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Molecular screening for the presence of *Streptococcus agalactiae*, *Escherichia coli* and *Listeria monocytogenes* in samples of milk from dairy herds

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This study aims to verify the predominance of contamination with pathogenic microorganisms in dairy herds. In order to validate the initially used methodology, an artificial contamination was conducted in commercially acquired whole UHT milk, with strains of *Listeria monocytogenes*, *Streptococcus agalactiae* and *Escherichia coli*, in final concentrations from 2.10^{-7} to 2.10^{0} CFU/mL, which were submitted to a DNA extraction protocol and to a later amplification using the polymerase chain reaction (PCR) technique. The 702 bp fragments were identified, 884 and 524 bp corresponding, respectively, to *L. monocytogenes*, *E. coli*, *S. agalactiae*. In order to verify the presence of these pathogens in *in natura* milk, the samples were obtained directly from the teats of 125 cows from the dairy herds of four producers, and from the cooling tanks of eight producers, being submitted to DNA extraction, and posterior PCR analysis. The data were analyzed with the Chi-squared test (χ^2) and different sensibility and specificity values were obtained for each microorganism. In cooling tanks, a prevalence of 37.5% of contamination with *S. agalactiae* and of 31.25% by *E. coli* was found. Regarding samples obtained from cow teats, we observed the presence of *S. agalactiae* and *E. coli* in 16.2 and 47.5% of the samples. No sample tested positive for *L. monocytogenes.* The results obtained indicate that the isolation protocol of bacterial DNA directly from the milk, and the PCR technique were efficient to detect the analyzed microorganisms, and may be incorporated as part of routine tests. Moreover, PCR may be an important mechanism to evaluate the quality of milk to be consumed.

Key words: Milk, microorganism, predominance, polymerase chain reaction (PCR).

INTRODUCTION

Milk is one of the most complete and nutritive foods, and is necessary to the human diet. Its high nutritional value is due to the great amount of proteins and dietary elements, characteristics that make it susceptible to contamination by microorganisms.

Milk contamination may occur inside the mammary gland

of the cows with clinical and subclinical mastitis; and during milking and storage, due to ineffective cleaning of udder, teats and milking equipment (Huck et al., 2007). These contamination risks are all associated with improper herd and milk management; therefore, the Brazilian Agriculture Ministry launched the National Program for Milk Quality Improvement. Aiming to increase milk and dairy products safety, this program set higher standards for physicochemical and microbiological parameters of raw milk. Brazil is currently the fourth largest milk producer globally, producing approximately 32.3 billions of liters annually (FAO, 2014), thus improving milk quality is important both for public health and for the country's economy.

Contamination of milk with pathogenic microorga-nisms is a fact in Brazil. Among them, are prominent: *Streptococcus agalactiae*, *Escherichia coli* and *Listeria monocytogenes*. *S. agalactiae,* one of the pathogens associated with cattle and human infections and could be identified through a small quantity of tests as it presents specific biochemical characteristics (Santos et al., 2007). *E. coli* is part of the intestinal microbiome of several animals, also associated with mastitis, which, depending on the virulence factors, can be classified as enterohemorrhagic, enteropathogenic, among others (Bavaro, 2012). *L. monocytogenes*, is a psychotropic bacterium that reproduces at temperatures between -0.4 and 50°C (Donnelly, 2001) and may also be associated with mastitis (Dias, 2007).

Studies have shown the presence of several types of pathogenic microorganisms (*Staphylococcus aureus, Streptococcus* spp, *Corynebacterium bovis, Streptococcus agalactiae* and *Staphylococcus* spp.) in samples of milk in Brazil and other countries (Nornberg et al., 2010; Ribeiro et al., 2009; Arcuri et al., 2006; Bennedsgaard et al., 2006).

Due to the importance of these pathogens and the difficulty of obtaining a quick and precise diagnostic, some studies use molecular techniques to detect these microorganisms. However, they do so indirectly by sowing and enriching the milk with specific growth mediums (Borela et al., 1999; Perez et al., 2002; Zocche et al., 2009).

Among the techniques used, the polymerase chain reaction (PCR) technique has been the most used, since it does not need viable microorganisms in the samples, which enables analysis of samples submitted to improper conservation processes. This technique is also able to detect non-cultivable microorganisms and does not suffer interference by the presence of antibiotic in milk, providing quick, selective and specific results (Meiri-bendek et al., 2002; Ahmadi et al., 2010; Amagliani et al., 2012).

