAG which was not cultivated in some of the milk samples, as well as the negative effect of cellular and humoral immunity of the host on the viability of the pathogen causing mastitis [13]. Additionally, the analytical sensitivity and repeatability of qPCR to detect SAG in comparison to the plate count method was determined. Detection of SAG was performed by qPCR considering 10^{-4} to 10^{-7} cfu/mL dilutions (analytical sensitivity) and intra-assay variation $<5\%$ (repeatability). The repeatability of results was similar to Graber et al. [14] and, Botaro et al. [4], which reported a CV of $<5\%$ for *S. aureus*.

In the present study, average counts of SAG from CM and BTM were 4.31×10^3 and 1.90×10^4 cfu/mL, respectively. Our results of the average counts of SAG

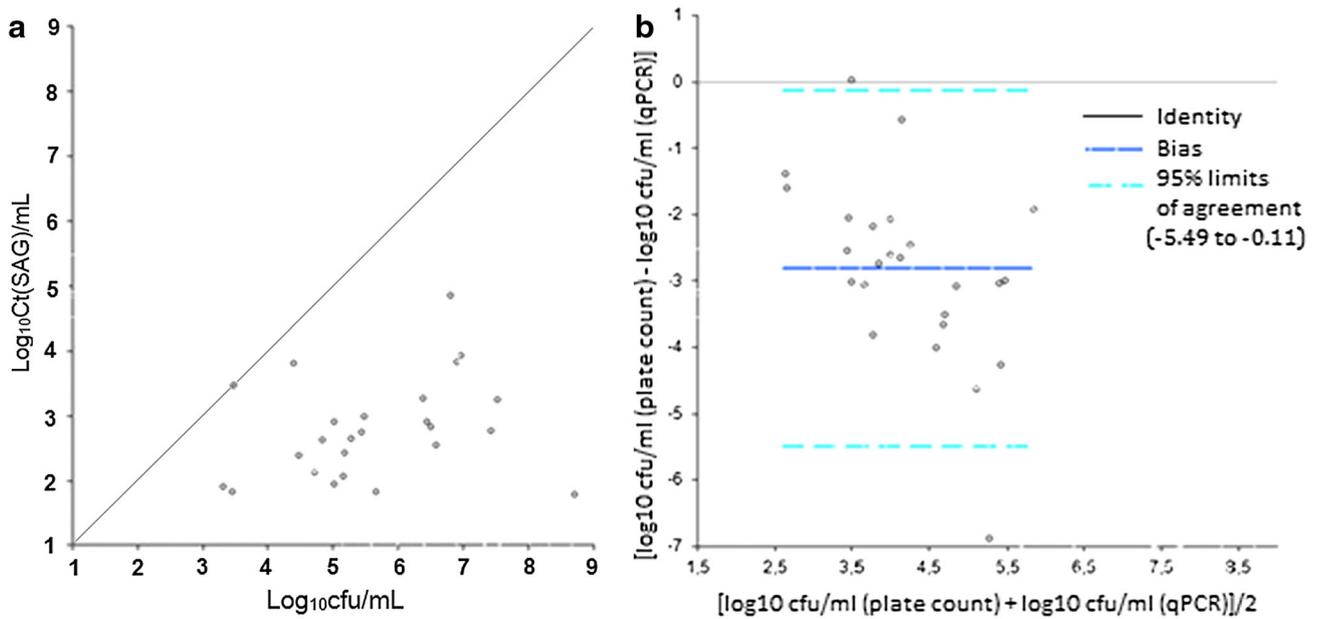


Fig. 3 **a** SAG enumeration (\log_{10} cfu/mL) obtained by real-time quantitative PCR [\log_{10} cfu/mL (qPCR)] and the plate count reference method [\log_{10} cfu/mL (plate count)] plotted on the identity curve. **b** Representative plot of the average of the results of counts (\log_{10} cfu/mL) as a function of the differences observed between both

methods [real-time quantitative PCR (qPCR) and plate count]. The bias (2.8) was the average difference between the values (\log_{10} cfu/mL) of both methods. Limits of agreement between the methods ranged from -5.49 to -0.11

