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Molecular screening of bovine raw milk for the presence of Shiga toxin-producing *Escherichia coli* (STEC) on dairy farms

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Abstract

Milkborne transmission of Shiga toxin-producing *Escherichia coli* (STEC) has raised considerable concern due to recent outbreaks worldwide and poses a threat to public health. The aim of this study was to develop a sensitive and specific multiplex PCR assay to detect the presence of STEC in bovine raw milk. To identify *E. coli* (ATCC 25922) contamination, the gene *uspA* was used, and PCR sensitivity and specificity were accessed by testing diluted samples ranging from 2 to 2.0×10^6 CFU/mL. To detect STEC, the *stx1* and *stx2* genes were selected as targets. After reaction standardization, the multiplex assay was tested in raw milk collected from 101 cows on dairy farms. PCR assay for *E. coli* detection had a specificity of 100% and sensitivity of 79% ($P < 0.0001$), with a lower detection limit of 2 CFU/mL. Multiplex PCR assay had 100% sensitivity for *E. coli* positive raw milk samples, and 31.1% were contaminated with STEC, 28.3% of *stx2*, and 1.9% of *stx1*. The multiplex PCR assay described in the present study can be employed to identify and screen *E. coli* harboring *stx1* and *stx2* genes in raw milk on dairy farms and in industries.

Keywords: shiga-toxin; cattle; multiplex PCR; milk.

1 Introduction

Escherichia coli is a Gram-negative, nonsporulating, rod shaped, flagellated, and facultative anaerobic bacteria belonging to the Enterobacteriaceae Family. Based on its virulence, this species is classified as enterotoxigenic *E. coli* (ETEC), enteropathogenic *E. coli* (EPEC), enterohemorrhagic *E. coli* (EHEC), attaching and effacing *E. coli* (AEEC), and Shiga toxin-producing *E. coli* (STEC) (Bavaro, 2012).

The Shiga toxin-producing *E. coli* are an important emerging group of foodborne pathogens. These strains harbor the phage-encoded genes *stx1* and *stx2*, coding for cytotoxins that cause severe tissue damage, specially STX2 that cause a variety of human illnesses ranging from diarrhea to hemorrhagic colitis (HC), thrombotic thrombocytopenia purpura (TTP), and hemolytic uremic syndrome (HUS) with fatal consequences (Gyles, 2007; Petruzzello-Pellegrini & Marsden, 2012; Walker et al., 2012). In Brazil, in a prospective clinical, observational, multicenter, non-controlled and non-randomized study, conducted between 2001 and 2005, 92.3% of patients with HUS were infected by STEC. These infected patients' ages ranged from 8 months to 6 years and 3 months, with an average age of 2 years and 5 months. However, almost half of these patients (46%) were less than 2 years old. These results show a higher risk for pediatric patients, also common victims of foodborne illnesses (Souza et al., 2011).

Ruminants, especially bovines, are the principal reservoir of STEC, and human contamination is often associated with consumption ground beef and direct contact with the animals or their environment (Savoye et al., 2011) STEC strains are

highly pathogenic to human in low infectious doses, causing food-borne diseases through consumption of contaminated water or food (Dweik et al., 2012). Bovine milk and other dairy products such as yogurt and cheese have been associated with diseases caused by STEC (Chapman et al., 1993; Prandel et al., 2008; Martin & Beutin, 2011). Outbreaks of milk-borne diseases associated with STEC, including pasteurized dairy products, have been reported worldwide emphasizing the importance of STEC control for public health (Morgan et al., 1993; Upton & Coia, 1994; Seghal et al., 2008).

The identification of the presence of microorganisms in the food is the gold standard method to determine food poisoning sources. In most clinical laboratories, identification procedures are based on microbiological culture of milk and biochemical tests of the isolated bacteria. 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 negative culture may also be due to the presence of residual therapeutic antibiotics that may inhibit bacterial growth *in vitro*, more likely, sub lethally-injured or stressed bacteria may not grow directly on selective media, unless allowed to recover on selectively first (Riffon et al., 2001). The detection of STEC strains are labor-intensive, and the total time required for strain characterization is typically 72 h, involving methods such as cytotoxicity assays and Sorbitol MacConkey Agar plating. On the other hand, molecular methods are sensitive, specific, and rapid approaches for detection and characterization of microbiological contaminants in food.

