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Standardization of a rapid quadruplex PCR method for the simultaneous detection of bovine, buffalo, *Salmonella* spp., and *Listeria monocytogenes* DNA in milk

[*Padronização de um método rápido de PCR quadriplex para a detecção simultânea de DNA de bovino, bubalino, Salmonella spp. e Listeria monocytogenes em leite*]

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ABSTRACT

The objective of the present study was to Standardize a Polymerase Chain Reaction (PCR) protocol for the authentication of bovine and buffalo milk, and to detect the presence of *Salmonella* spp. and *Listeria monocytogenes*. For this, the target DNA was extracted, mixed, and subjected to a PCR assay. Milk samples were defrauded and experimentally contaminated with microorganisms to assess the detection of target DNA at different times of cultivation, bacterial titers, and concentration of genetic material. In addition, the protocol was tested with DNA extracted directly from food, without a pre-enrichment step. The proposed quadruplex PCR showed good accuracy in identifying target DNA sequences. It was possible to simultaneously identify all DNA sequences at the time of inoculation (0h), when the samples were contaminated with 2 CFU/250mL and with 6h of culture when the initial inoculum was 1 CFU/250mL. It was also possible to directly detect DNA sequences from the food when it was inoculated with 3 CFU/mL bacteria. Thus, the proposed methodology showed satisfactory performance, optimization of the analysis time, and a potential for the detection of microorganisms at low titers, which can be used for the detection of fraud and contamination.

Keywords: fraud, food safety, molecular techniques, food pathogens

RESUMO

O objetivo do presente estudo foi padronizar um protocolo de reação em cadeia da polimerase (PCR) para a autenticação de leite bovino e bubalino e a detecção da presença de Salmonella *spp. e Listeria monocytogenes. Para isso, o DNA-alvo foi extraído, misturado e submetido ao ensaio de PCR. Amostras de leite foram fraudadas e contaminadas experimentalmente com os micro-organismos, para se avaliar a detecção do DNA-alvo em diferentes tempos de cultivo, os títulos bacterianos e a concentração de material genético. Além disso, o protocolo foi testado com DNA extraído diretamente do alimento, sem a etapa de pré-enriquecimento. A PCR quadriplex proposta mostrou boa precisão na identificação de sequências de DNA-alvo. Foi possível identificar simultaneamente todas as sequências de DNA no momento da inoculação (0h), quando as amostras estavam contaminadas com 2 UFC/250mL, e com seis horas de cultura, quando o inóculo inicial foi de 1 UFC/250mL. Também foi possível detectar diretamente as sequências de DNA do alimento quando este foi inoculado com 3 UFC/mL de bactérias. Dessa forma, a metodologia proposta apresentou desempenho satisfatório, otimização do tempo de análise e potencial para detecção de micro-organismos em baixos títulos, podendo ser utilizada para detecção de fraude e contaminação.*

Palavras-chave: fraude, segurança alimentar, técnicas moleculares, patógenos alimentares

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INTRODUCTION

Currently, concerns regarding the quality and safety of food have increased due to scenarios in which several products, especially milk, are targets of contamination by pathogenic microorganisms and adulterations (Souza *et al*., 2011; Mullan, 2019; Farah *et al*., 2021). Food fraud is a widely discussed issue due to economic losses and potential health risks to consumers (Zhang and Xue, 2016; Soon *et al*., 2019). According to current Brazilian legislation (Brasil, 2020), all raw materials or products that are partially or totally deprived of any of their characteristic components, including ingredients or substances of any nature, are considered as fraudulent; these are often used in order to conceal, hide changes, quality deficiencies, manufacturing defects, and increased product volume or weight.

Many products of animal origin are subject to adulteration. Among them, milk and dairy products are the biggest targets of fraud (Zhang and Xue, 2016; Hansen and Holroyd, 2019). In addition, several authors (Spink and Moyer, 2011; Zhang and Xue, 2016) have pointed out that financial motivation has been one of the primary factors associated with fraudulent practices. Milk is considered as a healthy food, an essential component of a balanced diet from a nutritional and functional point of view, which can be used to make various dairy products and which has a positive impact on consumer's health (Verruck *et al*., 2019; Hansen and Holroyd, 2019).

However, in addition to bovine milk, milk from other species, such as buffalo milk, has gained market share (Araújo *et al*., 2011; Pignata *et al*., 2014). However, limitations regarding its availability and economic appeal make this food a potential target for fraud, mainly due to the incorporation of bovine milk (not mentioned on the label on the product), which is cheaper and more widely available (Darwish *et al*., 2009) and that it can modify the nutritional indices of the products (Viana *et al*., 2020). In addition to authentication, another concern regarding milk quality is contamination by pathogenic bacteria (Mullan, 2019), especially those belonging to the genus *Salmonella* and the specie *Listeria monocytogenes*. According to data from the Brazilian ministry of health, *Salmonella* spp. are among the most common agents related to cases of foodborne diseases between 2000 and 2018 (Brasil, 2019) and different serotypes have been detected in dairy products, with pathogenic potential and resistance to several antibiotics (Cunha-Neto *et al*., 2019). Contamination by *L. monocytogenes*, on the other hand, has become highly worrisome due to its high mortality rate, ranging from 30% to 75%, especially in high-risk groups (Liu, 2006; Nayak *et al*., 2015).

