ACCURACY OF A MULTIPLEX PCR PROTOCOL FOR *Staphylococcus aureus*, *Streptococcus agalactiae* AND *Escherichia coli* DETECTION IN BULK TANKS

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ABSTRACT

The objective of the present study was to evaluate the accuracy of a multiplex PCR (mPCR) protocol for *Staphylococcus aureus, Streptococcus agalactiae* and *Escherichia coli* detection in bovine milk samples collected from bulk tanks. Twenty dairy farms in mid-west of São Paulo State, Brazil were enrolled in the study. Milk samples collected from each bulk tank were evaluated by mPCR and microbiological culture (gold standard test). *S. aureus*, *S. agalactiae* and *E. coli* were isolated by culture in 30%, 10% and 40% respectively, and detected by mPCR in 0%, 10% and 35% bulk tank milk samples respectively. mPCR has presented 93.2% specificity and 37.5% sensitivity values, with 78.3% accuracy. This mPCR protocol was efficient for *S. agalactiae* and *E. coli* detection in the evaluated bulk tank milk samples, but failed to detect *S. aureus*. Despite the high specificity values obtained by mPCR, the sensitivity levels were low, indicating that this molecular method should only be recommended as a complementary diagnosis test for milk quality monitoring in bulk tanks.

Keywords: *Staphylococcus aureus, Streptococcus agalactiae, Escherichia coli,* microorganisms, molecular diagnosis, dairy, bulk tank, milk.

ACURÁCIA DE PROTOCOLO DE MULTIPLEX PCR PARA DETECÇÃO DE *Staphylococcus aureus*, *Streptococcus agalactiae* E *Escherichia coli* EM TANQUES DE EXPANSÃO

RESUMO

O objetivo do presente estudo foi avaliar a acurácia de um protocolo de multiplex PCR (mPCR) para detecção de *Staphylococcus aureus, Streptococcus agalactiae* e *Escherichia coli* em amostras de leite bovino obtidas de tanques de expansão. Vinte propriedades leiteiras localizadas no centro-oeste do estado de São Paulo foram envolvidas no estudo. As amostras de leite colhidas de cada tanque de expansão foram examinadas por mPCR e por cultivo microbiológico (padrão ouro). *S. aureus*, *S. agalactiae* e *E. coli* foram isolados em 30%, 10% e 40% das amostras avaliadas, respectivamente, e detectados por mPCR em 0%, 10% e 35% amostras de leite, respectivamente. O protocolo de mPCR utilizado apresentou 93,2% de especificidade e 37,5% de sensibilidade, com 78,3% de acurácia. O mPCR foi eficiente para a detecção de *S. agalactiae* e de *E. coli* nas amostras de leite de tanques de expansão avaliadas, porém não possibilitou a detecção de *S. aureus.* Apesar da alta especificidade, a sensibilidade da mPCR foi baixa, indicando que esta técnica molecular poderia ser recomendada unicamente como teste diagnóstico complementar para o monitoramento da qualidade do leite em tanques de expansão.

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Palavras-chave: *Stapylococcus aureus, Streptococcus agalactiae, Escherichia coli,* micro-organismos, diagnóstico molecular, propriedade leiteira, tanque de expansão, leite.

ACURACIA DE UN PROTOCOLO DE MULTIPLEX PCR PARA LA DETECCION DE *Staphylococcus aureus*, *Streptococcus agalactiae* Y *Escherichia coli* EN TANQUES ENFRIADORES