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The development of PCR-based methods for the detection of pathogens or virulence factors (Shahi et al., 2013; Branquinho & Ferreira, 2012; Gandra et al., 2011) can be used for the initial screening of the presence of microorganisms in samples of milk and dairy products. Furthermore, the present study, through direct isolation of bacterial DNA in milk samples, aimed to develop the multiplex PCR to assess the prevalence of *E. coli* and STEC in samples of fresh milk.

2 Materials and methods

2.1 Collection of raw milk samples

This study was carried out on three dairy farms located in Rio Grande do Sul State, Brazil. For the microbiological and molecular biology analyses, 15 mL of raw milk samples of 101 cows were collected. The milk was drawn by hand directly into sterile test tubes and transported to the laboratory in a cooler containing ice.

2.2 Microbiological analysis

To determine contamination of *E. coli* in milk, Brazilian federal normative (IN62) of the Ministry for Agriculture, Livestock and Supply was followed and used as the standard method (Brasil, 2003). Milk samples were cultured in Violet Red Bile agar (Oxoid Cambridge, UK) at 36°C for 24 h. From the samples that tested positive for *E. coli*, three colonies were transferred to Brilliant Green medium (Acumedia Lansing, USA) and incubated at 36°C for 48 h. One loopful of cultures presenting gas production was streaked onto a EC broth (Acumedia Lansing, USA) and incubated at 36°C for 48 h. The samples presenting gas formation were cultured at 36°C in Eosin Methylene Blue Agar (EMB, Oxoid Cambridge, UK.). After 24 h, *E. coli* colonies were counted with standard technique.

2.3 Preparation of template DNA

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 x g, 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 x g, 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 x g, 5 min). A total volume of 26.5 µL of 2M 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 x g, 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.

2.4 Molecular characterization of STEC in milk samples

A multiplex PCR was designed to determine the occurrence of *E. coli* harboring *stx1* and/or *stx2* virulence genes in the raw milk samples. Milk DNA samples were used as templates for PCR assays. All primers were from Invitrogen (Carlsbad, CA, USA), and the molecular biology reagents were from Biotools (Madrid, Spain). The names of specific primer sequences, predicted fragment size, and reaction concentrations are presented in Table 1. The *uspA* gene encoding the universal stress protein was used to detect *E. coli* DNA (Chen & Griffiths, 1998). To determine the presence of STEC, primers based on *stx1* (Genebank accession number AB083043.1) and *stx2* (Genebank accession number AB030484.1) genes were designed for this study. Multiplex PCR conditions were 1.75 mM MgCl₂, 20 mM Tris-HCl, 50mM KCl (pH 8.4), 500 µM dNTPs, 1.5 U *Taq* DNA polymerase, and 100 ng of template DNA; 2% DMSO (Sigma-Aldrich, St Louis, MO, USA) was added to a total volume of 25 µL. Amplification reactions were carried out using Techne® TC-512 thermocycler (Techne® Barloworld Scientific, Stone, Staffordshire, UK) as follows: 5 min at 94°C, 35 cycles each consisting of 2 min at 94°C, 1 min at 58°C, 1 min at 72°C, and a final extension step of 10 min at 72°C. Characterization of presence of *E. coli* producer of Shiga I and/or Shiga II toxins were performed by agarose (Invitrogen, Carlsbad, CA, USA), gel electrophoresis stained with ethidium bromide (Sigma-Aldrich, St Louis, MO, USA). *E. coli* O157:H7 Edl 933 strain (kindly provided by Dr. Caroline de Nardi from Instituto Federal de São Paulo, Brazil) was used as positive control; PCR without template DNA and/or DNA polymerase was performed to be used as negative control.

To determine PCR sensitivity and specificity, sterile whole milk was artificially contaminated with 2 to 2.0 × 10⁶ CFU/mL of *E. coli* (ATCC 25922) culture in stationary phase grown in brain heart infusion (BHI) broth (Sigma-Aldrich, St Louis, MO, USA) for 18-24 h at 36°C (Guido, 2003). The

Table 1. Primer sequences for detection of STEC in raw milk samples.

