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ORIGINAL ARTICLE

Assessment of a multiplex detection method for *Salmonella enterica*, *Escherichia coli* O157:H7, and *Listeria monocytogenes* in cow milk

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Abstract

Raw cow milk is considered one of the most important vehicles for pathogenic bacteria like *Salmonella* spp., *Escherichia coli* O157:H7, and *Listeria monocytogenes*. These three bacteria are responsible for foodborne diseases. Routine microbiological methods to detect these microorganisms in cow milk can be complicated and time consuming. The aim of this work was to evaluate a method to simultaneously detect *Salmonella* spp., *Escherichia coli* O157:H7, and *Listeria monocytogenes* in experimentally contaminated cow milk. The assessed method combined a standard microbiological culture step, using a pre-enrichment medium that favors the growth of the three focal microorganisms: SEL broth, followed by a single PCR assay. A total of 43 interference bacterial strains were used to evaluate the method's specificity. The detection rate for the microbiological method with standard culture media was 10 UFC/mL, and that of the PCR detection, following pre-enrichment in SEL broth, was 10 UFC/mL for *S. enterica* and *L. monocytogenes* and between 1 and 5 UFC/mL for *E. coli* O157:H7. The PCR method showed specificity for the reference strains. Simultaneous detection by multiple PCR using SEL broth was successful for the detection of *S. enterica*, *E. coli* O157:H7, and *L. monocytogenes* in samples of experimentally contaminated cow milk, featuring both a high detection rate and a high specificity. This approach promises to be a feasible routine procedure when testing milk samples in industry and public health control setups.

Keywords: Foodborne diseases; food safety; hygienic quality of milk.

Introduction

The main components of cow milk are water, proteins, fat, carbohydrates, and minerals. These components exhibit quantitative variation both within and between individuals and their proportions in milk chiefly depend on cow race, feed, age, lactation period, time of the year, and milking system [1].

Along its production chain, cow milk quality and safety might be threatened by biological, physical, and chemical hazards. Inadequate milking and manipulation practices, lack of boiling, and insufficient cooling methods during raw milk harvesting may lead to microbial growth in a very short period, thus increasing the risk of food poisoning and foodborne infections in consumers [2].

The most important biological contaminants of raw cow milk include bacteria like *Salmonella* spp., *Escherichia coli* O157:H7, and *Listeria monocytogenes* [3]. These bacteria have been associated to food poisoning cases in the United States [4]. In 2014, a total of 140 food poisoning outbreaks were associated to *Salmonella*, 23 to Shiga toxin-producing *E. coli*, and 9 to *L. monocytogenes*; 80 out of the 140 *Salmonella* outbreaks were originated by consumption of food of animal origin. Among these, 15 cases were associated to the intake of unpasteurized dairy products, 3 cases due to consumption of pasteurized milk products, and 1 case involved a dairy product lacking information on hygienic protocols [4]. In Colombia, various investigations have demonstrated the presence of pathogenic microorganisms such as *Brucella* spp., *L. monocytogenes*, and *Staphylococcus aureus* in cow milk, and public health surveillance bodies have reported outbreaks of *E. coli* and coagulase-positive *Staphylococcus* associated with milk consumption [5, 6].

Commonly employed microbiological identification methods for *Salmonella enterica*, *E. coli* O157:H7, and *L. monocytogenes* in milk and dairy products rely on growing samples in selective media [7, 8]. Depending on the culture medium used, presence/absence of colonies is evaluated. Then, biochemical phenotyping and serotyping assays are performed. These culture-based microbiological detection methods take several days to identifying a given pathogen. In industry and public health surveillance setups, efficient and accurate testing methodologies are needed to ensure the timely release of consumer-safe milk and dairy products into the market. Molecular techniques are fast and efficient to detect pathogenic bacteria in food, particularly in milk. By combining both methodologies, the milk industry can have reliable test results over a short time period [7-10].

A promising molecular method for food testing is the multiplex PCR (mPCR). This method uses a group of target-specific primers to amplify different DNA fragments in a single reaction. With this technique, multiple microbial species can be simultaneously detected since the pool of primers consists of sequences targeted to a given bacterial species [11, 12]. This approach has been successfully applied to detect *S. enterica*, *E. coli* O157, and *L. monocytogenes* in milk [13], meat [14], cereals [15], and kimchi [16], offering the possibility to implement it as a routine technique.