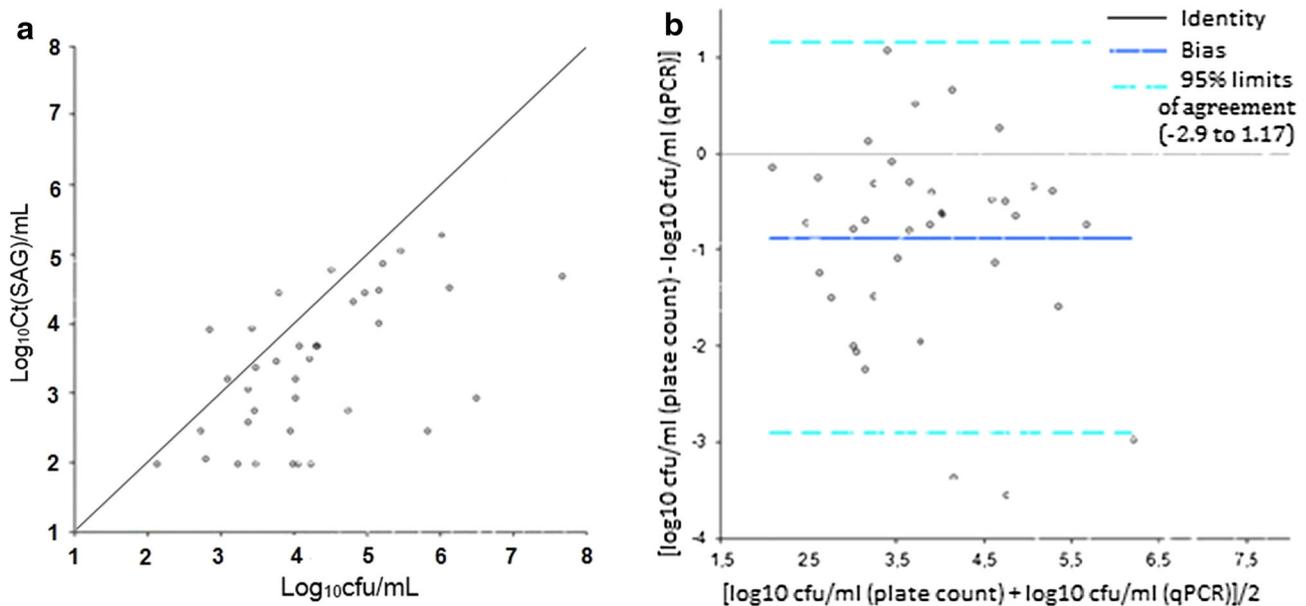


Fig. 4 **a** SAG enumeration (\log_{10} cfu/mL) obtained by real-time quantitative PCR [\log_{10} cfu/mL (qPCR)] and the plate count reference method [\log_{10} cfu/mL (plate count)] plotted on the identity curve. **b** Representative plot of the average of the results of counts (\log_{10} cfu/mL) as a function of the differences observed between

both methods [real-time quantitative PCR (qPCR) and plate count]. The bias (0.86) is the average difference between the values (\log_{10} cfu/mL) of both methods. Limits of agreement between the methods ranged from -2.9 to 1.17

from BTM were different than those found by Elias et al. [9], which reported 7.77×10^2 cfu/mL. Differences associated with composition from different types of milk

samples (BTM and CM) may have influenced the efficiency of DNA extraction directly from milk samples. Different types of milk samples were used to observe how

rapidly qPCR could detect SAG from BTM and diagnose at the herd level SAG mastitis. Diagnosis of mastitis caused by SAG from BTM by qPCR is rapid and has some advantages because is only one representative milk sample and consequently requires less analysis time. Additionally, SAG is an obligate parasite of the mammary gland, which supports the idea of using a BTM sampling to detect SAG-positive cows in the herd [23]. Plate counts of SAG when matched to SCC may be an indication if mastitis cases are latent, unspecific, or subclinical. By the way, immune response intensity in the mammary gland due to the reaction of mastitis cases is highly correlated to specific causative agent [28] and the intensity with which these agents colonize the mammary gland. Quantification and detection of microorganisms from BTM samples is important for SAG screening of herds [5].