RESUMEN

El objetivo del presente estudio fue evaluar la acuracia de un protocolo de multiplex PCR (mPCR) para La detección de *Staphylococcus aureus, Streptococcus agalactiae* y *Escherichia coli* en muestras de leche bovina obtenidas de tanques enfriadores. Veinte haciendas lecheras localizadas en el centro-oeste de la provincia de San Pablo fueran involucradas en el estudio. Las muestras de leche obtenidas de cada tanque enfriador fueron examinadas por mPCR y por cultivo microbiológico (gold standard). *S. aureus*, *S. agalactiae* y *E. coli* fueron aislados en 30%, 10% y 40% de las muestras evaluadas, respectivamente, y detectados por mPCR en 0%, 10% y 35% muestras de leche, respectivamente. El protocolo de mPCR utilizado presentó 93,2% de especificidad y 37,5% de sensibilidad, con 78,3% de acuracia. El mPCR fue eficiente para la detección de *S. agalactiae* y de *E. coli* en las muestras de leche de tanques enfriadores evaluadas, todavía no posibilitó la detección de *S. aureus.* Mientras los elevados índices de especificidad, los valores de sensibilidad de la mPCR fueron bajos, indicando que esta técnica molecular podria ser recomendada unicamente como test diagnóstico complementar para el monitoreo de la calidad de la leche en tanques enfriadores.

Palabras clave: *Stapylococcus aureus, Streptococcus agalactiae, Escherichia coli,* microorganismos, diagnóstico molecular, hacienda lechera, tanque enfriador, leche.

INTRODUCTION

The Normative Instructions n.51 (1) and n.62 (2) published by Brazilian Ministry of Agriculture represent technical regulatory determinations for milk production, identity, quality, sampling and transport, in order to improve milk quality. These legal requeriments have being started in July 2005 and have determined a intensive demand for fast, cost feasible and highly accurate diagnosis methods, in order to process a high number of milk samples, in milk quality control programs (3). Despite these regulations were recently approved in Brazil, the concept of mastitis control, milk quality and food safety is very well structured in other countries, especially in North America and Europe. In these regions, scientific studies using molecular techniques for pathogens detection in milk products and milk samples have been developed since 1990's (4). Molecular biology has significatively contributed for an accurate detection of microorganisms in milk samples, individually collected from cows' quarters, or composed obtained from bulk tanks.

Some inhibitors factors that normally difficult the microorganisms detection by routine microbiological culture methods like antimicrobial residues, low number of microorganisms in samples, and contamination with environmental pathogens, have no interference when using molecular techniques. The direct detection of pathogen DNA and/or specific genes usually determines high sensitivity and specificity values (5).

First molecular trials were based on the detection of one target DNA (one genus or specie) by simplex PCR protocols using one pair of primers. In the past 10 years, the researchers have been adjusting the DNA extraction and amplification protocols, in order to obtain optimized conditions to detect more then one target DNA, as duplex PCR (two target DNAs) and multiplex PCR (mPCR), for the identification of more than two target DNAs (6).

Recent studies using different mPCR protocols reported results in individual or composed milk samples, also contributing with epidemiological evaluations (7). However, there are some intrinsic characteristics of milk composition and also some microorganisms' particularities that directly affect mPCR's accuracy (8).

The aim of the present study was to use an mPCR protocol for *S. aureus, S. agalactiae* and *E. coli* detection in milk samples collected from bulk tanks in Sao Paulo, Brazil, and to evaluate the accuracy of this method in order to verify its applicability in milk quality control programs. The chosen of *S. aureus, S. agalactiae* and *E. coli* as target microorganisms is due their importance as main mastitis pathogens in Brazil and also because of their pathogenic potential for public health.

MATERIAL AND METHODS

Dairy farms selection

Twenty dairy farms sited in mid-west cities of Sao Paulo State, Brazil, were enrolled randomly, as follows: four dairies in Botucatu; three dairy farms in Itatinga; two dairies in Nova Odessa and other two farms in Sao Pedro; and one dairy farm in each of the following cities: Areiópolis, Pardinho, Lençóis Paulista, Porto Feliz, Lins, Agudos, Araras, Santa Rita do Passa Quatro and Conchas. The selection criteria used for farm selection were: farms with milking machine systems and individual milk bulk tanks (obligatory items for inclusion in the study). Dairies had 19 to 970 cows in lactation (median 92) with 18.3 L milk daily production (median).

Samples

Milk samples from bulk tanks were collected until 24 hours after milking and after 5 minutes homogenization. A total of 250 mL of milk were collected from each bulk tank. Samples were kept in sterile glass recipients and transported under refrigeration (4-8°C) to the laboratory for analysis.

