Genetic heterogeneity of *Escherichia coli* isolated from pasteurized milk in State of Paraná, Brazil

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Food contamination caused by enteric pathogens is a major cause of diarrheal disease worldwide, resulting in high morbidity and mortality and significant economic losses. Bacteria are important agents of foodborne diseases, particularly diarrheagenic *Escherichia coli*. The present study assessed the genetic diversity and antimicrobial resistance of *E. coli* isolates from pasteurized milk processed in 21 dairies in northwestern State of Paraná, Brazil. The 95 *E. coli* isolates were subjected to antimicrobial susceptibility testing according to the recommendations of the Clinical and Laboratory Standards Institute and assessed genotypically by Enterobacterial Repetitive Intergenic Consensus-Polymerase Chain Reaction (ERIC-PCR). The highest rate of resistance was observed for cephalothin (55.78%). ERIC-PCR revealed high genetic diversity, clustering the 95 bacterial isolates into 90 different genotypic patterns. These results showed a heterogeneous population of *E. coli* in milk samples produced in the northwestern region of Paraná and the need for good manufacturing practices throughout the processing of pasteurized milk to reduce the risk of foodborne illnesses.


A contaminação de alimentos por patógenos entéricos é uma das principais causas de doenças diarréicas em todo o mundo, resultando em altas taxas de morbidade e mortalidade e perdas econômicas significativas. As bactérias são importantes agentes de doenças de origem alimentar, particularmente *Escherichia coli* diarreogênicas. O presente estudo teve como objetivo avaliar a diversidade genética e a resistência a antimicrobianos de *E. coli* isoladas de leite pasteurizado, processados em 21 laticínios na região noroeste do Paraná - Brasil. Os 95 isolados de *E. coli* foram submetidos a testes de susceptibilidade aos antimicrobianos de acordo com as recomendações do *Clinical and Laboratory Standards Institute* e avaliados genotipicamente por ERIC-PCR (*Enterobacterial Repetitive Intergenic Consensus - Polymerase Chain Reaction*). O principal perfil de resistência encontrado entre os isolados foi resistência à cefalotina (55,78%). ERIC-PCR revelou alta diversidade genética, agrupando os 95 isolados bacterianos em 90 diferentes perfis genotípicos. Estes resultados mostraram uma população heterogênea de *E. coli* em amostras de leite produzido na região noroeste do Paraná e a necessidade de boas práticas na manipulação de todo o processamento de leite pasteurizado, a fim de reduzir o risco de doenças transmitidas por alimentos.


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INTRODUCTION

Food contamination caused by enteric pathogens is a major cause of diarrheal disease worldwide, resulting in high morbidity and mortality and significant economic losses (Centers for Disease Control and Prevention, 2013). Bacteria are important agents of foodborne diseases (Centers for Disease Control and Prevention, 2013), particularly Escherichia coli, which is widely distributed in nature. Despite being commonly found in the gastrointestinal tract of humans and other animals, it can cause a variety of diseases, some of which are life threatening in more vulnerable groups, such as the elderly, children, and immunocompromised individuals (FDA, 2012).

E. coli has been isolated from various foodstuffs, such as meat, vegetables, milk, and milk derivatives, which can serve as a vehicle for the transmission of diseases by food (Lee et al., 2009; Solomakos et al., 2009; Brooks et al., 2012; Maffei et al., 2013).

In Brazil, some studies have reported a high prevalence of E. coli in raw and pasteurized milk. E. coli was isolated from 41.1% of pasteurized milk samples evaluated by Silva et al. (2001), while in raw milk, this bacteria was found in 79.2% (Campos et al., 2006) and in 76% (Barreto et al., 2012) of milk samples analyzed. An important subject is the emergence of resistant foodborne pathogens that may be transmitted to humans as food contaminants. Outbreaks of foodborne diseases that involve resistant bacteria associated with food animal sources have been reported (Safe Food, 2010).

Milk may get contaminated with E. coli from various sources during different stages of production and processing, which could explain the genetic diversity of these bacteria in milk. The presence of E. coli in pasteurized milk may be due to inadequate pasteurization or contamination of the product after pasteurization process. Studies of the epidemiology of foodborne microorganisms, especially the source of contamination, have been conducted using various molecular techniques, such as ribotyping, multilocus sequence typing, randomly amplified polymorphic DNA, enterobacterial repetitive intergenic consensus-polymerase chain reaction, repetitive extragenic palindromic, and pulsed-field gel electrophoresis (Foley, Lynne, Nayak, 2009).