In the present study we assessed the effectiveness of the mPCR technique in detecting *S. enterica*, verotoxin-producing *E. coli* (*E. coli* O157:H7), and *L. monocytogenes* in experimentally contaminated cow milk. In parallel we conducted culture-based conventional microbiological identification tests, evaluating the use of a culture medium that favors the growth of the three focal bacteria.

Materials and methods

Bacterial strains

The bacterial strains used in this study were obtained from different reference collections. The *S. enterica* and *L. monocytogenes* reference strains were obtained from the American Type Culture Collection (strain codes ATCC13076 and ATCC19115, respectively; Microbiologics®, USA) and the *E. coli* O157:H7 strain was obtained from the Collection of Microorganisms at Universidad Javeriana (strain code CMDM0218; Colombia). In addition, 43 bacterial strains, across 11 genera, were used to assess the mPCR assay specificity (**Table 1**). These microorganisms were considered because they are also common contaminants of milk [5, 6, 17].

Culture-based approach: simultaneous detection of *Salmonella enterica*, *Escherichia coli* O157:H7 and *Listeria monocytogenes* with SEL broth.

For the simultaneous detection of *S. enterica*, *E. coli* O157:H7, and *L. monocytogenes* in cow milk, experimentally contaminated milk samples with these three microbes were cultured in SEL broth, as a pre-enrichment step, according to the method by Kim & Bhunia [18]. Briefly, seven different microbial concentrations in a range of 10^0 to 10^6 UFC/mL, of each microorganism, were used to experimentally contaminate 10 ml of commercial ultra-pasteurized (UHT) milk, which was previously confirmed free of pathogens by conventional microbiological methods and PCR. Each 10 ml contaminated milk sample was used to inoculate 90 mL of SEL broth. SEL broth cultures were incubated for 6 hours at 35 ± 2 °C. Later, each dilution was streaked in Hektoen agar, Mac Conkey sorbitol agar supplemented with cefixime and tellurite, and oxford agar [19-22]. All the plates were incubated at 37 °C for 24 hours.

In parallel, the set of experimentally contaminated milk samples were evaluated for microbial contamination in three different selective culture media according to FDA and INVIMA recommendations [19-22]: Lactose pre-enriched broth was used to grow *Salmonella* [21], EC broth supplemented with Novobiocin (EC-N) for *E. coli* O157:H7, and buffered *Listeria* medium (Oxoid® Thermo Fisher Scientific, USA) for *Listeria*. All cultures were

Table 1. Bacterial strains used to assess multiplex PCR assay specificity. **1**. Collection Universidad Javeriana; **2**. Bank of Germplasm, Bacteria and Virus (AGROSAVIA); **3**. Collection Universidad Colegio Mayor de Cundinamarca; **4**. INVIMA; **5**. Instituto Colombiano Agropecuario (ICA); **6**. Universidad Nacional; **7**. Work collection AGROSAVIA.

No	Bacterial strain	Source
1	*Bacillus cereus* CD DM 019	1
2	*Bacillus subtilis* CD DM 025	1
3	*Brucella abortus*	2
4	*Escherichia coli* B001	2
5	*Escherichia coli* ATCC 25992	1
6	*Escherichia coli* O 157:H7 CD DM 218	1
7	*Klebsiella ascorbata* CD DM 042	1
8	*Klebsiella pneumoniae*	3
9	*Klebsiella pneumoniae* CD DM 041	1
10	*Klebsiella* spp.	1
11	*Listeria inocua* ATCC 33090	4
12	*Listeria ivanovii* ATCC 19119	4
13	*Listeria monocytogenes* ATCC 35152	4
14	*Listeria monocytogenes*	1
15	*Listeria monocytogenes* ATCC 19115	7
16	*Listeria* spp.	7
17	*Listeria* spp.	7
18	*Listeria* spp.	7
19	*Micrococcus luteuos* CD DM 090	1
20	*Proteus vulgaris*	3
21	*Pseudomona* spp.	1
22	*Pseudomona aeruginosa* ATCC 27853	3
23	*Salmonella* spp.	6
24	*Salmonella* spp.	5
25	*Salmonella Enteritidis*	7
26	*Salmonella* spp.	1
27	*Salmonella* spp.	1
28	*Salmonella* spp.	1
29	*Salmonella* spp.	1
30	*Staphylococcus aureus*	4