Considering that DNA extraction of SAG directly from milk samples has been used for quantitative and qualitative qPCR method, it demands specific care during extraction procedures, such as the volume standardization of milk samples and reagents used for reactions. Cremonesi et al. [7] also extracted DNA directly from milk samples and similar to our results, reported difficulties associated with the extraction protocol. In our study, the main difficulties that possibly influenced DNA extraction were low concentration of DNA of SAG cells [39], factors associated with the level of cell lysis and exposure of nucleic acid, presence of inhibitory substances (calcium, proteinases, fat, and casein content) [41]. These difficulties were considered as critical points associated with DNA extraction directly from milk samples [7]. Furthermore, Elias et al. [9] reported a DNA extraction protocol directly from milk that was efficient in lysing bacterial cell walls without damaging the target DNA. For confirmation of the DNA extraction, amplification using the set of BMCB primers (control test) was used because DNA extracted by this protocol could not be read by NanoDrop, likely due to the presence of inhibitory substances that remained even after extraction [7].

Real-time PCR was suggested as a method to quantify microbial populations in food [37], which is different from the conventional PCR methodology that is only based on microorganisms detection. The objective of using qPCR in milk samples is due to its high sensitivity for the detection of mastitis causing pathogens [20]. In the present study, one control test (BMCB primers) was used to evaluate the protocol of DNA quantification. This test consisted of serial dilutions (10^{-1} to 10^{-10}) of DNA extracted from SAG ATCC 13813 as the objective to measure the DNA obtained by qPCR (CtSAG/mL). Threshold cycle (C_t) is the cycle number required to amplify the nucleic acid. The nucleic acid is detected by fluorescence and represents the quantification of DNA target [20]. Therefore, it was

possible by linear regression to observe that C_t is inversely proportional correlated to DNA concentration, namely the greater the C_t value the lower DNA concentration, which is in accordance with results described by Postollec et al. [37] and Katholm et al. [20].

The final determination of bacterial load by qPCR can be influenced by the number of rRNA operons of a given species. The ribosomal RNA (rRNA) genes (5S, 16S, and 23S) of bacteria and archaea are typically found in operons. Although many organisms have a single rRNA operon, the actual number is known to vary between 1 and 15 [24]. For example, according to Klappenbach [25], the rRNA operon copy number of *S. agalactiae* is seven. It has been suggested that *rrn* copy number is an index of microbial life histories [40]; due to the central role of ribosomal RNAs in the formation of peptide bonds, rRNA genes share regions of highly conserved sequence that are interspersed with more variable regions. An additional benefit of knowing the *rrn* copy number of a microorganism is derived from a positive correlation between the number of rRNA genes in an organism's genome and the capacity of that organism to respond to favorable growth conditions [29].

Klappenbach et al. [25] reported that rRNA operon copy number information has become increasingly valuable for studies evaluating methodologies such as quantitative qPCR. Due to redundancy of the rRNA genes in some microorganisms, the measured abundance of a rRNA gene might be attributed to few microorganisms with many rRNA genes or many microorganisms with few rRNA genes. This redundancy must be considered in studies that measure the abundance of rRNA genes, especially techniques such as quantitative PCR [29]. The importance of *rrn* copy number is recognized in studies worldwide, mainly for studies comparing different bacterial species, with different number of *rrn* copies.

A standard curve was created to evaluate the analytical sensitivity of the qPCR to detect SAG considering the conditions used in this study. This curve was used mainly to compare methodologies, i.e., SAG plate count (cfu/mL) and qPCR results (CtSAG/mL). The results of the standard curve and regression equation presented in this study were similar to those of Botaro et al. [4]. *S. agalactiae* plate counts ranged from 3×10^2 to 3.2×10^5 cfu/mL (dilutions 10^{-4} to 10^{-7}). However, different from the present study, Gillespie and Oliver [13] have constructed a standard curve based on multiplex qPCR methodology in which the detection sensitivity ranged from 10^0 to 10^8 cfu/mL.

Streptococcus agalactiae, *S. aureus*, and *Mycoplasma* species are the primary contagious microorganisms isolated from infected cows and bulk milk tanks. Justice-Allen et al. [19] and Botaro et al. [4] have constructed a standard curve for *Mycoplasma* spp. and *S. aureus*, respectively, in

order to compare bacterial counts (cfu/mL) found by the plate-counting methodology and qPCR. Although several studies have evaluated the detection of contagious pathogens using qPCR, none of them estimated the correlation between SAG plate counts and qPCR [9, 13, 26, 27].