Determining the authenticity of dairy products and detecting the presence of microorganisms in these foods may not be a simple task, since the existing techniques for this purpose are usually expensive and time-consuming. Thus, molecular assays, such as the polymerase chain reaction (PCR), have emerged as an alternative solution to this problem (Gonçalves *et al*., 2014; Velioglu *et al*., 2017). The PCR technique reduces the time and cost of the analyses, which makes diagnosis faster and more accurate, owing to the high sensitivity and specificity of the method (Ghovvati *et al*., 2009).

Some researchers have already been able to acknowledge, through PCR, the presence of *Salmonella* spp. (Marathe *et al*., 2012; Li *et al*., 2017) and *L. monocytogenes* (Peres *et al*., 2010), separately or together (Gonçalves *et al*., 2014), in addition to the detection of fraud by mixing matter cousin of bovine and buffalo origin in fluid milk and its derivatives (Darwish *et al*., 2009; Silva *et al*., 2015). However, the development of a PCR protocol that authenticates bovine and/or buffalo products and simultaneously detects contamination by *Salmonella* spp. and *L. monocytogenes*, as performed in the present study, has not yet been proposed, which would make it possible to implement an important tool to be used to guarantee milk quality. Therefore, the objective of this study was to standardize a quadruplex PCR for the simultaneous detection of bovine, buffalo, *Salmonella* spp., and *L. monocytogenes* in fluid milk samples.

MATERIAL AND METHODS

To standardize the proposed methodology, refrigerated raw milk of bovine (*Bos taurus*, Girolando breed) and buffalo (*Bubalus bubalis*, Murrah breed) origin, as well as standard strains of *Salmonella enterica* serovar Typhimurium (ATCC 14028) and *Listeria monocytogenes* (ATCC 7644), were used as positive controls. To

obtain the DNA of the controls, the standard strains were grown in brain-heart infusion broth (BHI) (HiMedia®, India), at 37°C for 24h. After bacterial growth, the culture purity was controlled through bacterioscopy and Gram staining. Then, the cultures were centrifuged (Hettich® MIKRO^{TM} 120 centrifuge, Germany) for 2min at 14,000 rpm, the supernatant was discarded, and the resulting pellet was used for DNA extraction, following the protocol by Darwish *et al*. (2009), with some modifications: the addition of 600µL lysis solution (10mM Tris-HCl; 100mM NaCl; 1mM EDTA, pH 8; 0.5% SDS) to the pellet and 3µL of proteinase K (Ludwig Biotec®, Brazil) (20 mg/mL), followed by incubation at 37°C overnight. The remaining steps were performed according to the original protocol.

In order to obtain bovine and buffalo DNA from milk samples, 5mL aliquots of each sample were centrifuged for 10min at 4,000 rpm. Afterwards, the supernatant was discarded and 5mL of Tris-HCl-EDTA (TE) buffer (pH 8) was added, and another centrifugation was performed under the same conditions. The pellet was then resuspended in 5mL of TE, and 200µL of the obtained material was used for extraction, which was also carried out according to the method described by Darwish *et al*. (2009), but with changes in the volume of proteinase K used (20µL).

All extracted DNA were eluted in 30µL of TE, separated via electrophoresis on a 0.8% agarose gel, stained with non-mutagenic dye (GelRed^{TM}, Biotium, Califorina-USA), and visualized under ultraviolet light in a transillumination device (Gel Documentation System, Del Doc^{TM}, Bio-Rad®, California-USA), with the aid of Total Lab TL Lab 5.2 Software. The quality and concentration of the extracted material was assessed via spectrophotometry (NanoDrop® ND-1000 UV-Vis equipment, Thermo Fisher Scientific Inc., Massachusetts, EUA), using 260nm and 280nm filters. The extracted DNA were used separately as a positive control and, starting from a common concentration, were also mixed in the same proportion (1:1:1:1) forming a pooled suspension for the standardization of quadruplex PCR.

In addition, a negative control test using DNA of other species (*Escherichia coli, Staphylococcus aureus, Capra hircus*) was performed to evaluate the possibility of cross-reactions and attest to the specificity of the primers used in the reaction. All tests were performed in triplicate to ensure better data reliability. In the PCR assays, the primers were used to amplify specific genomic regions in cattle (reverse 5'-AAATAGGGTTAGATGCACTGAATCCAT-3'), buffaloes (reverse 5'-TTCATAATAACTTTCGTGTTGTTGGGTGT-3'), and a region common to both species (forward 5'-CTAGAGTATAGATC-3'). These oligo-initiators have been described by López-Calleja *et al*. (2005) and amplified fragments of 220 base pairs (bp) for buffalo DNA and 346 bp for bovine DNA. For *Salmonella* spp., the primers used (ST11: 5'-GCCAACCATTGCTAAATTGGCGCA-3' and ST15: 5'-GGTAGAAATTCCCAGCGG GTACTGG-3') amplified fragments 429 bp long, as described by Aabo *et al*. (1993). For *L. monocytogenes*, the following primers were used: LM1 (5'-CTAAGACGCCAATCGAA-3') and LM2 (5'-AAGCGCTTGCAACTGCTC-3'), described by Border *et al*. (1990), and amplified a 702 bp product.