31	*Staphylococcus aureus* ATCC 29213	3
32	*Staphylococcus aureus* CD DM 080	1
33	*Staphylococcus aureus* NCI MB 12702 R2	5
34	*Staphylococcus* spp.	7
35	*Staphylococcus* spp.	7
36	*Streptococcus agalactiae*	3
37	*Streptococcus pyogenes*	3
38	*Streptococcus* spp.	6
39	*Streptococcus* spp.	6
40	*Streptococcus* spp.	6
41	*Streptococcus* spp. NCI MB 701348 R3	5
42	*Yersinia enterocolitica* ATCC 23715	7
43	*Yersinia pseudotuberculosis* ATCC 29833	7

incubated at 35 ± 2 °C for 24 hours. After incubation, an aliquot of each broth was streaked in selective agar broths: Hektoen agar, Mac Conkey sorbitol agar supplemented with cefixime and tellurite, and oxford agar. All the plates were incubated at 37 °C for 24 hours.

Molecular approach: simultaneous detection of *S. enterica*, *E. coli* O157:H7 and *L. monocytogenes* by mPCR

The employed DNA extraction protocol followed molecular methods for microbial food contaminants [23-25]. After pre-enrichment in SEL broth, three 1 mL aliquots were taken from each culture; then, bacterial cells pellets were obtained with centrifugation at 4 600 rpm for 5 minutes, followed by two washes with PBS buffer (137 mM NaCl; 2.7 mM KCl; 10 mM NaH_2PO_4; 2 mM KH_2PO_4, pH 7.4) with centrifugation at 4 600 rpm for 5 minutes each. Bacterial pellets were re-suspended in 100 μL Tris-EDTA lysis buffer (Tris 10 mM, EDTA 1 mM, pH 8.0; Buffer TE) plus lysozyme (20 mg/mL) and incubated at 37 °C for 30 minutes. Then, 467 μL of TE buffer, 30 μL of 10 % SDS, and 3 μL of proteinase K (20 mg/mL) were added to each sample and the mix was incubated at 65 °C for 60 minutes. Next, 100 μL of NaCl 5M and 80 μL of Hexadecyltrimethylammonium bromide (CTAB/NaCl) were added and incubated at 65 °C for 20 minutes. A volume of 700 μL of chloroform-isoamyl alcohol (24:1) was then added to each sample. Samples

were centrifuged at 9 000 rpm at 4 °C for 10 minutes and the resulting aqueous phase retained. A second extraction was completed adding 800 μL of phenol: chloroform: isoamyl alcohol (25:24:1) to the aqueous phase and spinning at 9 000 rpm at 4 °C for 2 minutes. Finally, DNA was precipitated with isopropanol and washed with 100 μL of 70 % ethylic alcohol and centrifuging at 4 250 rpm at 4 °C for 2 minutes. DNA samples were dried at room temperature and re-suspended in 50 μL of buffer TE and stored refrigerated until further use.

Multiplex PCR reactions were conducted at a final volume of 25 μl, containing PCR Buffer 1X, MgCl2 1.5 mM, dNTP 0.2 mM. 1.5 U of Taq Platinum® (Invitrogen, USA), and 5 μl DNA (~100 ng of DNA). The set of primers used in each reaction targeted highly conserved genes of *S. enterica* (gene *his*J) [26, 27], *L. monocytogenes* (gene *hly*A) [25, 28, 29], and *E. coli* O157:H7 (genes vt_1 and vt_2) [30]. Primers targeting *S. enterica* and *E. coli* O157:H7 genes were used at a final concentration of 5 pmol, and primers targeting the *L. monocytogenes* gene were used at a concentration of 40 pmol. All primer sequences are shown in **Table 2.**

The amplification program consisted of an initial denaturation step for 1 minute at 95 °C, followed by 30 cycles of 30 seconds at 94 °C, 30 seconds at 50.4 °C, and 1 minute at 74 °C. The program was completed with one final extension step for 5 min at 74 °C. DNA extracted from single reference strain cultures was used as positive control in every mPCR reaction. All mPCR products were visualized in 2 % agarose gels in 1X TAE buffer (40 mM Tris-acetate, 1 mM EDTA pH 8 ± 0.2) and stained with SYBR® Safe - DNA Gel Stain (Thermo Fisher Scientific, USA). Electrophoreses were run at 90V for 90 minutes.