Primers	Sequence (5' - 3')	Product size	Target gene	Concentration
uspAF	CCGATACGCTGCCAATCAGT	884 bp	<i>uspA</i>	0.5 µM
uspAR	ACGCAGACCGTAGGCCAGAT			
stx1F	GATTTCATCTGCATCCCCGTACG	346 bp	<i>stx1</i>	0.5 µM
stx1R	CTTACGCTTCAGGCACATACAG			
stx2F	GGTTAAGCCACTGCCTTTAACTTC	461 bp	<i>stx2</i>	1.5 µM
stx2R	CGGGTGAGTAACGCGTAGGTAA			

quantification was performed at this stage by plating on the depth of the microorganism suspensions of PCA (Plate Count Agar), followed by incubation at 36°C for 48 h. Dilution curves were repeated 8 times, and each point was analyzed in triplicate. DNA extraction and PCR were performed as described above.

2.5 Statistical analyses

All statistical analyses were performed using the Prism 5 (Graph Pad Software). The prevalence and frequency of contamination and sensitivity and specificity determination were accessed with exact 95% confidence interval (CI), Kruskal-Wallis test, Fisher's exact test and Chi-square; $p < 0.05$ was considered statistically significant.

3 Results and discussion

To validate the use of PCR to detect *Escherichia coli* contamination in raw milk, the sensitivity and specificity for the primers designed for the *uspA* gene were checked. The method was compared to microbiological analysis, which showed a total prevalence of contamination of raw milk by *E. coli* of 53.5% (against to 51.2% determined by PCR), with no difference ($P = 0.3623$) between the farms evaluated. However, the contamination level on farm C ($60,508.8 \pm 31,653.8$) was higher ($P < 0.0001$) than that on farms A and B, 442.8 ± 261.27 and 106.6 ± 27.3 , respectively (Figure 1).

100% specificity and 79% sensitivity were observed when PCR was performed with DNA obtained from milk artificially contaminated with 2 CFU/mL ($P < 0.0001$) (Table 2). When PCR was compared to microbiological analysis, 100% specificity (CI: 0.9328 to 1.000) was observed ($P < 0.001$); however, lower sensitivity (52.48%; CI: 0.4230 to 0.6251) was found. These results may be related to occurrence of samples with

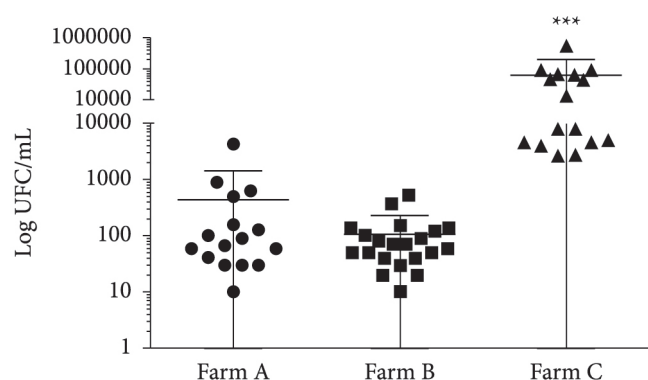


Figure 1. Comparison of milk contamination by *E. coli* on dairy farms A, B, and C. Results are presented as Log UFC/mL. Asterisks (***) denote statistical difference ($P < 0.001$, Kruskal-Wallis test).

contamination less than 2.0×10^5 CFU/mL since the limit for 100% sensitivity was 2.0×10^6 CFU/mL.

The molecular characterization of STEC was accessed by means of multiplex PCR. The combination of the three sets of primers allowed the detection of the genes of interest as they produce different sized PCR specific products. Hence, after thorough trials (data not shown), the primer pairs to detect the *stx1* and *stx2* genes were found to be highly specific (Figure 2).

The multiplex assay detected that among all *E. coli* positive samples, 31.1% were contaminated with STEC. The *stx2* fragment was found in 15 samples (28.3%), and *stx1* in only one sample (1.9%), which was not *stx2* positive. Environmental contaminations and poor herd and milking managements are important causes of reduction of milk quality. The high frequency of *stx2* in faecal samples collected from buffalo, cows, and goats described elsewhere (Islam et al., 2008; Stephan et al., 2008; Bandyopadhyay et al., 2011, 2012) might explain the prevalence of *stx2* positive STEC in raw milk. In humans, epidemiologic data suggest that *E. coli* O157 strains that express *stx2* are more important than *stx1* in the development of HUS, and that strains that express *stx2* alone are more likely to be associated with the progression to HUS than strains that produce both *stx1* and *stx2* (Griffin, 1995).

