academicJournals

Vol. 11(47), pp. 1669-1675, 21 December, 2017
DOI: 10.5897/AJMR2017.8677
Article Number: 57A72DE55259
ISSN 1996-0808

http://www.academicjournals.org/AJMR

African Journal of Microbiology Research

Full Length Research Paper

Genotypic characterization of *Escherichia coli* strains isolated from dairy cattle environment

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Received 13 August, 2017; Accepted 15 November, 2017

The aim of this study was to characterize the diversity of *Escherichia coli* strains involved in the dispersion of virulence genes. 152 *E. coli* strains originated from dairy cattle environment were evaluated through phenotypic and proteomic assays. These samples were investigated for the presence of virulence genes (*eae*A, *stx*I, *stx*II, *ST*, *LT*, *eagg*, *ial*) and biofilm related genes (*fim*H, *csg*A, *flu*). Eighteen profiles were obtained and 30 isolates were selected for macrorestriction assay by *Pulsed-Field Gel Electrophoresis* (PFGE) technique. PFGE patterns of *Xba*I-digested performed to determine the clonal relatedness of *E coli* isolates. A total of 27 pulsotypes of *E. coli* were identified with a low percentage of genetic similarity (≤68%) demonstrating a high genetic diversity in the isolates tested. Furthermore, the presence of biofilm-associated related gene (*fim*H) can contribute to the dispersion and persistence of this pathogen into the milk environment. Also, all of the pulsotypes (n = 8) with genetic similarity from 95% were present in milk samples, feces and liner were found in different seasons of the year, sharing at least one gene associated with biofilm formation. So the characterization of the genetic diversity of *E. coli* is important for understanding its dispersion in order to assist in the implementation of measures to prevent the spread of these agents in milk production environment.

Key words: circulating clones, diversity, *Escherichia coli*, persistence, Pulsed-Field Gel Electrophoresis (PFGE), virulence gene.

INTRODUCTION

Escherichia coli have been isolated from a wide range of animal hosts and in mammals. However, this specie is able to adapt to various environmental conditions, being highly diversified with commensal and pathogenic strains that can colonize and persist in humans, animals and abiotic environments (Wirth et al., 2006; Tenaillon et al., 2010).

The pathogenic *E. coli* subtypes have caused diseases

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worldwide and the main route of human infection is through bovine meat and water contaminated with bovine fecal material (Leclerc et al., 2001). This specie may persist in the intestines of the animals and can be excreted in the environment through the feces, ensuring the spread of this pathogen in the animal production environment (Lambertini et al., 2015). Hussein and Sakuma (2005) discussed the importance of dairy cattle as reservoirs of STEC and its significant importance to health risk to humans. The authors demonstrated that fecal testing of dairy cattle worldwide showed wide ranges of prevalence rates for *E. coli* O157: H7 (0.2 to 48.8%). In animal residues, pathogenic *E. coli* can survive from 50 to more than 300 days, depending on environmental factors such as temperature, aeration and nutrient availability (Rogers and Haines, 2005).

In addition, the bovine gastrointestinal tract has been considered as a natural reservoir for commensal and pathogenic *E. coli* of high phylogenetic and genotypic diversity with the putative ability to cause mastitis (Houser et al., 2008). Thus, it has been proposed that several *E. coli* genotypes with specific phenotypes are more suitable to incite bovine mastitis than others (Shpigel et al., 2008; Blum et al., 2008; Blum et al., 2013).

A severe outbreak of O104: H4 in Germany in 2011 emphasized this versatility and adaptability of pathovar STEC (shiga - toxin producing *E. coli*), as it was caused by a newly developed hybrid strain that combines virulence properties of enterohemorrhagic *E. coli* (EHEC) and enteroaggregative *E. coli* (EAEC) (Denamur, 2011). Recently, Eichhorn et al. (2015) have shown that some atypical *E. coli* strains (aEPEC) and EHEC share the same phylogeny and are called post-pre-EHEC aEPEC isolates.

The constant horizontal transfer of genes allows triggering new strains STEC and EHEC with unpredictable risk potential for humans. Thus, STEC clones that persist in animals or farms over long periods of time, which are equipped with certain virulence and virulence-associated genes (VAGs), can serve as reservoirs of genes for the evolution of these new strains (Hoffman et al., 2006). However, the persistence of distinct EHEC subpopulations such as O157: H7 was shown to be related to the colonization properties and, for example, to the presence of the large EHEC virulence plasmid or long polar fimbriae (Lim et al., 2007).