The sensitivity of the mPCR was assessed using DNA extracted from milk inoculated with the six concentrations of *S. enterica*, *E. coli* O157:H7, and *L. monocytogenes*. The specificity of the mPCR assay was assessed with DNA isolated from the 43 bacterial strains other than the focal bacterial trio (Table 1). These bacteria were grown in media without selective bacterial inhibitors and expanded in BHI broth, then 1 mL of each culture was used to prepare DNA with a standard phenol-chloroform protocol [24]. DNA extraction of Gram-positive bacteria was performed with lysozyme at a concentration of 2 mg/mL at 37 °C for 30 minutes [24, 25]. Multiplex PCR amplification was performed with primers designed for the selected genes for *S. enterica*, *E. coli* O157:H7, and *L. monocytogenes* strains, as described in the preceding section. A sample of 10 mL UHT milk sample incubated in SEL broth was used as negative control.

Table 2. Primer sequences used for the multiplex PCR (mPCR) detection of *Salmonella enterica*, *Escherichia coli* O157:H7 and *Listeria monocytogenes*.

Bacteria	Target Gen	Nucleotide Sequence 5´- 3'	Position in the gen	Melting Temperature Tm	Reference
L. monocytogenes	*hly*A F	caaacgttaacaacgcagta	527 - 546	51.2	[25]
L. monocytogenes	*hly*A R	tccagagtgatcgatgttaa	1275 - 1256		
***E. coli* O157:H7**	vt_1 F	gaagagtccgtgggattacg	1191 - 1210	55	[30]
***E. coli* O157:H7**	vt_1 R	agcgatgcagctattaataa	1301 - 1320		
***E. coli* O157:H7**	vt_2 F	ttaaccacacccacggcagt	416 - 445	59.3	[30]
***E. coli* O157:H7**	vt_2 R	gctctggatgcatctctggt	752 - 771		
***Salmonella* spp.**	*his*J F	actggcgttatccctttctctggtg	2464516 - 2464540	52	This study
***Salmonella* spp.**	*his*J R	atgttgtcctgcccctggtaagaga	2465010 - 2464986		

Results

Assessment of the SEL broth for the simultaneous detection of *S. enterica*, *E. coli* O157:H7, and *L. monocytogenes*

The SEL broth allowed growth of the three microorganisms in all seven concentrations evaluated. **Fig. 1** depicts the growth from the concentrations 10^4 UFC/mL, 10^3 UFC/mL, 10^2 UFC/mL, and 10^1 UFC/mL in differential selective agars. The detection limit for *S. enterica*, *E. coli* O157:H7 and *L. monocytogenes* in the SEL broth and in conventional broths was 10^1 UFC/mL showing that this broth allows the growth of the three microorganisms. No bacterial growth was observed in any of the selective broths.

Multiplex PCR sensitivity

The mPCR assay protocol led to successful, simultaneous amplification of the fragments diagnostic of *S. enterica* (*his*J), *E. coli* O157:H7 (vt_1 and vt_2) and *L. monocytogenes* (*hly*A). With this approach, we detected *S. enterica* and