The PCR reagent mixture was prepared to a final volume of 25µL, containing 0.4 pmol of each primer, 2.4mM $MgCl_2$, 100mM Tris-HCl (pH 8.5), 500mM KCl, (2× Buffer), 0.48mM 0.48mM of each deoxynucleotide triphosphate (DNTP), 2,5 U of Taq DNA Polymerase (all mixture reagents produced by Ludwig Biotec®, Brazil), approximately 80ng of template DNA (2µL), and sterile Milli-Q water. The reactions were carried out in a thermocycler (Applied Biosystems Veriti^{TM} Thermal Cycler, California, USA), programmed for the following steps: initial denaturation at 94°C for 5min, 35 cycles of denaturation (94 °C for 30 s), annealing (56 °C for 45 s), and extension (72 °C, for 45 s), and a final extension at 72 °C for 5min.

The PCR products were subjected to electrophoresis in a 1.5% agarose gel (Inlab^{TM}, Brazil), run in 0.5% TBE buffer, stained with non-mutagenic dye (GelRed^{TM}), using a 100 bp molecular standard as a marker (Ludwig Biotec®, Brazil). The results were read under ultraviolet light using a transillumination device (Gel Documentation System, Del Doc^{TM}, Bio-Rad®, California-USA). In order to determine the detection limit of the technique, fraud and experimental contamination was carried out. For this, 25mL samples of previously pasteurized, for 15 seconds, at 72 °C (High Temperature Short

Time Pasteurization -hTST) bovine milk were experimentally defrauded by the addition of buffalo milk (1%, 3%, 5%, 10%, 50% and controls with only bovine and buffalo milk).

Then three samples (containing a 1: 1 ratio of milk from each animal source) were contaminated with 1, 2, and 3 colony forming units (CFU) - treatments A, B, and C, respectively, of *S.* Typhimurium and *L. monocytogenes*, previously grown on Xylose Lysine Deoxycholate (XLD) (HiMedia®, India) and Polymyxin Acriflavin Lithium Chloride Ceftazidime Asculin Mannitol (PALCAM) (HiMedia®, India). In addition, a negative contamination control was also carried out from the cultivation of the milk sample without the inoculation of the microorganisms. The samples were grown at 37°C and collections of 2mL were performed in quadruplicate, at 0h, 6h, 12h, 18h, and 24h, in two replicates of the experimental contamination.

For each culture sample collected, the concentration of microorganisms present was verified from titration. For this, 1mL of each sample was diluted in 9mL of 0.85% saline and serial dilutions were performed (labeled 10^{-1} to 10^{-12}). The dilutions were seeded by spreading on XLD and PALCAM agar plates. In parallel, the samples collected were subjected to DNA extraction and the previously standardized quadruplex PCR, according to the procedures already described. In addition, experimentally defrauded milk samples were contaminated with 1 to 5 CFU of each bacterium per mL of milk and submitted to the extraction and PCR protocol without the enrichment step in the culture medium. Here, we sought to evaluate the efficiency of the proposed methodology directly from food samples. The data obtained from the replicates of the experiment were treated using descriptive statistics, adopting measures of central tendency (arithmetic mean) and variability (standard deviation).

RESULTS AND DISCUSSION

From the DNA extraction performed on milk samples and bacterial cultures, the genetic material obtained was of good quality and in sufficient concentration for molecular assays. The proposed quadruplex PCR was effective and accurate for the detection of bovine, buffalo, *Salmonella* spp., and *Listeria monocytogenes* DNA separately (positive controls) and together (pooled). In addition, there was no cross-reaction with the genetic material of other species used as a control during the assay, as observed in Figure 1.