Molecular typing techniques have been used to investigate several *E. coli* subtypes as O157 and PFGE is currently considered the gold standard for fingerprint DNA of these strains (Swaminathan et al., 2001). The ecology and epidemiology of this organism in cattle appear to be very complex, often involving multiple clones on a single farm (Renter et al., 2002; Liebana et al., 2003). Previous studies have reported on persistence on the farm in different livestock production systems. However, information on persistence in individual animals is very scarce (Renter et al., 2003; LeJeune et al., 2004).

Thus, the objective of this study was to characterize the diversity of *E. coli* of major importance in the dispersion of virulence genes within the milk production system using the technique of pulsed field gel electrophoresis.

MATERIALS AND METHODS

Ethics statement

This study was conducted according to ethical standards and approved by the Ethics Committee and Biosafety of the institution under protocol number: CEUA-3664040915. The samples used in the current study were obtained from samples submitted to routine veterinary diagnosis.

Sample collection

The present study was performed in the town of Barra do Piraí, Rio de Janeiro, Brazil between 2014 and 2015 (Figure 1). A pool of milk samples from 94 cows tested positive by the California Mastitis Test (CMT), collected over three consecutive weeks. Thus, 282 milk samples were obtained.

A total of 94 rectal feces samples from these same lactating cows were also collected. Representing milk line samples (n=48) were also collected: 10 samples from workers' hands, 10 nasal samples from workers, 20 milking machine samples and 8 nasal samples of pets (dogs and cats present in the milking parlor). Finally, one sample each from farm water supply was collected from well, weir, faucet, drinking fountain and brook, making a total of 19 water samples.

Bacterial Identification

The bovine milk and milk line samples were first inoculated on blood agar enriched with 5% sheep blood (MicroMed®, Rio de Janeiro, Brazil 2098), while the fecal and water samples were inoculated on Eosin Methylene Blue (EMB) agar (Acumedia®, Lansing, Michigan 48912) and incubated at 35°C (±2°C) for 24 h. Then, the isolates were submitted to routine microbiological diagnostics, including inoculation in selective medium for analysis of cultural propertie (Rodrigues et al., 2017).

The Gram negative bacteria identification was followed according to Koneman et al. (2012), glucose and lactose fermentation with gas production, H_2S (hydrogen sulfide), indole, motility, acetoin and mixed acid production and citrate were assayed. All tests were carried out in triplicate. The strain *E. coli* ATCC25922 was used as positive control.

Maldi-Tof MS used to confirm the species

All Enterobacterial strains previously identified by biochemical tests were evaluated for, Matrix-Assisted Laser Desorption Ionization–Time of Flight Mass Spectrometry (MALDI-TOF MS) technique to confirm their species. Assay was performed in Laboratory of Investigation in Medic Microbiology of the Institute of Microbiology Paulo Góes, Federal University of Rio de Janeiro (UFRJ). The samples were inoculated in Brain Heart Infusion (BHI) agar (Merck KGaA®, Darmstadt, Germany 64271) at 37°C for 24 h and each culture was transferred to a microplate (96 MSP, Bruker - Billerica, USA).

The bacterial sediment was covered by a lysis solution (70% formic acid, Sigma-Aldrich). Furthermore, 1 µL aliquot of matrix

Figure 1. Map of study area, Barra do Piraí City.

solution (alpha-ciano-4-hidroxi-cinamic acid diluted in 50% acetonitrile and 2.5% trifluoracetic acid, Sigma-Aldrich) was added to each sediment. The spectra of each sample were generated in a mass spectrometer (MALDI-TOF LT Microflex, Bruker) equipped with a 337-nm nitrogen laser in a linear path, controlled by the FlexControl 3.3 (Bruker) program. The spectra were collected in a mass range between 2,000 to 20,000 m/s, and then analyzed by the MALDI Biotyper 3.1 (Bruker) program, using the standard configuration for bacteria identification by which the spectrum of the sample is compared to the references in the database. The results vary on a 0 to 3 scale, where the highest value means a more precise match and reliable identification. In this study, we accept values for matching greater than or equal to 2, as proposed by the manufacturer (Rodrigues et al., 2017).