Microbiological culture

For identification of microorganisms strains, all milk samples collected from bulk tanks were diluted to 10^{-1} and 10^{-2} and one mL of each diluted sample was inoculated into blood agar (5%) and MacConkey agar and incubated at 37°C during 72 hours. The bacterial strains were identified according to Quinn et al. (9).

Standard bacterial strains

Staphylococcus aureus (ATCC 25923) and *Escherichia coli* (ATCC 11229) standard strains from Culture Collection of Adolfo Lutz Institute, Sao Paulo, and *Streptococcus agalactiae* (ATCC 13813) standard strain from National Institute for Health Control Quality (INCQS), Rio de Janeiro, were used as positive controls in tests.

Staphylococcus aureus, Streptococcus agalactiae and *Escherichia coli* DNA detection

DNA extraction. The DNA extraction from Milk samples and from bacterial standard strains was carried out using the Milk Bacterial DNA Isolation Kit (NorgenBiotek Corporation, Ontario, Canada), according to supplier's reccomendation.

Primers. For *S. aureus, S. agalactiae* and *E. coli* detection, primers SAU1 and SAU2 (for *S. aureus*), SAGA1 and SAGA2, SIP3(F) and SIP4(R) (for *S. agalactiae*), Ecoli1 and Ecoli2 (for *E. coli*) were used (10). These primers amplify specie-specific DNA regions that codify the 16S and 23rRNA regions of these microorganisms.

mPCR. Each mPCR reaction was performed using 17.5µL milli-Q water; 2.5µL buffer (10mM TrisHClpH 8.0, 50mM KCl); 0.75µL of $MgCl_2$ (1.5mM); 0,5µL of dNTP (0.2mM); 2.0µL of each primer (10ρM); 0.5µL(0.2 U) of *Taq Platinum* DNA polimerase (Invitrogen,USA), and 3µL of genomic DNA (10ng). Termocycler conditions were: 96°C for 5 min; 30 cycles of 96°C for 1 min, 55°C for 1 min and 72°C for 2 min, and a final extension of 72°C for 8 min. Positive and negative controls were the ATCCs strains and milli-Q water, respectively (10).

Visualization of amplified products. The amplified material was visualized by electrophoresis in agarose gel (1.5%) added with 1.0µL/mL of SYBR Safe DNA gel stain (Invitrogen - USA). Electrophoresis was carried out in horizontal cube containing TBE 1X (89 nMTris-HCl, 89mM boric acid and 20mM EDTA) solution at 65V. mPCR products were visualized in an image analyser (GelDoc-IT™ Imagin System - UVP, USA) by using VisonWorks®LS. It were used 8µL of amplified DNA added to 2µL of stained buffer. 4µL of 100bp ladder was used as marked (Invitrogen, USA).

The expected mPCR products were: *S. agalactiae*: 293 and 590 bp; *E. coli*: 660 bp and *S. aureus*: 1.300 bp (10), as presented in Figure 1.

Analytical sensitivity and specificity tests. The concentration of DNA extracted from ATCCs was measured by NanoVue (GE Healthcare®, USA). These samples were diluted at 1.000ρg/µL, 100ρg/µL, 10ρg/µL, 1 ρg/µL, 0.1ρg/µL and 0.01ρg/µL with milli-Q water and after were processed by mPCR. The results were analyzed by electrophoresis.

For primers specificity test it were used standard strains (ATCCs) of *Streptococcus intermedius, S. epidermidis, S. uberis, S. dysgalactiae* and *Pseudomonas aeruginosa*, from INCQS, Rio de Janeiro, Brazil.

mPCR reproducibility test. Five samples randomly selected were processed by mPCR during five consecutive days in order to evaluate the reproducibility of test.