The SAG plate counting (cfu/mL) was compared to SAG counts estimated by qPCR (CtSAG/mL) using a similar methodology as used by Botaro et al. [4]. Mean differences of colony counts from SAG plate counting and qPCR from CM and BTM were 103.26 % (4.453×10^3 cfu/mL) and 26.78 % (2.412×10^4 cfu/mL), respectively. These differences may be due to the higher sensitivity of qPCR [7, 20, 36] when compared to conventional plate-counting methodology, because microbiological culture only counts viable cells.

In the present study, CM samples used were not from the same farms as the BTM samples. Furthermore, viable bacterial DNA was not measured using qPCR, as suggested by Nogva et al. [34]. These factors may have contributed to the non-equivalence of results between the two methods used, which could be considered as limitations of the present study. It is recommended that future studies about qPCR combined with DNA extraction directly from milk strategically count only viable cells, as suggested by Nocker et al. [33] and Taskin et al. [42], in which they proposed the use of ethidium bromide monoazide to inhibit PCR amplification of DNA from dead bacteria.

Conclusion

Real-time PCR may be used to detect *S. agalactiae* in bulk tank and in composite milk samples from cows with sub-clinical mastitis. However, qPCR combined with DNA extraction directly from milk samples was not equivalent to conventional plate-counting method.

Acknowledgments The authors are grateful to José Garcia Moreno Franchini, Lucinéia Mestieri, Lígia Garcia Mesquita, and Daniele Cristine Beuron for technical assistance and Roberta Lyman who reviewed the English writing.

Conflict of interest The authors declare that they have no conflict of interest.

References

- Altman DG (1991) Practical statistics for medical research. Chapman and Hall, London
- Barreiro JR, Ferreira CR, Sanvido GB, Kostrzewa M, Maier T, Wegemann B, Bottcher V, Eberlin MN, dos Santos MV (2010) Short communication: identification of subclinical cow mastitis pathogens in milk by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. J Dairy Sci 93(12):5661–5667. doi:10.3168/jds.2010-3614
- Bland JM, Altman DG (2010) Statistical methods for assessing agreement between two methods of clinical measurement. Int J Nurs Stud 47(8):931–936. doi:10.1016/j.ijnurstu.2009.10.001
- Botaro BG, Cortinhas CS, Março LV, Moreno JFG, Silva LFP, Benites NR, Santos MV (2013) Detection and enumeration of *Staphylococcus aureus* from bovine milk samples by real-time polymerase chain reaction. J Dairy Sci 96(11):6955–6964. doi:10.3168/jds.2013-6559
- Brito MAVP, Brito JRF, Souza HMd, Vargas OL (1998) Avaliação da sensibilidade da cultura de leite do tanque para isolamento de agentes contagiosos da mastite bovina. Pesquisa Veterinária Brasileira 18:39–44
- Bustin S (2004) A-Z of quantitative PCR. International University Line, La Jolla
- Cremonesi P, Castiglioni B, Malferrari G, Biunno I, Vimercati C, Moroni P, Morandi S, Luzzana M (2006) Technical note: improved method for rapid DNA extraction of mastitis pathogens directly from milk. J Dairy Sci 89(1):163–169. doi:10.3168/jds.S0022-0302(06)72080-X
- Duarte RS, Miranda OP, Bellei BC, Brito MA, Teixeira LM (2004) Phenotypic and molecular characteristics of *Streptococcus agalactiae* isolates recovered from milk of dairy cows in Brazil. J Clin Microbiol 42(9):4214–4222. doi:10.1128/jcm.42.9.4214-4222.2004
- Elias AO, Cortez A, Brandao PE, da Silva RC, Langoni H (2012) Molecular detection of *Streptococcus agalactiae* in bovine raw milk samples obtained directly from bulk tanks. Res Vet Sci 93(1):34–38. doi:10.1016/j.rvsc.2011.07.016
- Épizooties Old (2010) The Animal Health and Production Compendium: Manual of Diagnostic Tests and Vaccines for Terrestrial Animals. <http://www.cabi.org/ahpc/default.aspx?site=160&page=3323>. Accessed 4 Jan 2012
- Farnsworth RJ (1987) Indications of contagious and environmental mastitis pathogens in a dairy herd. In: Proceedings 26th Annual Meeting of the National Mastitis Council O, FL, pp 151–5
- Giannechini R, Concha C, Rivero R, Delucci I, Moreno Lopez J (2002) Occurrence of clinical and sub-clinical mastitis in dairy herds in the West Littoral Region in Uruguay. Acta Vet Scand 43(4):221–230
- Gillespie BE, Oliver SP (2005) Simultaneous detection of mastitis pathogens, *Staphylococcus aureus*, *Streptococcus uberis*, and *Streptococcus agalactiae* by multiplex real-time polymerase chain reaction. J Dairy Sci 88(10):3510–3518. doi:10.3168/jds.S0022-0302(05)73036-8
- Graber HU, Casey MG, Naskova J, Steiner A, Schaeren W (2007) Development of a highly sensitive and specific assay to detect *Staphylococcus aureus* in bovine mastitic milk. J Dairy Sci 90(10):4661–4669. doi:10.3168/jds.2006-902
- Hassanin A, Douzery EJ (1999) The tribal radiation of the family Bovidae (Artiodactyla) and the evolution of the mitochondrial cytochrome b gene. Mol Phylogenet Evol 13(2):227–243. doi:10.1006/mpev.1999.0619
- Hogan JSSK (1992) Using bulk tank milk cultures in a dairy practice. Workshop Mastitis Microbiology Diagnostics, National Mastitis Council, Arlington
- Jayaroo BM, Doré JJ, Baumbach GA, Matthews KR, Oliver SP (1991) Differentiation of *Streptococcus uberis* from *Streptococcus paruberis* by polymerase chain reaction and restriction fragment length polymorphism analysis of 16S ribosomal DNA. J Clin Microbiol 29(12):2774–2778
- Jayaroo BM, Pillai SR, Sawant AA, Wolfgang DR, Hegde NV (2004) Guidelines for monitoring bulk tank milk somatic cell and bacterial counts. J Dairy Sci 87(10):3561–3573. doi:10.3168/jds.S0022-0302(04)73493-1