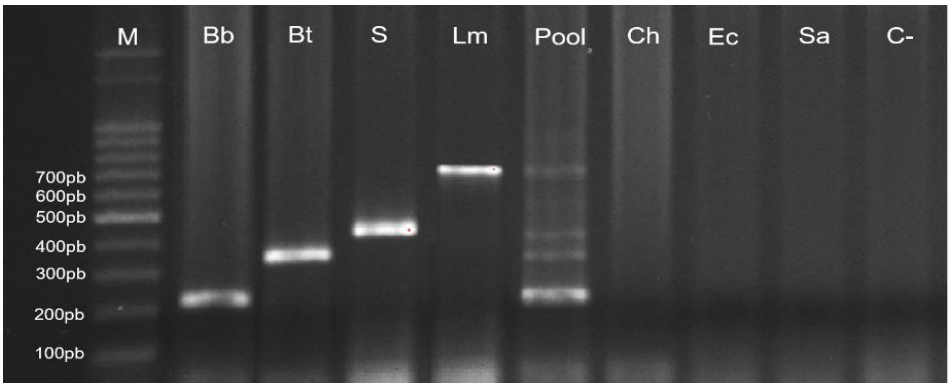

Figure 1. 1.5% agarose gel showing the presence of DNA fragments of bovine (346 bp), buffalo (220 bp), *Salmonella* spp. (429 bp) and *Listeria monocytogenes* (702 bp) origin, obtained from quadruplex PCR amplification. M: 100 bp molecular size marker; Bb: reaction only with buffalo DNA (*Bubalus bubalis*); Bt: reaction with bovine DNA (*Bos taurus*); S: reaction with *Salmonella* sp.; Lm: reaction with *Listeria monocytogenes* DNA; Pool: reaction with the mixture of DNAs obtained from each species; Ch: reaction with *Caprahircus* DNA; Ec: reaction with *Escherichia coli* DNA; Sa: reaction with *Staphylococcus aureus* DNA; C-: negative reaction control.

Molecular tests are quick, practical, sensitive, and can be used for several purposes. In food analysis, PCR can be successfully performed both for fraud investigation and the detection of microorganisms, as previously reported by other authors (Darwish *et al*., 2009; Tao *et al*., 2017; Chin *et al*., 2017). However, a methodology that simultaneously detects the presence of fraud and microbial contamination with high sensitivity, besides being a novelty, is important because it is economically viable, since it saves reagents and analysis costs.

The methodologies for verifying the presence of adulterations by incorporating raw material from different species and even for the identification of bacteria are difficult and time-consuming. In addition, little is known or discussed about the fact that a type of fraudulent practice predisposes to other problems such as microbial contamination, since producers/manipulators of malicious or uncompromised foods are unlikely to guarantee the authenticity and quality of their products probably do not perform the control of the milk they use for fraud. Thus, the development of a single reaction that simultaneously discriminates the presence of DNA of bovine, buffalo, *Salmonella* spp., and *Listeria monocytogenes* origin is an important advancement in the inspection of products of animal origin and in public health.

In the present study, fraud by mixing milk from different species was detected from the lowest percentage tested. The technique was able to detect since 1% of addition of bovine milk incorporated to the buffalo. The adulterating practice does not only harm the consumer from an economic point of view, but it also brings potential health risks. Individuals who choose to consume milk and buffalo derivatives because of certain food restrictions to bovine milk, such as allergies, can develop serious complications (Fiocchi *et al*., 2010). Darwish *et al*. (2009) developed and applied PCR to check the presence of bovine DNA in buffalo milk. These authors mixed the milk of the two species in different proportions and managed to amplify the genetic material, with the minimum 0.5% of the incorporation of bovine milk.

Then, they applied this protocol to commercial products, in which, of the 21 samples evaluated, 10 were in fact buffalo milk, 8 were fraudulent with cow's milk, and in 3 of the samples there was fraud arising from a total replacement of the raw material, concluding that fraud is a common practice in the dairy market.In the experimental contamination of milk, good microbial growth was observed in the treatments inoculated with 1, 2, and 3 CFU (Table 1). After 6h of cultivation, *L. monocytogenes* reached a concentration of 10^6 CFU/mL, reaching a maximum of 10^{15} CFU/mL at the end of the experiment (24h of cultivation). *Salmonella* sp. presented concentrations of 10^9 CFU/mL at 6h of culture and reached a maximum final concentration of up to 10^{16} CFU/mL.

From the PCR performed after the collection of the experimental contamination, it was possible to observe the amplification of the DNA of the four species after 6h of culture, when the samples were contaminated with 1 CFU of each bacterium. When the samples were contaminated with 2 and 3 CFU of each bacterium, there was an amplification of the DNA of all species from 0h of culture. Concerning the detection limit, the lowest concentration of DNA detected from the four species occurred simultaneously in the sample contaminated with 2 CFU/250mL of each bacterium in the collection performed at the time of inoculation (0h), when the concentration of genetic material was 70 ng/μL (Table 1).

In the present study, it was found that milk is an excellent substrate for the proliferation of *L. monocytogenes* and *Salmonella* sp., since the bacterial titers were high and both bacteria grew concomitantly without significantly inhibiting the proliferation of the other. However, higher concentrations of *Salmonella* sp. than *L. monocytogenes* over the cultivation period. It has been stated in the literature that there is a certain difficulty in the growth of *L. monocytogenes* in the face of the competing microbiota (Omiccioli *et al*., 2009; Peres *et al*., 2010). Peres *et al*. (2010) experimentally contaminated milk with this microorganism in sterile food samples and with different aerobic and mesophilic counts. In some of these treatments, there was no growth of *L. monocytogenes*.