Several studies have used ERIC-PCR to assess the genetic similarity of E. coli isolates from different sources. Rantsiou, Alessandria and Cocolin (2012) assessed the genetic diversity of Shiga toxin-producing E. coli from meat and dairy products using ERIC-PCR. Wenz et al. (2006) described genetic variability among E. coli isolates from dairy cattle with mastitis using ERIC-PCR. Ibenyassine et al. (2006) used the same technique and evaluated the similarity of isolates from plants infected by irrigation water. Other authors have used ERIC-PCR to compare E. coli isolates from humans and animals (Leung et al., 2004; Sabate et al., 2008; Tramuta et al., 2011; De la Fé Rodriguez et al., 2012). Moreover, Casarez, Pillai, Di Giovanni, (2007), Duan et al. (2009), and Wan et al. (2011) showed that ERIC-PCR could discriminate E. coli from water and environmental samples. Knowledge about the epidemiology of diarrheagenic E. coli in food is important for understanding its distribution and transmission, besides developing and implementing control measures in food production.

To the best of our knowledge, no studies have used ERIC-PCR to assess the diversity of E. coli in pasteurized, ready-to-consume cow milk. Thus, the aim of the present study was to evaluate the genetic diversity and antimicrobial resistance of E. coli isolates from cow milk pasteurized in the northwestern region of Paraná, Brazil, using ERIC-PCR.

MATERIAL AND METHODS

Bacterial isolates

Ninety-five E. coli isolates from pasteurized, ready-to-consume cow milk samples were analyzed. The isolates were stored in Tryptic Soy Broth (TSB; Difco, Le Pont de Claix, France) with glycerol at -20°C and kept at the Laboratory of Food Microbiology, Department of Clinical Analysis and Biomedicine, Maringá State University. The milk samples were obtained from 21 dairies in Paraná, Brazil, from March 2006 to November 2008 in a previous study conducted in our laboratory that evaluated the presence of coliforms in pasteurized milk (Zanella et al., 2010). The different dairies were identified by the abbreviations AA, APRO, CAIÚÁ, CC, CIA, COC, COOP, GM, L, LAC, LOU, MOU, MP, NE, P, PN, S, SN, SO, TB, and U.

Antimicrobial susceptibility testing

Antimicrobial susceptibility testing was performed using the disc diffusion technique according to the method described by the Clinical and Laboratory Standards Institute (2009). The antimicrobial agents (Oxoid, Hampshire, England) were the following: amikacin (30 μg), amoxicillin-clavulanic acid (10-20 μg), ampicillin (10 μg), aztreonam (30 μg), ceftazidime (30 μg), cefepime (30 μg), cefotaxime (30 μg), cefoxitin
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(30 μg), ceftazidime (30 μg), ciprofloxacin (5 μg), chloramphenicol (30 μg), gentamycin (10 μg), imipenem (10 μg), sulphamethoxazole-trimethoprim (1.25-23.75 μg), tetracycline (30 μg), and tobramycin (10 μg). The *E. coli* ATCC 25922 and *E. coli* ATCC 35218 isolates were used as controls.

**ERIC-PCR**

Genomic DNA of *E. coli* isolates was extracted from overnight bacterial growth on Nutrient Agar (Difco, Le Pont de Claiix, France; Swanenburg *et al.*, 1998). DNA quantification was performed using NanoDrop 2000.

The amplification reaction was performed by adding 100 ng of bacterial DNA to a PCR reagent mixture that contained 1 μM of the primers ERIC1R (5’-ATGTAAGCCTGCTGATTAC-3’) and ERIC2 (5’-AAGTAAGTGACTGGGTGAGCG-3’; (Versalovic, Koeuth, Lupski, 1991), PCR buffer (20 mM Tris-HCl, pH 8.4, 50 mM KCl), 1.5 mM MgCl₂, 200 μM of each nucleotide (dATP, dCTP, dGTP, and dTTP), 1 U of DNA polymerase (Taq DNA polymerase; Invitrogen – Life Technologies, Brazil), and sterile deionized water to a final volume of 25 μl. The amplification was performed in a thermocycler (Gene Amp PCR System 2400, Perkin Elmer, Roche, Branchburg, NJ, USA) with denaturing at 94 ºC for 7 min, followed by 35 cycles at 94 ºC for 30 s, 52 ºC for 1 min, and 72 ºC for 8 min and a final extension at 72 ºC for 16 min. The PCR product underwent 1.5% agarose (Amersham Pharmacia Biotech AB, Uppsala, Sweden) gel electrophoresis at 7-10 V/cm² for 2 h and visualized under UV light. The spectral band analysis was performed using BioNumerics software (version 4.45, Applied Maths, Sint-Martens-Latem, Belgium). The Dendrogram was constructed using the Dice coefficient, and the phylogenetic distance was determined using the Unweighted Pair Group Method with the Arithmetic Mean algorithm. Isolates with ≥95% similarity were considered closely related. The discriminatory power of ERIC-PCR was calculated based on Simpson’s diversity index as indicated by Hunter and Gaston (1988).