19. Justice-Allen A, Trujillo J, Goodell G, Wilson D (2011) Detection of multiple Mycoplasma species in bulk tank milk samples using real-time PCR and conventional culture and comparison of test sensitivities. *J Dairy Sci* 94(7):3411–3419. doi:10.3168/jds.2010-3940
20. Katholm J, Bennedsgaard TW, Koskinen MT, Rattenborg E (2012) Quality of bulk tank milk samples from Danish dairy herds based on real-time polymerase chain reaction identification of mastitis pathogens. *J Dairy Sci* 95(10):5702–5708. doi:10.3168/jds.2011-5307
21. Keefe G (2012) Update on control of *Staphylococcus aureus* and *Streptococcus agalactiae* for management of mastitis. *Vet Clin North Am Food Anim Pract* 28(2):203–216. doi:10.1016/j.cvfa.2012.03.010
22. Keefe G CA, Jaramillo M, Londoño M, Chaffer M, Toro M (2011) Effects of *Streptococcus agalactiae* on the Columbian Dairy Industry. In: Proceedings of International Symposium. Mastitis and Milk Quality, 3rd National Mastitis Council, St. Louis, MO, pp 155–159
23. Keefe GP (1997) *Streptococcus agalactiae* mastitis: a review. *Can Vet J* 38(7):429–437
24. Klappenbach JA, Dunbar JM, Schmidt TM (2000) rRNA operon copy number reflects ecological strategies of bacteria. *Appl Environ Microbiol* 66(4):1328–1333. doi:10.1128/aem.66.4.1328-1333.2000
25. Klappenbach JA, Saxman PR, Cole JR, Schmidt TM (2001) rrndb: the ribosomal RNA operon copy number database. *Nucleic Acids Res* 29(1):181–184
26. Koskinen MT, Holopainen J, Pyorala S, Bredbacka P, Pitkala A, Barkema HW, Bexiga R, Roberson J, Solverod L, Piccinini R, Kelton D, Lehmusto H, Niskala S, Salmikivi L (2009) Analytical specificity and sensitivity of a real-time polymerase chain reaction assay for identification of bovine mastitis pathogens. *J Dairy Sci* 92(3):952–959. doi:10.3168/jds.2008-1549
27. Koskinen MT, Wellenberg GJ, Sampimon OC, Holopainen J, Rothkamp A, Salmikivi L, van Haeringen WA, Lam TJ, Pyorala S (2010) Field comparison of real-time polymerase chain reaction and bacterial culture for identification of bovine mastitis bacteria. *J Dairy Sci* 93(12):5707–5715. doi:10.3168/jds.2010-3167
28. Le Roux Y, Laurent F, Moussaoui F (2003) Polymorphonuclear proteolytic activity and milk composition change. *Vet Res* 34(5):629–645. doi:10.1051/vetres:2003021
29. Lee ZM-P, Bussema C, Schmidt TM (2009) rrnDB: documenting the number of rRNA and tRNA genes in bacteria and archaea. *Nucleic Acids Research* 37(Database issue):D489–D493. doi:10.1093/nar/gkn689
30. Makovec JA, Ruegg PL (2003) Results of milk samples submitted for microbiological examination in Wisconsin from 1994 to 2001. *J Dairy Sci* 86(11):3466–3472. doi:10.3168/jds.S0022-0302(03)73951-4
31. McDonald WL, Fry BN, Deighton MA (2005) Identification of *Streptococcus* spp. causing bovine mastitis by PCR–RFLP of 16S–23S ribosomal DNA. *Vet Microbiol* 111(3–4):241–246. doi:10.1016/j.vetmic.2005.10.012