Table 1. Limit of detection of PCR in samples experimentally contaminated with 1, 2, and 3 CFU of *Salmonella* Typhimurium and *Listeria monocytogenes* subjected to enrichment in a culture medium

Treatments	[DNA]* ng/µL X±SD**	Purity X±SD	*Salmonella* sp.	*Listeria monocytogenes*	PCR***
A 0h	63.3±0.75	1.58±0.01	1 CFU/ 250mL	1 CFU/ 250mL	-
A 6h	410±1.00	1.86±0.01	4.96×10^{9} CFU/mL	4.00×10^{6} CFU/mL	+
A 12h	1050±1.00	1.90±0.06	1.13×10^{11} CFU/mL	1.20×10^{8} CFU/mL	+
A 18h	1189±0.57	1.60±0.00	1.04×10^{13} CFU/mL	2.13×10^{11} CFU/mL	+
A 24h	1450±0.86	1.90±0.02	1.59×10^{16} CFU/mL	1.36×10^{15} CFU/mL	+
B 0h	70±0.50	1.62±0.00	2 CFU/ 250mL	2 CFU/ 250mL	+
B 6h	423±1.00	2.00±0.12	4.36×10^{9} CFU/mL	2.00×10^{8} CFU/mL	+
B 12h	1150±1.73	1.77±0.01	1.89×10^{11} CFU/mL	1.20×10^{10} CFU/mL	+
B 18h	1275±0.28	1.63±0.02	2.32×10^{14} CFU/mL	1.23×10^{11} CFU/mL	+
B 24h	1500±1.00	1.77±0.02	1.60×10^{16} CFU/mL	4.00×10^{13} CFU/mL	+
C 0h	132.5±0.17	1.65±0.01	3 CFU/ 250mL	3 CFU/ 250mL	+
C 6h	386±0.86	1.90±0.05	5.12×10^{9} CFU/mL	8.00×10^{6} CFU/mL	+
C 12h	1615±1.15	1.69±0.06	1.88×10^{11} CFU/mL	8.00×10^{9} CFU/mL	+
C 18h	1335±1.15	1.65±0.01	2.40×10^{13} CFU/mL	4.00×10^{11} CFU/mL	+
C 24h	1432±0.57	1.74±0.02	7.20×10^{15} CFU/mL	1.20×10^{14} CFU/mL	+

Treatments: A = contamination with 1 CFU of each bacterium; B = contamination with 2 CFU of each bacterium; C = 3 CFU contamination of each bacterium. *[.] DNA concentration **X= arithmetic mean; SD= Standard Deviation; ***(+) Positive; (-) Without amplification of all DNAs; The result was considered positive when the DNA of the four species was amplified simultaneously.

These authors concluded that the accompanying flora can, in addition to hindering recovery, reduce the multiplication of the pathogen. This is a serious problem for the analysis of foods by conventional methodologies, in which contamination with pathogens will only be diagnosed if they are able to survive the competition and have viability for growth in the culture media. Thus, the importance of developing more sensitive alternative methodologies, such as the PCR technique proposed here, is emphasized.

The pre-enrichment stage favors the diagnosis of microorganisms, increasing the efficiency of PCR (Omiccioli *et al*., 2009), which was also observed in the present study. In this procedure, a portion of the food (25mL) is added to the culture medium. It is believed that when the sample is well-homogenized, the components are more dispersed, which facilitates the separation of the somatic milk cells which contain the DNA that identify the species in the fraud as well as other compounds like fat. Microorganisms are also associated with the culture medium, which favors microbial growth and a greater availability of the cells from which their DNA will be extracted. The pre-enrichment phase has already been identified as of great importance for the PCR protocol (Rissato *et al*., 2011, Ding *et al*., 2017).

Ding *et al*. (2017) developed a real-time PCR method with the addition of the pre-enrichment step for detecting pathogens in raw milk and obtained a detection limit of 14 and 10 CFU for *L. monocytogenes* and *Salmonella* spp., respectively. The setups they used included 25mL of milk added in 225mL of BHI and incubated for 4h; without pre-enrichment, direct microbial detection from the food happened only from a concentration of 10^2 CFU/mL of each bacterium. For these authors, the identification of pathogens in food samples with low levels of contamination can be difficult and time-consuming, requiring the incorporation of an enrichment step. This step optimizes the procedures by increasing the concentration of the target microorganism and recovering injured cells, reducing false-negative results.

Sheng *et al*. (2018) artificially contaminated milk with *L. monocytogenes* and collected samples at intervals of 4, 6, 8, 10, and 12h after cultivation. These authors detected bacteria with a minimum cultivation time of 6h, when the sample was inoculated with 8.6×10^3 CFU/mL. After 8h of culture, when the bacterial titer was 8.6×10^3 and when the sample was inoculated with the lowest concentration of pathogens tested (8.6×10^2) it was only possible to detect *L. monocytogenes*

after 12h of culture. On the other hand, Chin *et al*. (2017) identified *Salmonella* spp. using conventional PCR after an enrichment of 12h when the bacterial titer was 10^5 CFU/mL. On the other hand, in our work, it was possible to detect fraud and the two bacteria in a lower cultivation time and with a lower concentration of microorganisms. This makes our protocol a more practical, quick, and sensitive alternative for the investigation of adulterations and contamination by these pathogens.