Thus, the aim of the present study was to validate a PCR protocol to detect DNA from *S. agalactiae*, *E. coli* and *L. monocytogenes* directly from milk samples and to evaluate the predominance of raw milk contamination for these microorganisms in dairy herds in Brazil.

MATERIALS AND METHODS

Sample preparation

Artificial contamination was conducted in commercially acquired UHT milk, with *S. agalactiae* (ATCC 13813), *E. coli* (ATCC 25922) and *L. monocytogenes* (ATCC 19114) strains. The dilutions were made from the stationary phase of each microorganism, cultivated in 18-24 h brain heart infusion (BHI) in a heater at 36°C. Quantification in this phase was made by plating suspensions of the microorganism in depth in plate count agar (PCA), and incubation at 36°C for 48 h. For the artificial contamination we used 1.8 mL of UHT milk, and 200 uL of dilutions, with final concentrations from 2.10^7 to 2.10° CFU/mL. 12-16 curves were built, with each point in triplicate.

Samples of raw milk (15 mL) were collected from the cooling tanks of eight properties in July and December, 2011. A sample from each producer per month was collected, with a total of 56 samples. In addition, we performed an analysis of samples of milk (15 mL) collected directly from the teats of 125 cows from dairy herds of three producers in September and October, 2012. All samples were submitted to the same DNA extraction protocol and PCR reactions described below.

DNA isolation

For DNA extraction, 200 µL of raw milk sample were used. Briefly, 20 µL of Tween-20 were added to the samples, followed by centrifugation (12000 xg, 15 min). Pellets were suspended in 60 µL extraction buffer (100 mM Tris-HCl, 100 mM EDTA, 250 mM NaCl, pH 8.0) (Invitrogen, Carlsbad, CA, USA), 30 µL 10% SDS (Sigma-Aldrich, St Louis, MO, USA), 15 µL proteinase K (Ambion®, Austin, TX, USA) (20 mg/mL), and 195 µL ultrapure water, and they were incubated for 1 h at 37°C. Subsequently, 100 µL of buffered phenol (ANRESCO, Solon, OH, USA) were added, and the samples were centrifuged (12000 xg, 5 min); the supernatant was collected and mixed with 100 µL of phenol-chloroform-isoamyl alcohol (25:24:1) (Sigma-Aldrich, St Louis, MO, USA) and centrifuged (12000 xg, 5 min).

A total volume of 26.5 µL of 2 M sodium acetate (Sigma-Aldrich,St Louis, MO, USA) was added to the supernatant, followed by addition of 400 µL of absolute ethanol (Merck, Darmstadt, GER) and overnight incubation at 4°C. The samples were centrifuged (12000 xg, 20 min) and the pellets with DNA were resuspended in TE buffer (10 mM Tris-HCL, 1 mM EDTA, pH 8.0), and stored at -20°C until use. The optical density ratio (260/280 nm) of DNA preparations was considered suitable of downstream applications when greater than 1.6.

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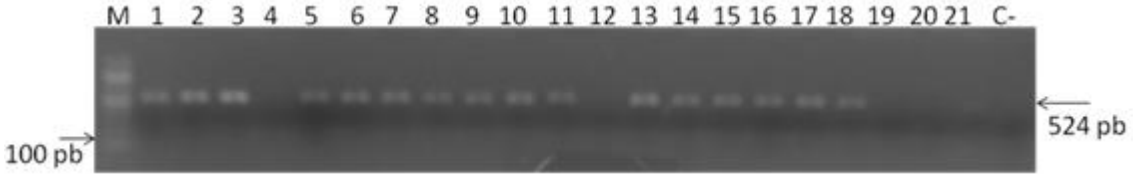

Figure 1. Detection curve for *S. agalactiae*. Samples in triplicate and in CFU/mL: 2 x 10^6 (lanes 1 to 3), 2 x 10^5 (lanes 4 to 6), 2 x 10^4 (lanes 7 to 9), 2 x 10^3 (lanes 10 to 12), 2 x 10^2 (lanes 13 to 15), 2 x 10^1 (lanes 16 to 18), 2 (lanes 19 to 21). C - negative control. M - Molecular weight marker 100 bp (New England Biolabs®, UK). Agarose gel stained with ethidium bromide.