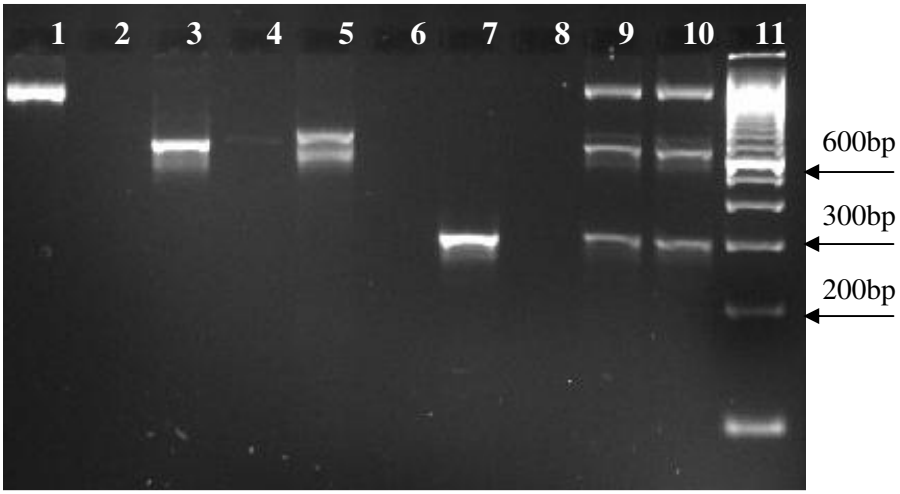

Figure 1. mPCR products obtained from the ATCC strains. 1: *S. aureus* (1.300bp); 3: *E.coli* (660bp); 5: *E. coli* and *S. agalactiae* (590bp); 7: *S. agalactiae* (293bp); 2, 4, 6 and 8: negative controls; 9 and 10: *S. aureus, E. coli and S. agalactiae*; 11: 100bp DNA ladder (Invitrogen).

Statistical analysis

S. aureus, *S. agalactiae* and *E. coli* total isolates and percentage frequencies were evaluated according to microbiological culture and mPCR results. mPCR's accuracy, sensibility and specificity evaluation, considering the microbiological culture as gold standard

were calculated using Mackinnon spreadsheet (11). All tests were analyzed using a significancy level of 5%.

Ethical Comitee Approval

The present study was approved by Ethical Comitee for Animal Experimentation of FMVZ-Unesp/Botucatu-SP - Brazil, by Process n° 133/2008-CEEA.

RESULTS AND DISCUSSION

Frequencies of *Staphylococcus aureus*, *Streptococcus agalactiae, Escherichia coli* and other bacterial strains isolated from bulk tank milk samples are presented on Table 1.

Non-haemolytical streptococci and staphylococci, common pathogens involved in contagious mastitis cases in dairy cows, were also frequently isolated in the evaluated samples, reinforcing the importance of these bacterial pathogens on final milk quality losses.

Table 1. Frequency of bacterial isolates from bulk tanks milk samples (n=20) from dairy farms of Sao Paulo State, Brazil.

Microorganism	Total Frequency (n)	Percentage Frequency (%)
General microorganisms†	16	28.5
Streptococcus sp.	12	21.4
Staphylococcus sp.	9	16.0
E. coli	**8**	**14.2**
S. aureus	**6**	**10.7**
S. agalactiae	**2**	**3.5**
Candida sp.	2	3.5
Corynebacterium sp.	1	1.7
TOTAL	56	100

†Non-target microorganisms, like some enterobacterial species (*Klebsiella* sp.; *Enterobacter* sp.), *Pseudomonas* sp., etc.

Results regarding the target microrganisms detection by culture and by mPCR are shown in Table 2. Accuracy levels of mPCR compared to microbiological culture are presented on Table 3.

The detection levels of *S. agalactiae* and *E. coli* presented by mPCR were very similar to the results obtained by culture. mPCR showed high specificity (88%) and accuracy (80%) values for *S. agalactiae* detection. The same occurred for *E. coli* -mPCR which had 75% sensitivity; 91.7% specificity and 85% accuracy, with good analytical agreement with microbiological culture. These results were similar to the obtained by Moussa et al. (12), which used an mPCRprotocol for shiga toxigenic *E. coli* (STEC) detection and characterization in milk and fecal samples from a dairy herd in Saudi Arabia. The serogroup-specific mPCR detected *E. coli* DNA in all positive samples by microbiological culture. Moreover, four strains (7.98%) O157:H7 and three strains (5.36%) O111 of fecal samples collected from diarrheic animals, as two samples (8.33%) O111 from assymptomaticanimals those were negative by microbiological culture, were positive by mPCR. Similarly, a negative sample by culture was positive by mPCR, demonstrating a good sensitivity of the molecular technique for *E. coli* detection.