32. Meiri-Bendek I, Lipkin E, Friedmann A, Leitner G, Saran A, Friedman S, Kashi Y (2002) A PCR-based method for the detection of *Streptococcus agalactiae* in milk. *J Dairy Sci* 85(7):1717–1723. doi:10.3168/jds.S0022-0302(02)74245-8
33. Nocker A, Cheung CY, Camper AK (2006) Comparison of propidium monoazide with ethidium monoazide for differentiation of live vs. dead bacteria by selective removal of DNA from dead cells. *J Microbiol Methods* 67(2):310–320. doi:10.1016/j.mimet.2006.04.015
34. Nogva HK, Dromtorp SM, Nissen H, Rudi K (2003) Ethidium monoazide for DNA-based differentiation of viable and dead bacteria by 5'-nuclease PCR. *BioTechniques* 34(4):804–808, 810, 812–803
35. Oliver SPGR, Hogan JS, Jayarao BM, Owens WN (2004) Microbiological procedures for the diagnosis of bovine udder infection and determination of milk quality. National Mastitis Council, Verona
36. Phuektes P, Mansell PD, Browning GF (2001) Multiplex polymerase chain reaction assay for simultaneous detection of *Staphylococcus aureus* and streptococcal causes of bovine mastitis. *J Dairy Sci* 84(5):1140–1148. doi:10.3168/jds.S0022-0302(01)74574-2
37. Postollec F, Falentin H, Pavan S, Combrisson J, Sohier D (2011) Recent advances in quantitative PCR (qPCR) applications in food microbiology. *Food Microbiol* 28(5):848–861. doi:10.1016/j.fm.2011.02.008
38. Santos EMPBM, Lange C, Brito JRF, Cerqueira MMOP (2006) *Streptococcus* and related genera as etiological agents of bovine mastitis. *Acta Scientiae Veterinariae* 35(1):17–27
39. Sears PM, Smith BS, English PB, Herer PS, Gonzalez RN (1990) Shedding pattern of *Staphylococcus aureus* from bovine intramammary infections. *J Dairy Sci* 73(10):2785–2789. doi:10.3168/jds.S0022-0302(90)78964-3
40. Stoddard SF, Smith BJ, Hein R, Roller BRK, Schmidt TM (2015) rrnDB: improved tools for interpreting rRNA gene abundance in bacteria and archaea and a new foundation for future development. *Nucleic Acids Res* 43(Database issue):D593–D598. doi:10.1093/nar/gku1201
41. Taponen S, Salmikivi L, Simojoki H, Koskinen MT, Pyorala S (2009) Real-time polymerase chain reaction-based identification of bacteria in milk samples from bovine clinical mastitis with no growth in conventional culturing. *J Dairy Sci* 92(6):2610–2617. doi:10.3168/jds.2008-1729
42. Taskin B, Gozen AG, Duran M (2011) Selective quantification of viable *Escherichia coli* bacteria in biosolids by quantitative PCR with propidium monoazide modification. *Appl Environ Microbiol* 77(13):4329–4335. doi:10.1128/aem.02895-10
43. Zadoks RN SH, Tikofsky-Garrison LL (2005) Molecular tools enhance the value of bulk tank milk monitoring. In: Proceedings of 44th Annual Meeting NMC pp 86–93