DNA extraction was performed directly from contaminated and experimentally defrauded milk, without the pre-enrichment step in culture medium, making it possible to amplify the four DNAs from 3 CFU for each mL of milk. Fraud was detected in all tests, and *Salmonella* sp. was identified in treatments with 1 and 2 CFU/mL, whereas the DNA of *L. monocytogenes* was only detected when inoculated in food at concentrations above 3 CFU. The lowest concentration of DNA for the detection of the four species simultaneously occurred when 135 ng/µL was obtained. Table 2 shows the DNA concentrations and purity of each treatment, as well as the result of each PCR assay adopted at this stage of the study.

Table 2. Limit of detection of PCR in samples experimentally contaminated with 1-5 CFU of each bacterium, after performing DNA extraction directly from the food

Treatments	[DNA]* ng/µL X±SD**	Purity X±SD	*Salmonella* sp.	*Listeria monocytogenes*	PCR***
1	70±1.52	1.53±0.02	1 CFU/mL	1 CFU/mL	-
2	85±0.28	1.60±0.01	2 CFU/mL	2 CFU/mL	-
3	135±2.51	1.58±0.03	3 CFU/mL	3 CFU/mL	+
4	290±1.04	1.58±0.01	4 CFU/mL	4 CFU/mL	+
5	300±3.00	1.63±0.01	5 CFU/mL	5 CFU/mL	+

*[.] DNA concentration; **X= arithmetic mean; SD= Standard Deviation; ***(+) Positive; (-) Without amplification of all DNAs; The result was considered positive when the DNA of the four species was amplified simultaneously.

The diagnosis of pathogens in foods without the pre-enrichment step decreases the analysis time; however, it must be done with caution because it reduces the DNA identification capacity of the microorganisms when they are in low concentrations in the sample (Peres *et al*., 2010). According to Aslam *et al*. (2003) there is a lot of difficulty in obtaining quality DNA from food samples due to their composition. Some components, such as fat, in the case of milk, can interfere with the test, as it makes access to genetic material difficult (Aslam *et al*., 2003). In addition, microorganisms can interact with fat, becoming associated with lipid globules, which represents, another obstacle to the molecular procedure.

Marathe *et al*. (2012) identified *Salmonella* spp. in samples of milk, fruit juice, and ice cream, without with the pre-enrichment stage. These authors discussed the existence of inhibitors of the PCR reaction present in foods, as in the case of milk with ahigh content of proteins and fat organized in micelles. They also endorsed the need to eliminate these inhibitors. In the current study, we made changes in the initial stages of the extraction protocol in an attempt to remove excess fat from milk. In addition, Marathe *et al*. (2012) achieved the amplification of *Salmonella* spp. when the concentration of bacteria was between 5 and 10 CFU/mL. Here, we were able to identify microorganisms directly from the food when their concentration is at least 3 CFU/mL.

Generally speaking, the dairy farming has economic importance worldwide and production indices are increasing (Dairy…, 2020). This activity generates great profitability if well conducted, but measures to control production are easier to be adopted by medium and large producers (Melo and Barros, 2014). Thus, for small producers, the challenges are to balance product quality, remain competitive in the market and guarantee subsidies for investing in business. Especially on buffalo milk and derivatives, many consumers looking for differentiated products pay up to twice as much as the commercial value of derivatives and bovine milk (Santini *et al*., 2013; Seixas *et al*., 2014), which demonstrates the high added value of this food.

Although buffalo dairy products have gained space in the market, the production chain needs organization and supervision, since many small companies operate underground (Seixas *et al*., 2014), which can favor the occurrence of adulterations and microbial contamination, especially in locations that operate without the adoption of good manufacturing practices and in incipient sanitary conditions. In this sense, the action of Organs inspection bodies is essential and that they have quick, practical and economically viable methodologies like the one proposed here.

CONCLUSIONS

The molecular assay proposed in the present study proved to be effective for the identification of the bovine, buffalo, *Salmonella* spp. e *Listeria monocytogenes*. The method allowed the identification of fraud and bacterial contamination, based on low concentrations of DNA obtained from samples enriched in culture medium and directly from food, in a short period of time. In this way, the quadruplex PCR protocol can be used as an important tool in the milk inspection service by inspection agencies.

REFERENCES

AABO, S.; RASMUSSEN, O.F.; ROSSEN, L. *et al*. *Salmonella* indentification by the Polymerase Chain Reaction. *Mol. Cell. Probes*, v.7, p.171-178, 1993.

ARAÚJO, T.P.M.; RANGEL, A.H.N.; SOARES, A.D. *et al*. Influência das estações do ano sobre a composição do leite de búfalas mantido em tanque de resfriamento. *Agropecuária Cient. Semiárido*, v.7, p.1-5, 2011.