Table 2. *Staphylococcus aureus*, *Streptococcus agalactiae* and *Escherichia coli* frequency in bulk tank milk samples (n=20) from dairy farms, evaluated by microbiological culture and mPCR. Botucatu, Sao Paulo State, Brazil.

Microorganism	Microbiological culture		mPCR	
	Positive	Frequency (%)	Positive	Frequency (%)
S. aureus	6/20	30	0	0
S. agalactiae	2/20	10	2/20	10
E. coli	8/20	40	7/20	35

Table 3. Sensitivity, specificity, positive and negative predictive values, accuracy, Kappa coefficient and *p* value obtained using a multiplex PCR protocol for *Staphylococcus aureus*, *Streptococcus agalactiae* and *Escherichia coli* detection in 20 bulk tank milk samples compared to microbiological culture. Botucatu, Sao Paulo, Brazil.

Test (cp/pp/tp)[(1)]	**Sens [(2)] (%)**	**Spec [(3)] (%)**	**Ppv [(4)] (%)**	**Npv [(5)] (%)**	**Ac [(6)] (%)**	**Kappa [(7)] (%)**	***p* [(8)] (%)**
mPCR (16/9/60)	37.5	93.2	66.7	80.4	78.3	0.3564	0.0016

(1) cp: number of positive results by culture; pp: number of positive results by mPCR; tp: total of exams; (2) Sensitivity estimative (% of true positives); (3) Specificity estimative (% of true negatives); (4) Predictive positive value; (5) Predictive negative value; (6) Accuracy value; (7) Kappa coefficient; (8)p value (obs.: p MacNemar = 0,0923)
Prevalence level: 26.7%

The absence of positive results for *S. aureus* in milk samples by mPCR reinforces the scientific experience that the sensitivity and specificity values of molecular methods for bacteria detection in milk are closely related to the microrganism characteristics. Cellular membrane complexity, for example, may difficults the lysis process during DNA extraction (13) and consequently the accuracy level of primers. A PCR protocol using Sa442- 1 and Sa442-2 primers for *S. aureus* detection in 100 milk samples obtained from bulk tanks of 50 dairy farms in Santa Catarina State (Brazil) had demonstrated 53.8% and 36.5% sensitivity and specificity values for S. aureus detection (8). These accuracy levels of the molecular technique were considered very low, when compared to the obtained by culture in Baird-Parker agar, that demonstrated 42% positive samples.

Better accuracy results for *S. aureus* detection from milk by molecular techniques was reported when lysostaphin – a peptidase produced by *Streptococcus simulans* that brokes the cellular membrane of *S. aureus* – is used in DNA extraction protocols (14). This enzyme provides a more specific DNA exposure, resulting in a better detection of the pathogen by PCR (15). In the present study, a commercial kit for DNA extraction was used and despite be indicated for bacterial genomic material isolation from milk samples, this kit does not contains lysostaphin in its composition. This fact may have interfered in the final *S. aureus* DNA amount after extraction process.

Previous studies have demonstrated that pre-incubation, centrifugation and freezing milk samples before DNA extraction may contribute for better sensitivity results in molecular diagnosis. In the first case, *S. aureus* multiplication is improved, and this increases the number of cells. In the second case, *S. aureus* concentration is increased on samples, because of the bacterial pellet, and in the third situation, *S. aureus* DNA exposure increases after defrosting process (16).