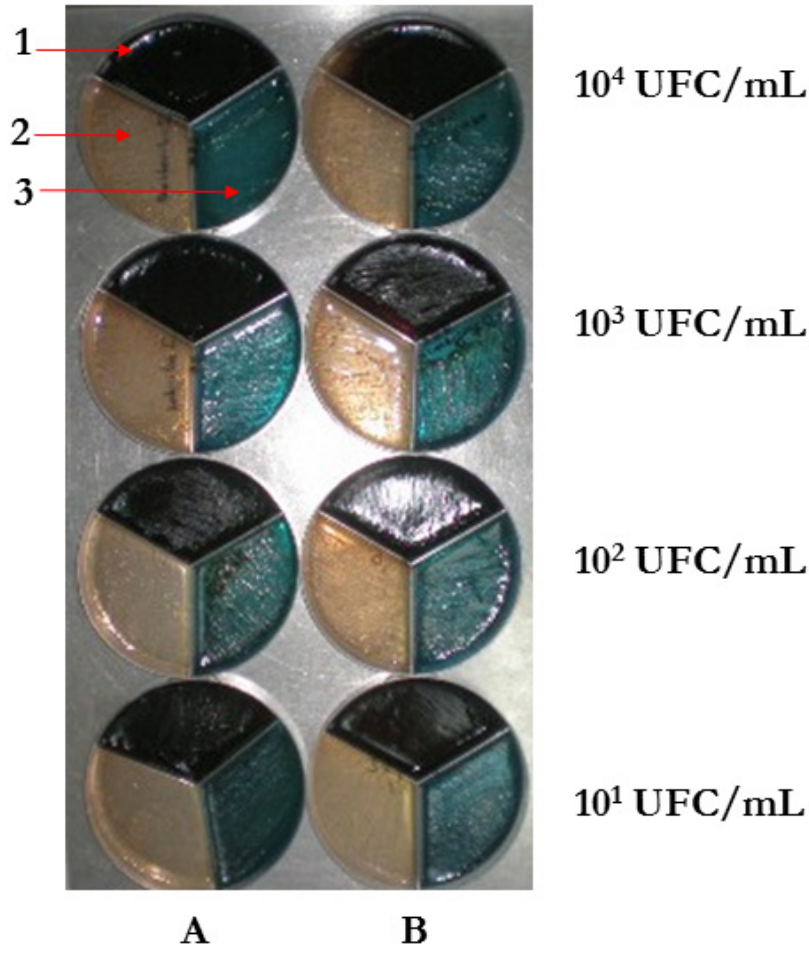

Figure 1. Detection rate for the three strains in the study **A**: Standard method; **B**: SEL broth. 1: Oxford (*L. monocytogenes*) 2: Sorbitol-MacConkey CT media (*E. coli* O157:H7) and 2: Hektoen (*Salmonella* spp.).

L. monocytogenes from contaminated milk samples and grown in SEL broth at a concentration as low as of 10^1 UFC/mL, for each microorganism and *E. coli* O157:H7 at a concentration as low as 10^0 UFC/mL (**Fig. 2**).

Multiplex PCR specificity

Fragment amplification with this mPCR assay was strain-specific for *L. monocytogenes*, *S. enterica*, and *E. coli* O157:H7. No amplicons were obtained with any of the DNA material from the additional 43 bacterial strains regardless of their taxonomic proximity to those of the focal trio (**Fig. 3**). For instance, amplification was negative for strains of other bacterial species in the genera *Listeria* (*Listeria inocua*) and *Escherichia* (non-STEC *E. coli*).