ASLAM, M.; HOGAN, J.; SMITH, K.L. Development of a PCR-based assay to detect Shiga toxin-producing *Escherichia coli*, *Listeria monocytogenes*, and *Salmonella* in milk. *Food Microbiol.*, v.20, p.345-350, 2003.

BORDER, P.M.; HOWARD, J.J.; PLASTOW, G.S.; SIGGENS, K.W. Detection of *Listeria* species and *Listeria monocytogenes* using Polymerase Chain Reaction. *Lett. Appl. Microbiol.*, v.11, p.158-162, 1990.

BRASIL. Ministério da Agricultura, Pecuária e Abastecimento. Regulamento de Inspeção Industrial e Sanitária de Produtos de Origem Animal – RIISPOA. Aprovado pelo Decreto nº 10.468, de 18 de agosto de 2020. *Diário Oficial da União*, Brasília, DF, 19 agosto 2020.

BRASIL. Ministério da Saúde. Surto de doenças transmitidas por alimentos no Brasil. Manual integrado de vigilância, prevenção e controle de doenças transmitidas por alimentos. 2019. Available in: http://bvms.saude.gov.br/bvs/publicacoes/manual_integrado_vigilancia_doencas_alimentos.pdf. Accessed in: Nov., 2020.

CHIN, W.H.; SUN, Y.; HOGBERG, J. *et al.* Direct PCR e A rapid method for multiplexed detection of diferente serotypes of *Salmonella* in enriched pork meat samples. *Mol. Cell. Probes*, v.32, p.24-32, 2017.

CUNHA-NETO, A.; CARVALHO, L.A.; CASTRO, V.S. *et al*. *Salmonella* Anatum, *S.* infantis and *S.* Schwarzengrund in Brazilian cheeses: occurrence and antibiotic resistance profiles. *Int. J. Dairy Technol.*, v.70, p.1-5, 2019.

DAIRY market review, March, 2020. Roma: FAO. Available in: http://www.fao.org/economic/est/est-commodities/dairy/milk-and-milk-products/en. Accessed in: Jan. 2021.

DARWISH, S.F.; ALLAM,h.A.; AMIM, A.S. Evaluation of PCR assay for detection of cow's milk in water buffalo's milk. *World Appl. Sci. J.*, v.7, p.461-467, 2009.

DING, T.; SUO, Y.; ZHANG, Z. *et al*. A multiplex RT-PCR assay for *S. aureus*, *L. monocytogenes* and *Salmonella* spp. detection in raw milk with pre-enrichment. *Front. Microbiol.*, v.8, p.1-11, 2017.

FARAH, J.S.; CAVALCANTI, R.N.; GUIMARÃES, J.T. *et al*. Differential scanning calorimetry coupled with machine learning technique: An effective approach to determine the milk authenticity. *Food Control*., v.121, p.1-9, 2021.

FIOCCHI, A.; BROZEK, J.; SCHÜNEMANN, H. *et al*. World allergy Organization giagnosis and rationale for action against Cow's Milk Allergy (DRACMA) Guidelines. *World Allerg. Organ. J.*, v.3, p.57-161, 2010.

GHOVVATI, S.; NASSIRI, M.R.; MIRHOSEINI, S.Z. *et al*. Fraud identification in industrial meat products by multiplex PCR assay. *Food Control.*, v.20, p.696-699, 2009.

GONÇALVES, J.S.; CHEIRUBIM, A.P.; BRITO, K.C.T.; BRITO, B.G. Detecção de *Salmonella* spp e *Listeria monocytogenes* através de técnica de PCR. *Arq. Ciênc. Vet. Zool.*, v.17, p.223-226, 2014.

HANSEN, P.W.; HOLROYD, S.E. Development and application of Fourier transform infrared spectroscopy for detection of milk adulteration in practice. *Int. J. Dairy Technol.*, v.72, p.321-331, 2019.

LI, F.; LI, F.; CHEN, B. *et al*. Sextuplex PCR combined with immunomagnetic separation and PMA treatment for rapid detection and specific identification of viable *Salmonella* spp., *Salmonella enterica* serovars Paratyphi B, *Salmonella* Typhimurium, and *Salmonella* Enteritidis in raw meat. *Food Control.*, v.73, p.587-594, 2017.

LIU, D. Identification, subtyping and virulence determination of *Listeria monocytogenes*, an important foodborne pathogen. *J. Med. Microbiol.*, v.55, p.645-659, 2006.

LÓPEZ-CALLEJA, I.; GONZALEZ, A.; FARJADO, V. *et al*. PCR detection of cow's milk in water buffalo milk and mozzarella cheese. *Int. Dairy J*., v.15, p.1122-9, 2005.

MARATHE, S.A.; CHOWDHURY, R.; BHATTACHARYA, R. *et al*. Direct detection of Salmonella without pre-enrichment in milk, ice-cream and fruit juice by PCR against hilA gene. *Food Control.*, v.23, p.559-563, 2012.