DNA extraction and detection of virulence genes

DNA extraction was performed by thermal lysis method described by Buyukcangaz et al. (2013) and the virulence genes were analyzed by Polymerase Chain Reaction (PCR) technique.
The PCR technique was performed with a thermal cycler (Bio Rad, T100TM Thermal Cicler, Singapore). The reaction mix contained 10× Buffer (10 mM Tris-HCl (pH 9.0) (Invitrogen), 2.0 mM of 20 mM MgCl2 (Invitrogen), 0.2 mM deoxynucleotide triphosphate (Invitrogen), 1U Taq DNA polymerase (Fermentas), 1 mM of each primer, to a total volume of 20 ul of reaction containing 20 ng of the extracted DNA.

All strains were investigated for molecular markers related to intimin (*eae*A) production, Shiga toxins (*stx*I and *stx*II), heat-labile enterotoxins (*LT*), heat-stable enterotoxins (*ST*), invasivity (*ial*) and enteroaggregative *E. coli* (EAEC) gene (*eagg*). Besides, they were analyzed for genes associated with adherence such as fimbriae F1 (*fim*H), curli fimbriae (*csg*A) and antigen 43 (*flu*). Base sequences and predicted sizes of the amplified products for the specific oligonucleotide primers used are shown in Table 1. The amplified products were evaluated for electrophoresis at 70 volts for 1 h in 1.5% agarose gel and developed with SYBR Green dye (Invitrogen), allowing visualization in UV light and documentation of the amplicons by image capture by the L- PIX EX (Loccus Biotechnology). The size of the fragments was estimated by comparison using the 100 bp molecular weight marker (Fermentas®).

PFGE assay

Preparation of genomic DNA for pulsed field gel electrophoresis

Bacteria were incubated in Soy Tripticase agar at 37°C for 16 to18 h. The cells were removed to microcentrifuge tubes containing 2 ml of Buffer (CSB) (100 mM Tris-HCl 100 mM EDTA pH 8) and cell density were regulated at 1unit. The cell suspension (245 µl) was transferred to microcentrifuge tubes with proteinase K (20-mg/ml stock solution) added to the final concentration of 1 mg/ml each.

An equal volume of molten 2% of Low Melting Point Agarose (BRL®) prepared in TE buffer (10mM Tris, 1mM EDTA, pH8) was added to the cell suspension. The mixture was transferred into two plugs mold and allowed to cool at 4°C for 20 min. The agarose plugs were transferred to 1275 µL of lysis buffer I (50 mM Tris, 50 Mm EDTA, pH 8.0 and 1% sodium lauryl sarcosine) with 75 µL of proteinase K (20-mg/ml stock solution), incubated at 50°C for 90 min. The lysis buffer solution was removed and the plugs were washed twice with water at 50°C for 15 min. After, the plugs were washed with 10 mL of TE buffer (10 mM Tris, 1 mM EDTA, pH8) for 4 times at 50°C for 15 min and stored in TE buffer at 4°C.

Digestion of genomic DNA in agarose plugs

Enzyme digestion was done by addition of the plug slice in microtubule containing restriction buffer (2 µl albumin, 20 µl Buffer 10×, 74 µl nuclease free water and 4 µl restriction enzyme *Xba*I).

Table 1. Primer sequence and predicted sizes of amplication products.

Target gene	Oligonucleotide Sequences of primers	Amplicon size (bp)	Reference*
*eae*A	5'-AGGCTTCGTCACAGTTG-3' 3'-CCATCGTCACCAGAGGA-5'	570	China et al., 1996
*stx*I	5'-AGAGCGATGTTACGGTTTG-3' 3-TTGCCCCCAGAGTGGATG-5'	388	CHINA et al., 1996
*stx*II	5'-TGGGTTTTTCTTCGGTATC-3' 3'-GACATTCTGGTTGACTCTCTT-5'	807	CHINA et al., 1996
ST	5'-ATTTTTCTTTCTGTATTGTCTT-3' 3'-CACCCGGTACAAGCAGGATT-5'	190	Lopez-Saucedo et al., 2003
LT	5'-GGCGACAGATTATACCGTGC-3' 3'-CGGTCTCTATATTCCCTGTT-5'	450	Lopez-Saucedo et al., 2003
ial	5'-GGTATGATGATGATGAGTCCA-3' 3'-GGAGGCCAACAATTATTTCC-5'	650	Lopez-Saucedo et al., 2003
eagg	5'-AGACTCTGGCGAAAGACTGTATC-3' 3'-ATGGCTGTCTGTAATAGATGAGAAC-5'	194	Pass et al., 2000
*fim*H	5'-TGCAGAACGGATAAGCCGTGG-3' 3'-GCAGTCACCTGCCCTCCGGTA-5'	508	Johnson & Stell, 2000
*csg*A	5'-GATCTGACCCAACGTGGCTTCG-3' 3'-GATGAGCGGTCGCGTTGTTACC-5'	178	Silva et al., 2014
flu	5'-CCGGCGGGCAATGGGTACA-3' 3'-CAGCTCTCACAATCTGGCGAC-5'	707	Restieri et al., 2007

