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Original

Molecular characterization and evaluation of antimicrobial susceptibility of enteropathogenic *E. coli* (EPEC) isolated from minas soft cheese

Caracterização molecular e avaliação da suscetibilidade antimicrobiana de cepas enteropatogenicas de E. coli (EPEC), isoladas de queijo minas frescal

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Abstract

Foodborne disease caused by microorganisms is a problem of public health. Minas soft cheese is a national product manufactured using simple technology; it has high level of acceptance in the country making its production an important economic activity. Many microorganisms may be present in foods including the bacterium *Escherichia coli* (*E. coli*). Overall, *E. coli* is a harmless commensal bacterium; however, some strains may have a pathogenic potential. Several outbreaks of foodborne diseases associated with consumption of contaminated cheese have been reported, and the presence of pathogenic strains of *E. coli* has increased. The objective of this study was to isolate, evaluate the antimicrobial susceptibility, and characterize, by Multiplex PCR, the pathogenic *E. coli* strains isolated from Minas cheese commercialized in Rio de Janeiro. Thirty samples were analyzed and five strains of *E. coli* (EPEC) were identified. The assessment of antimicrobial susceptibility revealed 40% of the isolates resistant to ampicillin and 40% with intermediate resistance to ampicillin-sulbactam combination. These findings are a warning signal to health authorities since Minas cheese is a ready to eat food product, and therefore should not pose health risks to the population.
Keywords: *minas soft cheese; EPEC; antimicrobial.*

Resumo

Doenças transmitidas por alimentos (DTA), causadas por microrganismos, representam um importante problema de saúde pública. O queijo Minas Frescal é um produto nacional, de tecnologia simples e elevada aceitação no País, tornando sua produção uma importante atividade econômica. Muitos microrganismos podem estar presentes neste alimento e, entre eles, destaca-se a bactéria *Escherichia coli* (*E. coli*). De modo geral, *E. coli* é um comensal inofensivo, entretanto, algumas cepas podem apresentar potencial patogênico. Diversos surtos de DTA, associados ao consumo de queijos contaminados, têm sido relatados e a presença de cepas patogênicas de *E. coli* cada vez mais observada. Este estudo teve como objetivo isolar, avaliar a suscetibilidade antimicrobiana e caracterizar molecularmente, através da Multiplex PCR, as cepas patogênicas de *E. coli*, isoladas de queijo Minas Frescal comercializado no município do Rio de Janeiro. Trinta amostras foram analisadas e cinco cepas de *E. coli* enteropatogênica (EPEC) identificadas. A avaliação da suscetibilidade antimicrobiana revelou 40% de resistência à ampicilina e 40% de perfil de resistência intermediária à associação ampicilina-sulbactam. Tais resultados representam um alerta para as autoridades sanitárias, uma vez que este alimento é um produto de pronto consumo e, portanto, precisa ser inócuo para que a saúde da população não seja colocada em risco.
Palavras-chave: *queijo minas frescal; EPEC; antimicrobianos.*

1 Introduction

In the last decades, human nutrition has been a reason of concern worldwide. Pursuit of healthy eating habits has led to increased consumption of milk and lactic derivatives (SILVA; SOUZA, 2006).

Minas soft cheese is a typical national product manufactured with simple technology production and has wide acceptance in the country (SILVA; SOUZA, 2006). This product contains a large amount of water (43%) and low pH (5.1–5.6) (FREITAS et al., 1993), and its microbiological standards have been established by the Technical Regulation of Identity and Quality of Minas Soft Cheese (BRASIL, 1997).

Despite the legal requirements for milk intended to be used for cheese production to be sanitized through appropriate methods and subjected to pasteurization or equivalent heat treatment (BRASIL, 1974), the commercialization requirements of Minas soft cheese are not thoroughly followed, especially by small industries and homemade cheese production (BRAMLEY; McKINNON, 1990). The handmade production of Minas soft

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cheese is considered an important source of income of farm families (EMPRESA…, 2004). Several outbreaks of foodborne diseases (FBD) associated with contaminated cheese have been reported worldwide (PASSOS et al., 2008).

The Technical Regulation which comprises food microbiological standards is Resolution nº 12/2001 (BRASIL, 2001). According to item 1.2.2 of appendix II, food which presents pathogenic microorganisms or toxins that pose risk to consumer health are considered improper to consumption. Water and food contamination by fecal bacteria represents a common and frequent issue resulting in impacts to public health and country economy (STEWART; SANTO DOMINGO; WADE, 2007).