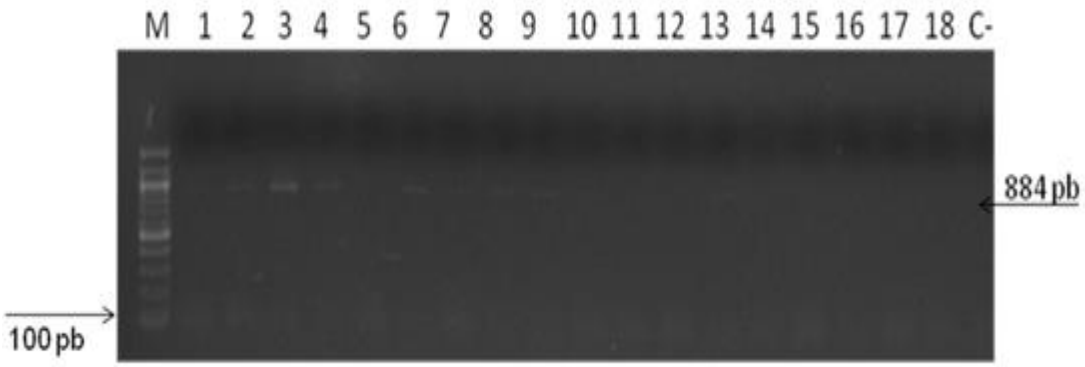

Figure 2. Detection curve for *E. coli*. Samples in triplicate and in CFU /mL: 2 x 10^5 (lanes 1 to 3), 2 x10^4 (lanes 4 to 6), 2 x10^3 (lanes 7 to 9), 2 x 10^2 (lanes 10 to 12), 2 x 10^1 (lanes 13 to 15), 2 (lanes 16 to 18). C - negative control. M - Molecular weight marker 100 bp (New England Biolabs®, UK). Agarose gel stained with 1.5% ethidium bromide.

PCR analysis

PCR was conducted using the primers for the 16S rRNA of *S. agalactiae* (GI: 386081764): Forward 5'CGGGTGAGTAACGCGTAGGTAA3' and reverse 5'GGTTAAGCCACTGCCTTTAACTTC3'. The conditions of the reaction were: 94°C for 30 s, 58°C for 45 s and 72°C for 45 s, during 30 cycles, providing a fragment of 524 bp corresponding to *S. agalactiae*. For *E. coli*, the primers: up 5´CCGATACGCTGCCAATCAGT3´ and down: 5´ACGCAGACCGTAGGCCAGAT3´ were used for the *uspA* gene (Chen and Griffiths, 1998). Reaction conditions were: 94°C for 2 min, 58°C for 1 min, and 72°C for 1 min, during 35 cycles producing a 884 bp fragment. For *L. monocytogenes*, primers for listeriolysin gene were used, generating an amplicon of 702 pb: LM1 5'CCTAAGACGCAATCGAA3' and LM2 5'AAGCGCTTGCAACTGCTC3' (Lawrence and Gilmour, 1994). Reaction conditions were: 94°C for 80 s, 50°C for 90 s and 72°C for 2 min during 30 cycles.

PCR reactions were performed on the thermal cycler (Techne® Barloworld Scientific, Stone, Staffordshire, UK) with a final volume of 50 µL using a PCR Buffer (20 mM of Tris HCL pH 8.4 and 50 mM of KCL), 1.5 mM of $MgCl_2$, 1.25 U of Taq polymerase, 0.4 µM of sense and antisense primers, and 0.2 mM of dNTP mix. The fragments generated were analyzed in agarose gel colored with ethidium bromide. The analysis of the gels was made with the image capture and photodocumentation system Gel Logic 200 (KODAK®RaytestGmbH, Straubenhardt, Germany).

Statistical analysis

All statistical analyses were performed using the Prism 5 (Graphpad®, California, EUA). The prevalence and frequency of contamination and sensitivity and specificity determination were accessed with exact 95% confidence interval (CI), Fisher's exact test and Chi-square; The gold method used was microbiological analysis. A $p < 0.05$ was considered statistically significant.

RESULTS

Sensibility and specificity of the PCR protocols tested were calculated based on the analysis of 16 curves for each of the microorganisms (Figures 1 to 3), and the PCR technique proved itself more sensible and specific for *S. agalactiae,* with a sensibility of 89.5% for a detection limit of 2 CFU/mL ($P < 0.001$, Table 1). For *E. coli*, the sensibility was 79.1% for a limit of 2 CFU/mL ($P < 0.001$, Table 2). The *L. monocytogenes* PCR protocol presented the lowest sensibility (16%) with a detection limit of 2 x 10^4 CFU/mL ($P = 0.0057$, Table 3).