*cycle.

The *Xba*I (Promega®) at concentration of 25 units U per plug was used for 3h at 37°C.

Electrophoresis, staining and destaining of agarose gel

PFGE was executed with the CHEF DR-III (Bio-Rad Laboratories, Hercules, C.A) system. Plug peaces were loaded and electrophoresed in 1% SeaKem gold agarose (SIGMA Chemical Company, USA) with 2 l of 0.5 X TBE (Tris Borate EDTA) running buffer. The electrophoretic criterion used: initial switch time, 2.2 s; final switch time, 54.2 s; run time, 19 h; angle, 120°; gradient, 6.0 V/cm; temperature, 14°C; ramping linear. The gels were stained after electrophoresis for 30 min in 150 ml of TBE buffer containing 6 µl of ethidium bromide (10 mg/ml).

A molecular size standard (*Salmonella* enterica serotype Braenderup H9812, CDC PFGE marker) was used at each run along with the *E. coli* isolates to be tested (Romero et al., 2004). The electrophoresis conditions adopted were those recommended by Pulsenet protocol for *E. coli* O157:H7.

The *Xba*I macrorestriction patterns were analyzed using BioNumerics 6.0 software (Applied Maths, A., USA). Clustering was created using unweighted pair group with arithmetic average UPGMA (Unweighted Pair Group Method with Arithmetic Mean) and Dice coefficient interprets according to Tenover et al. (1995). In the comparison of the similarity between clusters the cut tolerance was applied as 1.5%.

RESULTS AND DISCUSSION

The clustering analysis was conducted based on the eighteen profiles of the virulence genes selecting 30 isolates to the PFGE technique (Figure 2). The PFGE patterns of *Xba*I-digested were evaluated to determine the clonal relatedness of *E. coli* isolates in the dairy production during 2014 to 2015.

The detection of 28 different pulsotypes of *E. coli* with a low percentage of genetic similarity (≤ 68%) presents a high heterogeneity among the circulating clones within the dairy establishment studied. This high variability of pulsotypes may be related to the fact that, the target of the present study is strains that present only general characteristics of the species. The *E. coli* is a very diverse species considering virulence, resistance and other genes. Different panorama would be detected if a subgroup of such species had been selected as STEC and EHEC, among others. However, this selection was not possible due to the low prevalence of genes related to toxin production. On the other hand, regarding the presence of genes related to biofilm production, some results were interesting.

Two pulsotypes presented two strains with a clonal ratio of 100% similarity between them, being N46 and C59 belonging to pulsotype 1 and N66 and F86 to pulsotype 17. N46 and C59 strains were found respectively in milk and in the Liners at different periods (spring and summer) and obtained the same virulence profile (only positive to *fim*H). N66 and F86 strains isolated in autumn, presented the same virulence profile characterized by the presence of *fim*H and *csg*A both associated with biofilm formation.

4 strains (F64, F17, F76, C69) with pulsotypes being 97% of genetic similarity presented the identical biofilm-