In this study, the presence of STEC harboring the genes *stx1* and *stx2* in raw milk sample was detected. STEC harboring *stx1* and *stx2* genes were reported to be typical cattle colonizers (Brett et al., 2003; Vu-Khac & Cornick, 2008). Some authors have recently investigated 593 foodborne STEC strains for their serotypes and for nine virulence genes (*stx1*, *stx1c*, *stx1d*, *stx2*, *stx2b*, *stx2e*, *stx2g*, *E-hly* and *eae*), and they observed a significant association of *stx1* and *stx2* genes with bovine meat and milk products. These authors compared the properties of foodborne STEC with published data on faecal STEC from food producing animals, and found that virulence profiles and serotypes of STEC from food showed remarkable similarities to those of faecal STEC that were from the same animal species. Based on these results, it has been pointed out that food-producing animals represent the most important source for the entry of STEC in the food chain (Martin & Beutin, 2011).

It is noteworthy to mention the findings of Kumar et al. (2013), who showed the presence of pathogenic *E. coli* O157:H7 in pasteurized milk samples. These authors described a higher detection signal after enrichment, suggesting the presence of viable cells after pasteurization or post-pasteurization contamination. Therefore, fermented dairy products manufactured using raw milk contaminated with *E. coli* O157:H7, STEC, or other pathogenic strain can pose a threat to human health, demanding special attention of health and food agencies worldwide. Studies with emphasis

Table 2. Sensitivity and specificity of PCR assay for contamination of raw milk by *E. coli*. Each concentration was tested in 24 samples.

	<i>E. coli</i> concentration in milk samples (CFU/mL)					
	2.0×10^6	2.0×10^5	2.0×10^4	2.0×10^3	2.0×10^2	2.0
Sensitivity (%)	100	83.3	95.8	83.3	95.8	79.1
Specificity (%)	100	100	100	100	100	100

$P < 0.0001$ for all values. Fisher Exact test.

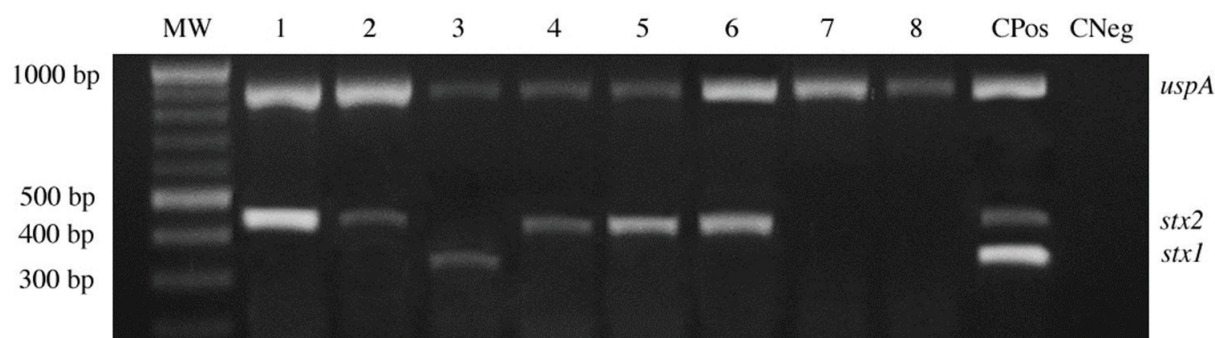


Figure 2. Specificity of multiplex PCR for detection of STEC in raw milk samples. Fragment identifications are presented on the right – *uspA* (884 bp), *stx1* (346 bp) and *stx2* (461 bp). Lanes: MW: 100 bp ladder; 1 to 8: milk samples; CPos: positive control (*E. coli* harboring *stx1* and *stx2* genes; CNeg: negative control, without template DNA. Agarose gel electrophoresis stained with ethidium bromide.

on detection of pathogens and their virulence factors in food sources may be useful to prevent infection and development of associated diseases.

Our findings on raw milk contamination are meaningful and should be considered since even one Shiga toxin-producing *E. coli* in a food sample may lead to gastrointestinal or urogenital disorder due to their multiplication in the body or the food itself during storage in poor conditions (Gyles, 2007). Therefore, strict hygiene and management practices for dairy herd and milk processing and storage must be adopted to avoid unwanted illness due contaminated milk and milk products consumption.

4 Conclusion

This report presents a quick, sensitive, and specific method for molecular characterization of STEC. In addition, the screening for STEC in three dairy herds showed a significant prevalence of Shiga toxin-producing *E. coli* contamination in raw milk.

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