**RESULTS**

All 95 *E. coli* isolates were found to be susceptible to amoxicillin-clavulanic acid, aztreonam, cefepime, cefotaxime, ceftazidime, ciprofloxacin, gentamycin, imipenem, and tobramycin. Sixty-two isolates (65.26%) were resistant to at least one of the antimicrobial agents tested. Among the resistant isolates, five were simultaneously resistant to three antimicrobial agents. The highest rates of resistance were observed for cephalothin (55.78%) and ampicillin (26.31%). Resistance to cefoxitin (5.26%), tetracycline (3.15%), amikacin (2.10%), chloramphenicol (2.10%), and trimethoprim-sulphamethoxazole (1.05%) was also observed. The *E. coli* isolates were grouped into 11 resistance profiles. The most common resistance profiles were to cephalothin (30 isolates) and cephalothin/ampicillin (14 isolates; Figure 1).

**DISCUSSION**

Pathogenic microorganisms have been isolated from milk and dairy products (D’Amico, Donnelly, 2010;...
FIGURE 2 - Dendrogram that represent the genetic relationship of ERIC-PCR, milk samples and antimicrobial resistance of 95 \textit{E. coli} isolated from pasteurized cow milk in State of Paraná, Brazil.
Costa Sobrinho et al., 2012). Among them, diarrheagenic *E. coli* is notable and can cause diseases with serious consequences, especially in children and the elderly (FDA, 2012). The analysis and comparison of *E. coli* isolates from different sources can generate important information about the origin and transmission of these bacteria (Foley, Lynne, Nayak, 2009).

Importantly, antimicrobial-resistant bacteria can act as reservoirs of resistance genes. Several studies of bacteria isolated from food have shown resistance to one or more antimicrobials, which is worrying because these microorganisms can be spread in the population as food contaminants (Lutgen et al., 2009; Solomakos et al., 2009; Dutil et al., 2010; Rahimi, Chaleshtori, Parsaei, 2010).

In the present study, 65.26% of the isolates showed resistance to at least one of the antimicrobial agents tested. Similar results were reported by Rahimi, Chaleshtori and Parsaei (2010), who found that 88.9% of the isolates were resistant to one or more of the tested antimicrobials. The highest rates of resistance were observed for cephalothin (55.78%) and ampicillin (26.31%). Paneto et al. (2007) evaluated *E. coli* isolated from cheese and provided indices of resistance to ampicillin and cephalothin that were similar to those observed in the present study. Rahimi, Chaleshtori and Parsaei (2010) found that 44.4% of the *E. coli* isolates from dairy products showed ampicillin resistance. Solomakos et al. (2009) found that all of the *E. coli* isolates from milk were resistant to ampicillin, whereas Campos et al. (2006) found increased resistance to ampicillin and cephalothin and resistance to tetracycline in *E. coli* isolated from milk and cheese.

With regard to resistance profiles, the *E. coli* isolates were differentiated into 11 profiles. Heterogeneous profiles of antimicrobial resistance were also described by Rangel and Marin (2009) and Solomakos et al. (2009) in *E. coli* isolated from milk.

In the present study, ERIC-PCR showed high genetic diversity among the 95 *E. coli* isolates, which were clustered into 90 profiles. Among the clusters generated by ERIC-PCR, eight clustered *E. coli* isolates from four milk samples processed in four different dairies, suggesting a single clone contaminant per milk sample (Figure 2). Nevertheless, one cluster that comprised two *E. coli* isolates from different milk samples processed in the same dairy could suggest a common source of contamination. These results indicate failures in pasteurization process or contamination in post-pasteurization processing and show the need for control measures to minimize *E. coli* contamination. The present results are consistent with Wenz et al. (2006), who studied *E. coli* isolates from dairy cattle with mastitis, and Rantsiou, Alessandria and Cocolin (2012), who used ERIC-PCR to assess the genetic similarity of *E. coli* isolated from meat products and unpasteurized dairy products. *E. coli* is widely distributed in nature, and the sources of milk contamination can be diverse, explaining the high genotypic diversity observed in the present study.

ERIC-PCR is effective in typing *E. coli* isolates from animals (Mohapatra, Broersma, Mazumder, 2007; Prabu et al., 2010; Wan et al., 2011; De la Fé Rodríguez et al., 2012) and water (Casarez, Pillai, Di Giovanni, 2007). However, some reports indicated that ERIC-PCR is not effective in typing *E. coli* isolated from humans, animals, and food (Giammanco et al., 2002; Leung et al., 2004; Costa et al., 2006).

The present study showed heterogeneous profiles of antimicrobial resistance and a high genotypic diversity using ERIC-PCR in *E. coli* isolated from dairy milk processed in northwestern Paraná. The high genetic diversity observed in the present study can be justified by several sources of contamination of cow milk by *E. coli* during the entire production process.

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