Escherichia coli (*E. coli*), which belongs to the *Enterobacteriaceae* family, is a gram-negative, facultative anaerobic, non-sporulating bacteria. It is widely distributed in intestinal microbiota of humans and warm-blooded animals and in the environment, when contaminated with fecal elimination (NATARO; KAPER, 1998; SMITH; WILLSHAW; CHEASTY, 2004).

Generally, *E. coli* is a harmless commensal; however, several strains may show pathogenic features (NATARO; KAPER, 1998). Due to its great genetic diversity, it has remarkable ecologic plasticity, which makes *E. coli* able to quickly adapt to different environments and therefore able to undergo a transition from free-living life organism to a commensal organism of the intestinal tract of warm-blood animals and finally to a deadly pathogen in humans and animals (SOUZA et al., 1999).

According to the virulence mechanisms and set of signs and symptoms of the diseases caused, pathogenic *E. coli* strains are classified into six different pathotypes: enteropathogenic *E. coli* (EPEC), enterotoxigenic *E. coli* (ETEC), Shiga toxin producer *E. coli* (STEC)/enterohemorrhagic *E. coli* (EHEC), enteroaggregative *E. coli* (EAEC), enteroinvasive *E. coli* (EIEC), and diffuse adhesion producer *E. coli* (DAEC) (NATARO; KAPER, 1998; LÓPEZ-SAUCEDO et al., 2003). There are many serotypes of Stx-producing *E. coli* (STEC), but only those that have been clinically associated with hemorrhagic colitis are designated as EHEC. These pathogenic strains are commonly associated to endemic diarrhea in children (HUILAN et al., 1991). However, cases of diarrhea and mortality are not exclusive of developing countries. In the USA, 48 million cases of FBD are notified per year resulting in 128,000 hospitalizations and 3,000 deaths (SCALLAN, 2011).

All pathogenic *E. coli* strains have already been directly related to foodborne disease outbreaks, including meat, vegetables, milk, and dairy products affecting mainly children and immunocompromised individuals in general. The presence of pathogenic *E. coli* strains in Minas soft cheese has been reported by several studies (NASCIMENTO; SABIONI; PIMENTA, 1988; PANETO et al., 2007).

Several studies suggest that every pathogenic *E. coli* pathotype carries out one or more specific genes or a combination of virulence factors distinguishing them from non-pathogenic strains and other pathotypes (SHPIGEL et al., 2008). Genes encoding virulence factors are conserved among isolated samples from different continentes (ARANDA et al., 2007; BRANDAL et al., 2007). A substantial progress and development has been achieved based on nucleic acids analysis; thus the amplification of specific DNA sequences of those genes has been used as an appropriate tool for the challenging task of finding pathogenic *E. coli* in clinical samples (GÓMEZ-DUART et al., 2009) and food samples (LÓPEZ-SAUCEDO et al., 2003). Furthermore, another concern is the probability of human intestinal mucosa colonization by microorganisms which are resistant to the antimicrobial frequently used to treat illnesses caused by ingestion of contaminated food (DIAS et al., 2010).

Cases of pathogenic *E. coli* strains showing different resistance patterns (COSTA et al., 2006) and cases of multi-resistant strains (GUERRA et al., 2003; DIAS et al., 2010) have been reported.

Foods of animal origin are remarkably known as an important factor in the FBD chain. This study aimed to isolate, evaluate the antimicrobial susceptibility, and molecularly characterize, by Multiplex PCR, pathogenic *E. coli* strains isolated from Minas soft cheese commercialized in the Rio de Janeiro.

2 Material and methods

2.1 Samples

In the period from 2007 to 2009, thirty samples of Minas soft cheese were acquired. Among those, 22 samples had gone through Sanitary Inspection (municipal, state, or federal), and 8 were handmade samples. The samples were purchased from supermarkets located in Rio de Janeiro and local homemade product stores. Following acquisition, the samples were stored in individual packages, sealed, and taken to the laboratory under refrigeration. The analyses were conducted at the food microbiology department of the *Instituto Nacional de Controle de Qualidade em Saúde* (INCQS) – FIOCRUZ.

2.2 Isolation, identification, and antimicrobial susceptibility evaluation of the pathogenic strains

Isolation of pathogenic *E. coli* strains was performed according to the methodology described by Feng, Weagant and Jinneman (2003) in the Bacteriological Analytical Manual. From each sample, approximately 160 typical or atypical *E. coli* colonies were selected for microbial identification by biochemical tests, antimicrobial susceptibility evaluation, and molecular characterization.