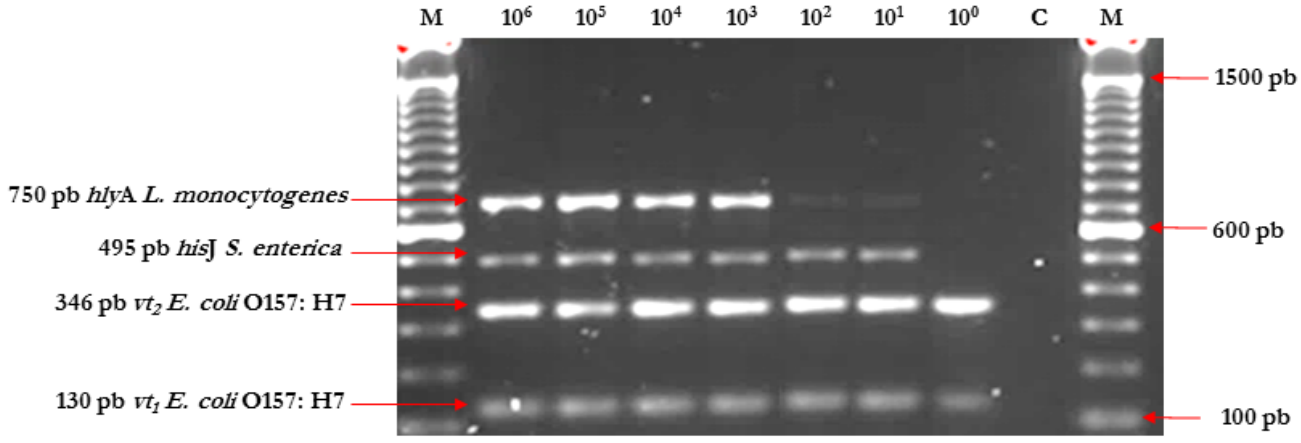

Figure 2. mPCR assay sensitivity results for *S. enterica*, *E. coli* O157:H7 and *L. monocytogenes*, using SEL broth. Lane M: Molecular size marker 100 bp (Invitrogen®). Lanes 1 and 10, M: Molecular size marker 100 bp (Invitrogen®); Lanes 2 to 8 correspond to PCR products from DNA material extracted from experimentally inoculated milk with pathogen concentrations: 10^6, 10^5, 10^4, 10^3, 10^2, 10^1, 10^0 UFC/mL; and Lane 9, C-: Negative control of the PCR reaction.

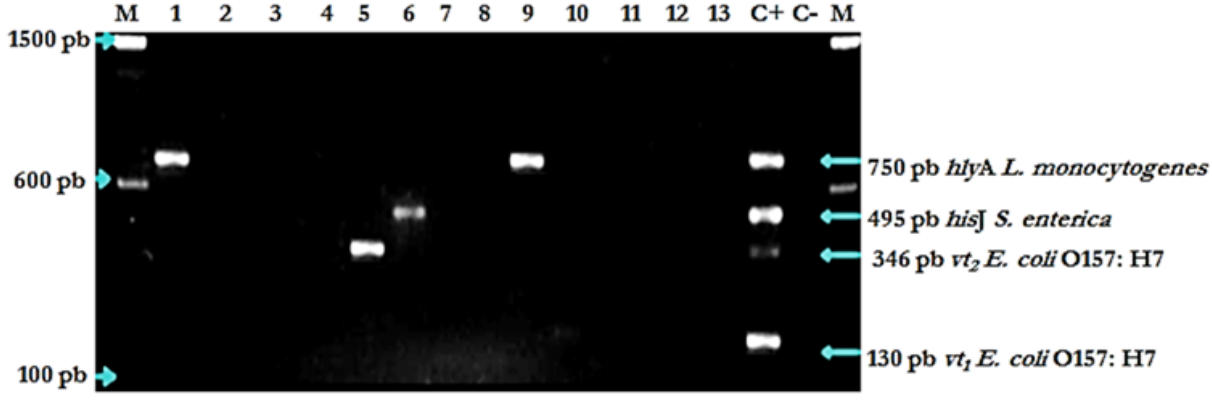

Figure 3. mPCR assay specificity evaluation results. Lanes: 1, *L. monocytogenes* strain 23; 2, *Staphylococcus aureus* CMDM080 strain 32; 3, *Klebsiella ascorbata* CMDM 042 strain 7; 4, *Brucella abortus* strain 3; 5, *Escherichia coli* B001 strain 4; 6, *Salmonella* spp. strain 23; 7, *E. coli* ATCC 25992 strain 5; 8, *Streptococcus* spp. strain 38; 9, *L- monocytogenes* strain 14; 10, *Staphylococcus aureus* strain 21; 11, *Micrococcus luteus* strain 19; 12, *Pseudomonas* sp. Strain 21; 13, *Streptococcus agalactiae* strain 36; C+: Positive control (*S. enterica*, *E. coli* O157:H7, *L. monocytogenes*); C-: Negative control of the PCR reaction; M: Molecular size marker 100 bp (Invitrogen®).

Discussion

Milk quality and safety are compromised when contaminated with microorganisms such as *S. enterica*, *E. coli* O157:H7, and *L. monocytogenes*. These microorganisms are likely to be present in raw milk and dairy products due to accidental contamination during the milking process or milk handling. These microbial milk contaminants pose a threat to public health if they remain undetected along the path from primary production to the consumer. Time-efficient pathogen detection methods ought to be developed to ensure constant supply of safe foods.