Molecular detection of *S. aureus* in milk samples was described to be more accurate when PCR simplex is used. Ymagishi et al. (17) evaluated 106 milk samples collected from quarters and from bulk tank in a dairy farm in Japan. *S. aureus* was identified in nine samples by PCR and only in three samples by microbiological culture, with a detection level of 1

CFU/mL. Faccioli (18) examined 104 milk samples obtained from bulk tanks by PCR for *S. aureus* detection. Sensitivity value was 99% with moderate analytical agreement with microbiological culture in positive samples – the routine method has resulted 176 (56.41%) negative samples that were positive by PCR. However, there were no differences between individual PCR and mPCR for *S. aureus* detection in the present study (Table 4), and similar results were obtained by Riffon et al. (19), which used mPCR to detect *S. aureus* and other five bacterial mastitis pathogens in milk samples collected from animal quarters. Detection level was $5x10^3$CFU/mL for all evaluated microorganisms by mPCR and individual PCR. Cremonesi et al. (13) examined 93 milk samples for *S. aureus* detection and 10 genes that codify staphylococcical toxins. The minimum detection level was equal (10 CFU/mL) between individual PCR and mPCR.

Table 4. *S. aureus*, *S. agalactiae* and *E. coli* DNA detection levels by individual PCRs and mPCR using SAU1 and SAU2, SIP3 and SIP4, and Ecoli1 and Ecoli2 primers.

	SAU1 and SAU2	SAGA1 and SAGA2	SIP3 and SIP4	Ecoli1 and Ecoli2
Individual PCR	100ρg	100ρg	1000ρg	10ρg
mPCR†	100ρg	UN	100ρg	100ρg
mPCR‡	100ρg	UN	100ρg	100ρg

† ATCCs individually evaluated

‡ ATCCs togheter

UN: unused because mPCR products size for *S. agalactiae* (590bp) is very similar to *E. coli* mPCR products (660bp), that would difficult the identification by electrophoresis.

ρg: picogram

Generally, despite the mPCR accuracy was 78.3% for simultaneous detection of *S. aureus*, *S. agalactiae* and *E. coli*, the sensitivity of molecular method was low (37.5%). These results were distinct from those obtained by Phuektes et al. (4), which used the mPCR for *S. aureus, S. agalactiae, S. dysgalactiae* and *S. uberis* detection in 117 milk samples collected from cows with subclinical mastitis. The mPCR showed better sensitivity values than microbiological culture for *S. aureus* and *S. uberis* detection, presenting 10^4CFU/mL and 10^3CFU/mL detection levels for each microorganism, respectively. However, no significant differences between mPCR and culture were verified for *S. agalactiae* and *S. dysgalactiae* detection.

Specificity value by mPCR of the present study was 93.2%, similar to the results reported in previous studies, ranging from 96.3% to 100% (3,7,20). Better detection levels for *S. agalactiae* were obtained by mPCR when compared to the individual PCR for this specific pathogen. These data are different from the ones obtained in other studies (4,6,21).

No differences between detection levels for *S. aureus*, *S. agalactiae* and *E. coli* were observed when mPCR was processed with the extracted DNAs from standard strains, either composed or individual. These data suggests that no interference between bacterial agents occurred, despite the different concentrations levels of each pathogen that normally occurs in milk samples.

After five repetitions of mPCR specificity tests using *Streptococcus intermedius, S. epidermidis, S. uberis, S. dysgalactiae* and *P. aeruginosa* standard strains (ATCCs), it was verified that no DNA of these microorganisms was amplified, suggesting the specific detection of *S. aureus, S. agalactiae* and *E. coli* DNA by mPCR. These results were similar to those obtained by Riffon et al. (19), which used a mPCR protocol to detect *E. coli, S. aureus, S. agalactiae, S. dysgalactiae, S. parauberis* and *S. uberis* in milk samples inoculated with standard strains of each microorganism, isolated from mastitis cases. Seven specie-specific pairs of primers were used and no other DNAs were detected, but only the specific target

ones. In the same way, after five repetitions of mPCR in random milk samples, the results observed in the present study were identical, demonstrating the test reproducibility.

CONCLUSIONS

According to the presented results, it is possible to conclude that the mPCR protocol was highly accurate for *E. coli* and *S. agalactiae* detection in bulk tank milk samples, but failed to detect *S. aureus.*

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