The biochemistry identification of species and antimicrobial susceptibility evaluation were carried out using GNI and GNS cards, respectively, performed by the VITEK_32 semi-automatized system (BioMerieux). Susceptibility evaluation was based on the Clinical Laboratory Standards Institute (CLSI). The antimicrobials tested and their minimum inhibitory concentration (MIC), in mcg/mL were: Amikacin (NA; 2), Ampicilin (AM; 32), Ampicilin-Sulbactan (MAS; 16), Azetreonam (AZM; 8), Cefepime (FEP; 4), Cefotaxime (TAX; 4), Cefoxitin (FOX; 2), Ceftazidime (TAZ; 8), Cefalotin (CF;

8), Ciprofloxacin (CIP; 0.5), Gentamicin (GM; 0.5), Imipenem (IMI; 4), Meropenem (MEM; 2), Piperacilin-Tazobactam (TZP; 8), and Trimethoprim-Sulfamethoxazole (STX; 10).

For this study, the selected *E. coli* colonies were streaked in "spots" onto trypticase soy agar (MERK) master-plates and incubated at 37 °C/24 hours for further DNA extraction, purification, and molecular analysis.

2.3 DNA extraction

Isolation and purification of genomic DNA was performed using the DNeasy Tissue kit (QIAGEN) according to the manufacturer instructions. DNA samples were stored at –20 °C.

2.4 Molecular detection of virulence genes

Purified DNA from isolated colonies previously extracted and stored at –20 °C was used for molecular analysis. Reagents and primers used for the molecular characterization in this study were acquired from "Invitrogen Life Technologies" (*São Paulo, SP, Brasil*).

2.5 Reference strains

Five reference strains were used as positive controls; each belonging to a group of pathogenic *E. coli*: strains INCQS 00181 (CDC 055 – EPEC), INCQS 00171 (CDC EDL – 933 – EHEC) and INCQS 00170 (CDC EDL – 1284 – EIEC), from the Reference Culture Collection of *Laboratório de Micro-orgamismos de Referência* – INCQS/FIOCRUZ; strain 258909-3 ETEC (CFA/I^{+} 0128:H –; LT+/ST+), originated from *Universidade do Estado do Rio de Janeiro* (UERJ), and strain 2391 – EAEC originated from *Instituto Adolfo Lutz* – SP (IAL). A PCR reaction mix without DNA was used as negative control.

2.6 Target genes and primers utilized

Selected target genes and primers used for each pathogenic *E. coli* category were respectively: *eae* and SK1/SK2 for EPEC; *stx* and Vtcom-u/Vtcom-d for STEC/EHEC; *est*/*elt* and EST-F/EST-R, ELT-F/ELT-R for ETEC; *aggR* and aggRks1/aggRskas2 for EAEC; *ipaH,* and ipaIII/ipaIV for EIEC (Table 1). The *eae* gene codifies for Intimin, a protein which causes attaching-and-effacing lesions; *stx* gene codifies the Shiga-like toxin; *elt* and *est* genes that respectively codify the Thermolabile and Thermoestable toxins of enterotoxigenic *E. coli*; *ipaH* gene is associated with invasion capacity, and it is present in multiple copies in the plasmids and in EIEC chromosomes; *aggR* gene is a transcriptional activator of aggregative adherence fimbriae.

2.7 Components and multiplex PCR conditions

The M-PCR reaction was carried out based on Toma et al. (2003) and optimized in this study to allow the simultaneous amplification of all target genes. It was used a final volume of 50 µL containing 50-100 ng of DNA sample, 1X PCR Buffer, $MgCl_2$ (3.0 mM), 1 U of *Taq* DNA Polymerase dNTP mix (0.3 mM), 1.250 µM of SK1 and SK2 primers, 0.250 µM of Vtcom-u and Vtcom-d primers, 0.500 µM of EST-F and EST-R primers, 0.250 µM of ELT-F and ELT-R primers, 0.100 µM of ipaIII and ipaIV primers and 0.250 µM of aggRks1, and aggRkas2 primers.

Multiplex PCR was assembled with 30 amplification cycles at 95 °C for 30 second; annealing at 50 °C for 30 second; extension at 72 °C for 1 minute, and a final extension at 72 °C for 10 minutes.