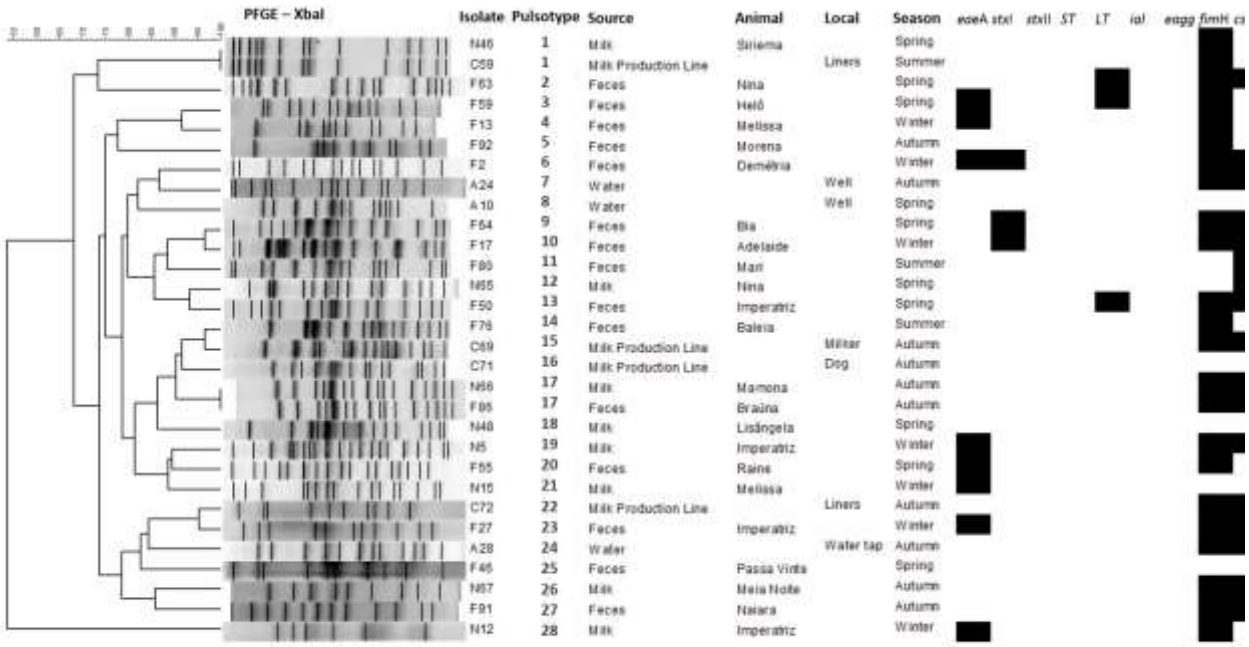

Figure 2. Dendrogram of 30 *E. coli* isolates digested with *Xba*I using the UPGMA method.

related virulence profiles. Furthermore, two of them (F64, F17) also amplify a gene related to shiga toxin (*sxt*I +) production, both isolates from feces samples. 2 strains (C72 and F27) belong to pulsotypes with 95% genetic similarity, they also presented the identical biofilm-related virulence profiles and additionally the F27 was positive for the *eae*A adhesion gene. The Figure 2 presents virulence profiles obtained with the PCR technique.

Dairy cattle and farms are known as reservoirs of several lineage of *E. coli* (Hussein and Sakuma, 2005; Gyles, 2007). This microorganism can enter a dairy farm environment through new animals inserted on herd; beyond the environmental medio such as air, water, and soil; wildlife; or organic materials, such as cattle feed and bedding (Lambertini et al., 2015). The prevalence of *E. coli* O157:H7 in cattle can be related with the seasonal influence obtaining highest rates of infection in the hottest months (Hancock et al., 1994). In this study, the clonal strains were observed in three different seasons: summer, spring and autumn that correspond to the hottest periods in Brazil.

Oliver and Page (2016) observed significant interactions between treatment and season when evaluating the effects of seasonal climatic variables on *E. coli* persistence in cattle feces through different treatments of open field exposure or with polytunnel protection. In their work the season had expressive impact *E. coli* persistence (P< 0.001) and in 2012, both seasons, summer and autumn, were observed higher in *E. coli* counts when compared to spring and winter for all treatments combined. Already in 2013, the highest *E. coli* counts (P< 0.001) was presented only in summer and the spring 2013 corresponded with the period whereby, counts of *E. coli* were considerably higher at the end of the experiment relative to day 0 (P<0.001).

Besides being present in different seasons, this microorganism was also found in different sources such as liners, milk and feces and these conditions can be related to factors that help in the persistence of *E. coli* in milk production environment. The persistence of this microorganism in mammary tissue is associated with virulence factors involving toxins which include enterotoxins that cause food poisoning and leukocidins that promote tissues/leukocyte destruction and also biofilms which enables the adherence and colonization of the mammary gland epithelium (Peacocok et al., 2002; Santos et al., 2003; Argudín et al., 2010). Although the dynamics and routes of introduction, colonization and persistence in animals and the farm environment are not well characterized, these virulence factors are important due to its intensification of the animal disease (Lambertini et al., 2015).