MELO, A.D.; BARROS, L.S.S. Perfil sócio-econômico dos produtores de leite, vinculados aos laticínios registrados no serviço de inspeção estadual (SIE) da Coordenadoria Regional/ADAB de Vitória da Conquista – BA. *Agropecu. Cient Semiárido*, v.10, p.1-11, 2014.

MULLAN, W.M.A. Are we closer to understanding why viable cells of Mycobacterium avium subsp. paratuberculosis are still being reported in pasteurised milk? *Int. J. Dairy Technol.*, v.72, p.332-344, 2019.

NAYAK, D.N.; SAVALIA, C.V.; KALYANI, I.H. *et al*. Isolation, identification, and characterization of *Listeria* spp. from various animal origin foods. *Vet. World*, v.8, p.695-701, 2015.

OMICCIOLI, E.; AMAGLIANI, G.; BRANDI, G.; MAGNANI, M.; A new platform for Rel-Time PCR detection of *Salmonella* spp., *Listeria monocytogenes* and *Escherichia coli* O157 in milk. *Food Microbiol.*, v.26, p.615-622, 2009.

PERES, N.D.; LANGE, C.C.; BRITO, M.A.V.P. *et al*. Detecção de *Listeria monocytogenes* pela técnica de PCR em leite contaminado artificialmente. *Arq. Bras. Med. Vet. Zootec.*, v.62, p.973-979, 2010.

PIGNATA, M.C.; FERNANDES, S.A.A.; FERRÃO, S.P.B. *et al.* Estudo comparativo da composição química, ácidos graxos e colesterol de leites de búfala e vaca. *Rev. Caatinga*, v.27, p.226-233, 2014.

RISSATO, D.P.; BORGO, A.P.; MOREIRA, J.P. *et al*. Detecção de *Salmonella* spp. em água de lavagem de carcaças de frango utilizando o método de Reação em Cadeia da Polimerase (PCR). *Rev. Saúde Pesqui.*, v.4, p.35-39, 2011.

SANTINI, G.A.; BERNARDES, O.; SCARPELLI, J.U. Análise das relações comerciais do segmento de processamento de leite e derivados de leite de búfala no estado de São Paulo. *Inform. Econ.*, v.43, 69-84, 2013.

SEIXAS, V.N.C.; CORREIA, L.F.M.; PERRONE, I.T. *et al.* Diagnóstico socioeconômico dos produtores de queijos do Marajó. *Rev. Inst. Laticínios Cândido Tostes*, v.69, p. 309-321, 2014.

SHENG, J.; TAO, T.; ZHU, X. *et al.* A multiplex PCR detection method for milk based on novel primers specific for *Listeria monocytogenes* 1/2a serotype. *Food Control.*, v.86, p.183-190, 2018.

SILVA, C.L.; SALES, G.A.; SANTOS NETO, J.G. *et al*. Detecção de fraude em amostras comerciais de queijo bubalino por adição de leite bovino por meio da técnica de Reação em Cadeia da Polimerase (PCR) multiplex. *Rev. Inst. Adolfo Lutz*, v.74, p.21-29, 2015.

SOON, J.M.; KRZYZANIAK, S.C.; SHUTTLEWOOD, Z. *et al*. Food fraud vulnerability assessment tools used in food industry. *Food Control.*, v.101, p.225-232, 2019.

SOUZA, S.S.; CRUZ, A.G.; WALTER, E.H. *et al*. Monitoring the authenticity of Brazilian UHT milk: a chemometric approach. *Food Chem.*, v.124, p.692-695, 2011.

SPINK, J.; MOYER, D.C. Defining the public health threat of food fraud. *J. Food Sci.*, v.76, p.157-163, 2011.

TAO, T.; CHEN, Q.; BIE, X. *et al*. Investigation on prevalence of *Listeria* spp. and *Listeria monocytogenes* in animal-derived foods by multiplex PCR assay targeting novel genes. *Food Control.*, v.73, p.704-711, 2017.

VELIOGLU, S.D.; ERCIOGLU, E.; BOYACI, I.H. Rapid discrimination between buffalo and cow milk and detection of adulteration of buffalo milk with cow milk using synchronous fluorescence spectroscopy in combination with multivariate methods. *J. Dairy Res.*, v.84, p.1-6, 2017.

VERRUCK, S.; BALTHAZAR, C.F.; ROCHA, R.S. *et al*. Dairy foods and positive impact on the consumer'shealth. *Adv. Food Nutr. Res*., v.89, p.95-164, 2019.

VIANA, M.P.; FERNANDES, S.A.A.; SILVA, A.G. *et al*. Fraud with the addition of cow's milk alters the lipid fraction of buffalo mozzarella. *Food Sci. Technol*., v.40, p.761-768, 2020.

ZHANG, W.; XUE, J. Economically motivated food fraud and adulteration in China: an analysis based on 1553 media reports. *Food Control.*, v.67, p.192-198, 2016.