In the present study we evaluated the use of pre-enriched SEL broth to simultaneously detect *S. enterica*, *E. coli* O157:H7, and *L. monocytogenes* in experimentally contaminated cow milk. Overall, bacterial growth in SEL broth was comparable to that achieved in strain-specific pre-enriched broths: lactose broth for *S. enterica*, EC-Novobiocin broth for *E. coli* O157:H7, and buffered Listeria broth for *L. monocytogenes*. Although in the present study the incubation period was shorter, our results agree with those by Kim & Bhunia [18]. Furthermore, growth of the three microorganisms was observed across most of the range of tested inoculum concentrations, per microorganism, (10^1 to 10^6 UFC/mL) after a six-hour incubation period at 37 °C. To contribute to the successful detection of several microorganisms in a single mPCR assay it was necessary to use an adequate pre-enrichment step. The universal pre-enrichment broth (UPB; DifcoLab, Sparks, MD®) is commercially available for multiple enrichment of pathogens; however, its lack of selectivity for certain pathogens, renders it unpractical for milk samples with high contamination levels [18]. Consequently, pre-enrichment in SEL broth culture is an efficient way to simultaneously recover the three pathogens in milk samples.

The mPCR assay appeared to be more sensitive in detecting *E. coli* O157:H7 than the other two focal bacterial strains at their lowest concentration level in contaminated cow milk. However, for a broad range of concentrations, 10^1 to 10^6 UFC, the mPCR assay's sensitivity for the three bacterial species was comparable. The mPCR assay was species-specific as revealed by the absence of amplicons when DNA of microorganisms such as *Staphylococcus* spp., *Streptococcus* spp. were used as templates. These results showed that this mPCR assay is a feasible technique to detect *S. enterica*, *E. coli* O157:H7, and *L. monocytogenes* in cow milk.

Current research aims at developing highly efficient bulk microbiological food testing methodologies favoring the use mPCR-based approaches [9, 13]. The detection of various pathogens in food via one single assay is an attractive

and financially feasible option; it reduces laboratory resources such as space, supplies, reagents, and the work required, optimizing both total cost and running time of the tests [19-22]. The use of a molecular platform for the simultaneous detection of *S. enterica*, *E. coli* O157:H7, and *L. monocytogenes*, with different sensitivity degrees, has been reported for different foods. Using a Real-Time PCR approach, these three microorganisms were detected in milk after an incubation period of 18 hours at 35 °C, with a sensitivity of 1 cell/mL, for each microorganism [8, 28, 29]. The same technique has been applied in vegetables with a detection sensitivity of 1-10 cells/mL for *Salmonella* and *E. coli* O157:H7 and 1 000 cells/mL for *L. monocytogenes* [13]. Interestingly, in this assay the universal pre-enrichment broth (UPB) was used. This broth was formulated for the simultaneous detection of *Salmonella* and *Listeria* in food after incubation at 37 °C for 15 hours. The mPCR approach has been used to detect the three microorganisms in other complex food matrixes. For example, in shrimp samples the detection sensitivity was 10^3 UFC/mL for *E. coli* O157:H7, 100 cells for *L. monocytogenes*, and 1-5 cells for *Salmonella*, using pre-enrichment broth No. 17 incubated for 24 h at 35 °C [9]. In egg samples, the sensitivity was determined at 10 cells/25 g for the three microorganisms, using tryptic soy pre-enrichment broth (TSB) after 15 hours of incubation.

PCR target genes, like specific virulence markers for *E. coli* O157:H7 and *L. monocytogenes* have been used to design simple PCR strategies for food and medical sample microbiological analysis. Two targets were chosen for *E. coli* O157:H7 falling into genes coding for two fractions of the verotoxins (*Stx* o *Vtx*). These two genes were chosen because they are highly conserved among enterohemorrhagic *E. coli* strains which, in turn, are the strains most often implicated in human toxic-infections worldwide. These two *E. coli* target genes have been extensively studied since 1990 and have been used in developing mPCR techniques for *E. coli* O157:H7 identification [20, 30].

The pair of primers used for the detection of *S. enterica* was designed to amplify a histidine transport protein-coding gen which is highly conserved within this genus [26-27]. The listeriolysin O-coding gene *hly*A (LLO) is a specific virulence factor for *L. monocytogenes*, and its amplification in PCR strategies is an excellent target for PCR-detection of *L. monocytogenes* contamination in meat and dairy products [28, 29].

Conclusions

The method for the simultaneous detection of *S. enterica*, *E. coli* O157:H7 and *L. monocytogenes* in cow milk samples, consisting of pre-enrichment in SEL medium and an mPCR assay is highly specific and sensitive. The

specificity of the mPCR assay relied on the choice of four species-specific targets corresponding to the gene *his*J in *S. enterica*, genes vt_1 and vt_2 in *E. coli* O157:H7, and gene *hly*A in *L. monocytogenes*. The detection level of this method was 10 UFC/mL for each microorganism. Moreover, this method is likely to be both time- and cost-effective.