In our study, the presence of biofilm-associated gene (*fim*H) in 21 strains (77.7%) was found and it was observed that, this virulence factor contribute to the dispersion of this pathogen into the milk environment. Researchers have reported that dispersion can be promoted by the interaction between biofilm formation and horizontal gene transfer (Madsen et al., 2012). High frequency of *fim*H gene, the adhesion-encoding gene is associated with epithelial cell invasion in uropathogenic *E. coli*. This gene, detected in 76.6% of isolates was similar with the findings of Dogan et al. (2006), by investigating the possibility that *E. coli* strains associated

with persistent intramammary infections are more capable of adhering, invading and surviving within cultured mammary epithelial cells. Although another study has shown 100% prevalence in mastitis *E. coli* isolates (Fernandes et al., 2011).

According to Madsen et al. (2012) both biofilm formation and horizontal gene transfer have been main areas of research in microbiology showing their relevance for bacterial adaptation and evolution. They observed that plasmid and biofilm community structure and functions are interconnected through numerous complex interactions, as community and genetic level, pointing towards a main role of the action of these activities in bacterial evolution and showing its relevance about this evidence of the connection between horizontal gene transfer and biofilm formation. The premise for interconnectedness between these two strands is that conjugation happens at higher frequencies in that, biofilm are dense communities that speed up the propagation of mobile genetic elements. This happens through a spatial and structural advantage though keeps the conjugative pili intact. Moreover the high horizontal transfer frequencies of mobile plasmids can possibly be the microorganism persistence as molecular parasites (Sorensen et al., 2005; Hausner and Wuertz, 1999; Sorensen et al., 2005). Therefore horizontal transfer can, however, still be an advantage to any mobile genetic element even though is not the main strategy.

Similarly, it has been shown in studies with *E. coli* and *Lactococcus lactis* that horizontal gene transfer within the biofilm community can introduce new dynamics, due to increased expression of clumping factors simultaneously as plasmid transmission which can also cooperate to biofilm formation by their new hosts (Ghigo, 2001; Luo et al., 2005). Conjugative plasmids are inserted to various incompatibility groups, expressing different types of conjugative pili which conferred in stimulatory effect on *E. coli* K-12 biofilm formation in study realized by Ghigo (2001). These results were supported by Reisner et al. (2006) who observed that, natural *E. coli* strains which housed conjugative plasmids was more frequent in biofilm formation and this virulence factor was more expressed during derepression of plasmids. Naturally repressed incF plasmids also are capable to form biofilm, even in a lower frequency. They believe that the expression of the conjugative pili occurs in biofilm priming but it appears that, the pili is not the main structure which directly facilitates the cell surface adherence. Such process is being initiated mainly by activating the host biofilm system (Madsen et al., 2012). This was also reported by May and Okabe (2010), who discussed the expression of colonic acid and curli in *E. coli* induced by a natural incF plasmid. The conjugative pili allow cell–cell contact while the induction of the acids production promote cell-surface adherence to global cohesion and structure of the biofilm. Also Madsen et al. (2012) demonstrated that, interactions are important in understanding the interconnectedness between biofilm arrangement and plasmid biology.

All pulsotypes (n = 8) with genetic similarity of 95% were present in samples of milk, feces and liner, found in different seasons of the year which share at least one gene associated with biofilm formation. The prevalence of biofilm-associated genes indicated the persistence of the microorganism in the production environment due to the presence of biofilm factor which was possible to observe the occurrence of bacterial interaction during milk collection, owing to the fact that they were found in samples which were evaluated as closely related to the animal.

Thus, through PFGE patterns it was possible to determine that this particular farm presented flaws in relation to the hygienic criteria, since it was proven persistence to Enterobacteria in this study in the production environment, during different seasons of the year as well as different sites of collection in a same station. Therefore, the characterization of the genetic diversity of *E. coli* is important in understanding the pathogen dispersion pattern within the environment of milk production, aiding in notes the measures of control and surveillance of these microorganisms in order to control and prevent diseases caused by them.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

ACKNOWLEDGEMENTS

Authors are grateful to the National Council for Scientific and Technological Development (CNPq), Carlos Chagas Filho Foundation for Research Support of the State of Rio de Janeiro (FAPERJ) and Coordination of Improvement of Higher Level Personnel (CAPES) for their support.

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