Short communication: Detection of *stx2* and *elt* genes in bovine milk by using a multiplex PCR system

R. D. S. Nandi, A. C. Campos, J. J. Puño-Sarmiento, R. P. Maluta, S. P. D. Rocha, R. K. T. Kobayashi, and G. Nakazato¹

Department of Microbiology, Center for Biological Sciences, Universidade Estadual de Londrina, Londrina CEP 86051-980, Brazil

ABSTRACT

The aim of this study was to detect 2 important toxin genes from diarrheagenic *Escherichia coli* (DEC) in bovine milk using a new multiplex PCR. To standardize the multiplex PCR, the *stx2* and *elt* genes were investigated for the detection of Shiga toxin-producing *Escherichia coli* (STEC) and enterotoxigenic *E. coli* (ETEC), respectively. The DNA template was prepared with a thermal procedure (boiling) and a commercial kit. Samples consisted of UHT and pasteurized milk, both skimmed, and STEC and ETEC were tested in concentrations between 10¹ and 10⁹ cfu/mL. With the thermal procedure, the multiplex PCR system detected both pathotypes of *E. coli* at 10⁹ cfu/mL in UHT and pasteurized milk. When the commercial kit was used for template preparation, STEC and ETEC could be detected at concentrations as low as 10⁴ cfu/mL in UHT and pasteurized milk. Negative controls (*Listeria monocytogenes*, *Salmonella* Typhimurium, *Salmonella* Enteritidis, and *Escherichia coli* strain APEC 13) were not amplified with the multiplex PCR. These results indicate that the multiplex PCR was a rapid (less than 6 h) and efficient method to detect STEC and ETEC in milk using different methods for DNA preparation; however, the commercial kit was more sensitive than the thermal procedure.

Key words: food, diarrheagenic, *Escherichia coli*, DNA extraction

Short Communication

Bovine milk is a nutrient-rich food consumed by people of all ages, especially children (Hou et al., 2015). Both milk and dairy products go through production, processing, and storage stages. Milk and its products possess intrinsic characteristics that may interfere with food quality. Their high nutritional value, neutral pH,

and high water activity are factors that favor the permanence and growth of biological contaminants (Cerva et al., 2014; Xu et al., 2015).

Biological contaminants include food spoilage microorganisms and potential pathogens, such as *Escherichia coli* strains associated with diarrhea (Fernández et al., 2015). These strains are termed diarrheagenic *E. coli* (DEC) and can be divided into 6 pathotypes: enteropathogenic *E. coli*, Shiga toxin-producing *E. coli* (STEC), enterotoxigenic *E. coli* (ETEC), enteroaggregative *E. coli*, enteroinvasive *E. coli*, and diffusely adherent *E. coli* (Kaper et al., 2004). Some characteristics that may be present in the strains allow differentiation of the pathotypes; for example, the presence of virulence genes, production of specific toxins, and the ability to bind and invade target cells (Williams et al., 2010; Fujioka et al., 2013).

Shiga toxin-producing *E. coli* is one of the most serious pathotypes associated with foodborne outbreaks. Some STEC strains are capable of causing hemorrhagic colitis and hemolytic uremic syndrome (Croxen et al., 2013), and STEC possess the ability to produce Shiga toxins (Stx1 or Stx2; Kaper et al., 2004). The STEC pathotype has been reported in milk and dairy products (Stella et al., 2012; Nobili et al., 2016), including the dangerous serotype O157:H7 (Guh et al., 2010).

Another important pathotype is ETEC, which is a major cause of “traveler’s diarrhea” in developing countries (Croxen et al., 2013). It is transmitted through contaminated water and food, and ETEC isolates have been detected in milk (Stella et al., 2012). The ETEC strains colonize the small intestine and produce heat-stable toxin (STa and STb) and heat-labile toxin (LT) (Croxen et al., 2013; Dubreuil, 2014).

Currently, molecular techniques have gained prominence for the detection and identification of bacterial strains. These techniques are quick and reliable approaches to detect and identify bacterial strains in milk and dairy products (Hou et al., 2015). Among these molecular techniques, PCR is a reliable methodology. It allows amplification of DNA fragments of target genes, which are subsequently electrophoresed and visualized

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¹Corresponding author: gnakazato@uel.br

under UV light (Gui and Patel, 2011; Law et al., 2015). Multiplex PCR is even more robust, because it allows the detection of multiple genes in a single reaction. The aim of this study was to evaluate a multiplex PCR system for the detection of STEC and ETEC genes in UHT and pasteurized milk artificially contaminated with *E. coli*.

The strains used in the study were positive controls for *stx2* (EHEC EDL 933 serotype O157:H7) and *elt* (ETEC H10407 serotype O78:H11), from the bacterial collection of the Laboratory of Basic and Applied Bacteriology, Department of Microbiology, Universidade Estadual de Londrina (Londrina, PR, Brazil).

Packages of UHT and pasteurized skim milk were obtained commercially and artificially contaminated with positive control strains at cell concentrations of 10¹ to 10⁹ cfu/mL by serial dilution. The DNA template from milk was obtained either with a thermal treatment (boiling for 10 min) or by using a commercial kit (PureLink Genomic DNA, Invitrogen, Carlsbad, CA).