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Conflict of interest

The authors declare having no conflict of interests.

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Determinación de un método de detección múltiple para *Salmonella enterica*, *Escherichia coli* O157:H7 y *Listeria monocytogenes* en leche de vaca

Resumen: La leche cruda de vaca se considera uno de los vehículos más importantes de bacterias patógenas como *Salmonella* spp., *Escherichia coli* O157:H7 y *Listeria monocytogenes*. Estas tres bacterias son responsables de enfermedades transmitidas por alimentos. Los métodos microbiológicos de rutina para detectar estos microorganismos en leche cruda de vaca pueden ser complicados y requerir mucho tiempo. El objetivo de este trabajo fue evaluar un método para detectar simultáneamente *Salmonella* spp., *Escherichia coli* O157:H7 y *Listeria monocytogenes* en leche de vaca contaminada experimentalmente. El método utilizado combinó una etapa de pre-enriquecimiento con cultivo microbiológico estándar utilizando un medio que favorece el crecimiento de los tres microrganismos focales (caldo SEL) seguido de un único ensayo de PCR. Se utilizaron 43 cepas de microorganismos de interferencia para evaluar la especificidad del método. La tasa de detección para el método de cultivo microbiológico estándar fue de 10 UFC/mL, y la de detección por PCR, después de pre-enriquecimiento en caldo SEL, fue de 10 UFC/mL para *S. enterica* y *L. monocytogenes* y entre 1 y 5 UFC/mL para *E. coli* O157:H7. El método PCR mostró especificidad para las cepas de referencia. La detección simultánea por PCR múltiple, luego de pre-enriquecimiento en caldo SEL fue exitosa para la detección de *S. enterica*, *E. coli* O157:H7 y *L. monocytogenes* en muestras de leche de vaca contaminada experimentalmente, mostrando tanto una alta tasa de detección como una alta especificidad. Esta aproximación promete ser un procedimiento de rutina factible cuando se analizan muestras de leche en la industria y en actividades de control de salud pública.

Palabras clave: enfermedades transmitidas por alimentos; inocuidad de los alimentos; calidad higiénica de la leche.

Determinação de um método de detecção múltipla para *Salmonella enterica*, *Escherichia coli* O157:H7 e *Listeria monocytogenes* em leite de vaca

Resumo: O leite de vaca cru é considerado um dos mais importantes veículos para bactéricas patogênicas como *Salmonella* spp., *Escherichia coli* O157:H7 e *Listeria monocytogenes*. Estas três bactérias são responsáveis por enfermidades transmitidas por alimentos. Os métodos microbiológicos de rotina para detectar estes micro-organismos em leite de vaca cru podem ser complicados e demandantes de tempo. O objetivo de este trabalho foi avaliar um método para detectar simultaneamente *Salmonella* spp., *Escherichia coli* O157:H7 e *Listeria monocytogenes* em leite de vaca contaminado experimentalmente. O método utilizado combinou uma etapa de pré-enriquecimento com cultura microbiológica padrão utilizando um meio que favorece o crescimento dos três micro-organismos focais (caldo SEL). Utilizaram-se 43 cepas de micro-organismos de interferência para avaliar a especificidade do método. A taxa de detecção para o método de cultura microbiológica padrão foi de 10 UFC/mL, e a de detecção por PCR, posteriormente ao pré-enriquecimento em caldo SEL, foi de 10 UFC/mL para *S. enterica* e *L. monocytogenes* e entre 1 e 5 UFC/mL para *E. coli* O157:H7. O método por PCR mostrou especificidade para as cepas de referência. A detecção simultânea por PCR múltiplex, logo do pré-enriquecimento em caldo SEL, foi exitosa para a detecção de *S. entérica*, *E. coli* O157:H7 e *L. monocytogenes* em amostras de leite de vaca contaminado experimentalmente, demostrando tanto uma alta taxa de detecção como uma alta especificidade. Esta aproximação promete ser um procedimento de rotina viável quando se analisam amostras de leite na indústria e nas atividades de controle de saúde pública.

Palavras-chave: enfermidades transmitidas por alimentos; inocuidade dos alimentos; qualidade higiênica do leite.

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