The present study also evaluated the predominance of contamination in milk samples from cooling tanks, and obtained directly from the teats of the cows by PCR

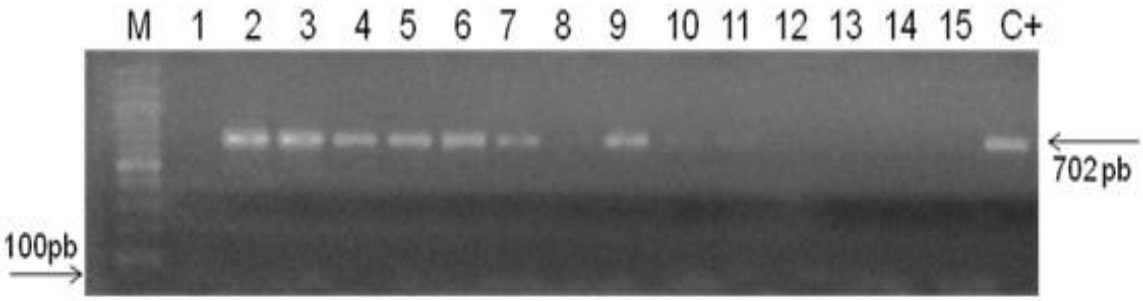

Figure 3. Detection curve for *L. monocytogenesi*. Samples in triplicate and in CFU /mL: 2 x 10^7 (lanes 1 to 3), 2 x 10^6 (lanes 4 to 6), 2 x 10^5 (lanes 7 to 9), 2 x 10^4 (lanes 10 to 12), 2 x 10^3 (lanes 13 to 15). C - negative control. C+ - positive control. M - Molecular weight marker 100 bp (New England Biolabs®, UK). Agarose gel stained with ethidium bromide.

Table 1. PCR sensitivity and specificity for *S. agalactiae* detection in raw milk. Each concentration was tested in 48 samples.

CFU/mL	2 x 10^6	2 x 10^5	2 x 10^4	2 x 10^3	2 x 10^2	2 x 10^1	2
Sensibility	100%	95.8%	100%	93.7%	97.9%	97.2%	89.5%
Especificity	100%	100%	100%	100%	100%	100%	100%
P-value	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001

Table 2. PCR sensitivity and specificity for *E. coli.* detection in raw milk. Each concentration was tested in 36 samples.

CFU/mL	2 x 10^6	2 x 10^5	2 x 10^4	2 x 10^3	2 x 10^2	2 x 10^1	2
Sensibility	100%	83.3%	95.8%	83.3%	95.8%	83.3%	79.1%
Especificity	100%	100%	100%	100%	100%	100%	100%
P-value	<0.0001	<0.0001	<0.0001	<0.0001	<00001	<0.0001	<0.0001*

Table 3. PCR sensitivity and specificity for *L.monocytogenes.*detection in raw milk. Each concentration was tested in 36 samples.

CFU/mL	2 x 10^7	2 x 10^6	2 x 10^5	2 x 10^4
Sensibility	83.3%	79,1%	50%	16.6%
Especificity	100%	100%	100%	100%
P-value	<0.0001	<0.0001.	<0.0001.	0.0057

technique. We verified that none of the 125 samples collected from the teats, as well as the 56 samples collected from the tanks, tested positive for *L. monocytogenes*. Regarding the samples collected from the tanks, 37.5% were positive for *S. agalactiae,* and 31.25% were positive for *E. coli*, and the analysis of the milk collected in the teats revealed that 47.5% of the samples tested positive for *E. coli,* and only 16.2% for *S. agalactiae.*

DISCUSSION

Raw milk is known as a carrier of pathogens, contributing to the acquisition of infectious diseases. In the USA, between 1998 and 2011, 2.384 diseases were associated with the consumption of raw milk and byproducts (CDC, 2013). The identification of the presence of microorganisms in the food is the gold standard method to determine food poisoning sources. Routinely, milk bacterial contamination is determined by means of microbiological culture of milk and biochemical characterization of isolated microorganisms. Despite being the gold standard method to determine food poisoning sources, the period necessary to obtain the

results by the current method is quite long. In average, it takes at least five days to confirm a negative result, and more than 10 days to confirm a positive result (Gasanov et al., 2005). Also, there are disadvantages associated with microbiological culture, such as: a milk culture may yield no bacteria from truly contaminated milk due to the presence of very low numbers of bacteria in the samples; and a result of negative culture may also occur due to the presence of residual therapeutic antibiotics that may inhibit *in vitro* bacterial growth.