The multiplex PCR system used in the study was developed by Vendruscolo et al. (2016), using 20 mol of each primer (Invitrogen) targeting the genes that encode for Stx2 (*stx2*) and LT (*elt*) as described in Table 1. We selected Stx2 as the target for STEC because of its higher virulence compared with Stx1. We chose LT for detecting ETEC because the multiplex PCR did not work well with STa or STb. Reagents (Invitrogen) for the reactions were used as following: 2.5 µL of 10× buffer, 2.2 mM MgCl₂, 0.46 mM each deoxynucleotide triphosphate (dNTP) and Taq DNA polymerase (3 U) in a 25-µL volume. The multiplex PCR reactions were performed in a thermocycler (ESCO-Swift MaxPro, Singapore), with the following conditions: 95°C/5 min; 15 cycles of 95°C/1 min, 62°C/2 min, and 72°C/2 min; 20 cycles of 95°C/1 min, 59°C/2.5 min, and 72°C/1 min; with a final extension of 72°C/7min. The products of the multiplex-PCR were discriminated on a 2.5% agarose (Invitrogen) gel with 0.01% Gel Red (Biotium, Fremont, CA) at 70 V for 90 min. The amplicons were compared with a 1-kb DNA ladder (Invitrogen) and the amplified fragments were visualized in an UV transilluminator.

With boiled samples, it was possible to detect *stx2* and *elt* at a concentration of 10⁹ cfu/mL in both UHT and

pasteurized milk. The remaining dilutions did not yield any bands (Figure 1). Purification of the DNA template with the commercial kit improved the sensitivity of the PCR. Purified samples generated amplified fragments at bacterial concentrations as low as 10⁴ cfu/mL, for both UHT and pasteurized milk (Figure 2). To test the specificity of the technique, UHT and pasteurized milk samples were spiked with *Listeria monocytogenes*, *Salmonella* Typhimurium, *Salmonella* Enteritidis, or *Escherichia coli* strain APEC 13 (negative controls). The DNA template was prepared by boiling or by using the commercial kit, with bacterial concentrations of 10⁹ or 10⁴ cfu/mL, respectively. No PCR amplification was detected in the negative controls.

A common methodology for the detection of DEC is the association of culture on agar plates, and subsequent PCR with a few isolates. After sample inoculation and spreading onto agar plates, it takes at least 16 to 18 h of incubation time for bacterial growth, plus about 20 min for DNA template preparation, 3.5 h for PCR, and 1.5 h for electrophoresis. In contrast, the multiplex PCR described herein does not require a long incubation time, since there is no culture. Also, multiplex PCR, directly from milk samples, is, theoretically, more sensitive than the common methodology. The sensitivity is increased because total microbial DNA is tested by this multiplex PCR in contrast to the common methodology that evaluates the DNA of only a few isolates.

In a previous work, Abdelhai et al. (2016) compared the efficiency of a thermal method and the TaKaRa MiniBEST Bacterial Genomic DNA extraction kit (TaKaRa, Kudatsu, Japan) for DNA template preparation. *Escherichia coli* O157:H7 was added to 0.5, 1, or 1.5 mL of sterile saline (0.85% NaCl), and these inoculated solutions were used as samples for the detection of genes by PCR. The researchers found no differences between the methods. In our study, multiplex PCR following the thermal method (boiling) was less sensitive than the commercial kit. This decreased sensitivity could be due to substances present in the milk that disturbed the detection of STEC and ETEC genes.

Currently, PCR techniques are widely used to detect pathogens. Saxena et al. (2015) presented a description of methods for the detection of *E. coli* O157:H7 in water

Table 1. Description of primers and amplicons used in a multiplex PCR

Gene (GenBank accession no.)	Primer (5'→3')	Amplicon size (bp)	Reference
<i>stx2</i> (AY633471.1)	TTAACCACACCCCACCGGGCAGT GCTCGGATGCATCTCTGGT	346	Leotta et al., 2005
<i>elt</i> (EU113248.1)	AGGCGTATACAGCCCTCACCCA ACCTGAAATGTTGCGCCGCTCT	530	Vendruscolo et al., 2016

Figure 1. Products of multiplex PCR in 2.5% agarose gel for the *elt* and *stx* genes in (A) UHT and (B) pasteurized milk, with DNA prepared using a thermal procedure (boiling). L = 1-kb DNA ladder (Invitrogen, Carlsbad, CA); CN = no-template control; CP = positive control.

Figure 2. Products of multiplex PCR in 2.5% agarose gel for the *elt* and *stx* genes in (A) UHT and (B) pasteurized milk, with DNA prepared using a commercial kit (PureLink Genomic DNA, Invitrogen, Carlsbad, CA). L = Invitrogen 1-kb DNA ladder; CN = no-template control; CP = positive control.

and food that highlighted the importance of the PCR technique, because it gives greater sensitivity and specificity and a shorter detection time. This demonstrates the importance of standardizing PCR techniques for the detection of *E. coli*, especially STEC and ETEC, which are found in milk and dairy products.

Several studies have demonstrated the presence of these *E. coli* pathotypes in water and food throughout the world. Trevisani et al. (2014) detected STEC in dairy farms in Italy, in both milk samples and milk filters used during milking. A study by Canizalez-Roman et al. (2013) evaluated the presence of DEC in food and beverages consumed in Mexico. Of all the foods and beverages tested, dairy products were more likely to be contaminated with DEC, including STEC and ETEC strains.

The multiplex-PCR technique standardized in our study can be used, in a timely manner, for the detection of STEC and ETEC in milk. Using a commercial kit to prepare template DNA is recommended because of its increased sensitivity compared with the boiling method.

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