Molecular techniques have been used as an alternative to microbiological methods for the detection of pathogens in food; however, regarding milk, most molecular studies isolate the microorganisms after sowing them in growth mediums, and not directly from the milk (Borela et al., 1999; Perez et al., 2002; Zocche et al., 2009). This probably happens due to the presence of inhibitory substances of the PCR in milk composition or because milk fat could cover bacterial surface and make lysis more difficult, thus lowering sensibility to PCR (Kim et al., 2001; Aslan et al., 2003).

The present study demonstrated a high sensibility and specificity for the PCR technique when detecting *S. agalactiae* and *E. coli*. A study obtained inferior results, with 85% sensibility and 82% specificity when detecting *S. agalactiae* in milk samples obtained from expansion tanks (Elias et al., 2012). The specificity and sensibility value achieved by this study is quite relevant since they demonstrate the capacity of the test to correctly identify samples that were really infected, and the samples that were really not infected, showing 100% reliability. Similarly, the detection limits achieved in this study were superior to those of Martinez et al. (2001) who achieved a sensibility limit of 100 CFU/mL for the same pathogen. Moreover, a detection limit of 2 CFU/mL was verified, being similar to the one of Kumar et al. (2013) that detected *E. coli* producers of the Shiga toxin (STEC) in samples of milk without pre-enriching. Meiri-Bendek et al. (2002) conducted a contamination of milk with different concentrations of *S. agalactiae* for later detection by PCR. Without enrichment, detection capacity varied from 10^4 to 10^5 CFU/mL, and its detection capacity was lower than the one presented in the present study. It is noteworthy to mention that enrichment of milk for microorganism detection increases the sample analysis time from 6 to 12 h. This contrasts with the need for fast and reliable methods aimed to be used in industrial routine.

L. monocytogenes has been a great concern for the food industry, since it is responsible for a significant percentage of food-transmitted diseases around the world (Gandhi and Chikindas, 2007). The detection of *L. monocytogenes* in food through traditional microbiological methods is time consuming, requiring the use of specific cultivation mediums for isolation (Vanegas et al., 2009). Therefore, the development of new ways for screening milk contamination by *L. monocytogenes* is of great interest. In the conditions tested, we observed a high sensibility only in contamination higher than 2×10^7 CFU/mL. Aznar and Alarcón (2003) also detected, through the PCR technique, *L. monocytogenes* artificially inoculated in different foods obtaining a sensibility from 1 to 10 CFU/mL. However, differently from the present study, the bacterial DNA was isolated directly from the cultivation plate after enrichment in a specific liquid for Listeria (LEB). Another study compared the PCR technique with conventional microbiology for the detection of *E. coli* O157, *Salmonella* spp. and *L. monocytogenes* in milk obtained from cooling tanks. Results revealed that the real time PCR was more sensitive in the detection of *E. coli* O157 than the culture method; while both methods were equally efficient in detecting *L. monocytogenes* (Amagliani et al., 2012). The diversity of results found in studies may occur due to the variation in quality and quantity of the DNA obtained with the use of different protocols. Furthermore, DNA extraction usually is performed using culture plates, and not directly from the milk or its byproducts.

The present study also evaluates the prevalence of contamination in samples of milk. Such scenario differs from others recently published in developed and developing countries. A study evaluated a total of 446 samples of raw milk, obtained from tanks of Iranian producers, in order to verify the presence of Listeria species. *Listeria* spp. was isolated in 18.6% of the samples, and the most found species was *Listeria innocua* (57.8%); followed by *L. monocytogenes* (21.7%); *Listeria welshimeri* (12%) and *Listeria seeligeri* (8.4%) (Jamali et al., 2013). In contrast, in the USA, a low prevalence of *L. monocytogenes* was observed, present in only three (2.3%) of the 113 samples collected from 11 dairy farms (D'amico et al., 2008).

Conclusion

The PCR methods here proposed for detection of milk contamination by *S. agalactiae* and *E. coli* have high sensibility and specificity. Providing quick and reliable results, the use of molecular methods can optimize and increase milk quality control in the dairy industry. Also, an important occurrence of contamination of milk prior industrial processing indicates milking and herd management problems. Industrial milk processing eliminates viable bacterial contamination; however in developing countries, the consumption of raw milk and dairy products is common, putting part of the population at risk of milk-borne infectious diseases.

Conflict of interest

The authors did not declare any conflict of interest

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