PCR products were differentiated by molecular weight through a 2% agarose gel electrophoresis at 80 V for 2 hours in 1X TBE Buffer (Tris HCl 100 mM, boric acid 90 mM, EDTA 1 mM, pH 8,0). A 100 bp DNA ladder (Invitrogen, Carlsbad, CA, USA) was used as molecular weight pattern; the gels were stained with ethidium bromide (0.5 $\mu g.mL^{-1}$), visualized, and digitalized using Video Documentation System and analyzed with the ImageMaster software (Amersham Pharmacia Biotech).

3 Results and discussion

The data obtained in this study indicate the unsatisfactory hygienic-sanitary conditions of Minas soft cheese commercialized in the city of Rio de Janeiro. Since it is a ready-to-eat product made with raw components, this kind of cheese should be innocuous and not a potential health risk.

Table 1. Genes, primers and sequences used in the Multiplex PCR.

Gene[a]	"Primer"	Sequence (5'-3')	Amplicon (bp)	Anel Temp. (°C)	Reference
eae	SK1	CCCGAATTCGGCACAAGCATAAGC	881	50	Oswald et al. (2000)
	SK2	CCCGGATCCGTCTCGCCAGTATTCG			
stx	Vtcom-u	GAGCGAAATAATTTATATGTG	518	50	Yamasaki et al. (1996)
	Vtcom-d	TGATGATGGCAATTCAGTAT			
est	EST-F	ATTTTTMTTTCTGTATTRTCTT	190	50	López-Saucedo et al. (2003)
	EST-R	CACCCGGTACARGCAGGATT			
elt	ELT-F	GGCGACAGATTATACCGTGC	450	50	López-Saucedo et al. (2003)
	ELT-R	CGGTCTCTATATTCCCTGTT			
ipaH	ipaIII	GTTCCTTGACCGCCTTTCCGATACCGTC	619	**50**	Sethabutr et al. (1993)
	ipaIV	GCCGGTCAGCCACCCTCTGAGAGTAC			
aggR	aggRks1	GTATACACAAAAGAAGGAAGC	254	**50**	Ratchtrachenchai et al. (1997)
	aggRkas2	ACAGAATCGTCAGCATCAGC			

1 - 100 bp DNA ladder; 2 - positive control.

Isolated colonies from 100% of samples exhibited phenotypic characteristics which suggest *E. coli* identification. Similar data were described by Araujo et al. (2002) and Paneto et al. (2007), who reported extremely high contaminations of *E. coli* in Minas soft cheese, with isolation rates of 97% and 96% of samples respectively.

The visualization of simultaneous amplification of *eae* gene for EPEC; *est* and *elt* for ETEC; *stx* for STEC; *aggR* for EAEC, and *ipaH* for EIEC used as positive controls can be observed in Figure 1. The amplification of *eae* gene for positive strains can be observed in Figure 2.

The molecular method used in the present study showed to be highly sensitive and specific to detect pathogenic *E. coli* strains originated from the studied samples, as documented by Paneto et al. (2007) and Stephan et al. (2008). From this Multiplex PCR, 5 enteropathogenic *E. coli* strains were molecularly identified.

According to the WHO (WORLD..., 2002), worldwide authorities have been intensifying their efforts to improve food security; however, pathogenic *E. coli* strains are still found in foods. After a foodborne outbreak caused by EPEC, the presence of this microorganism in cheeses has acquired a special significance (MARIER et al., 1973; PANETO et al., 2007).

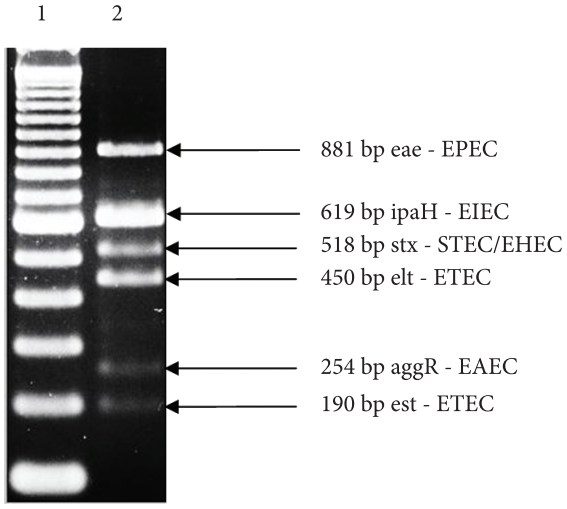
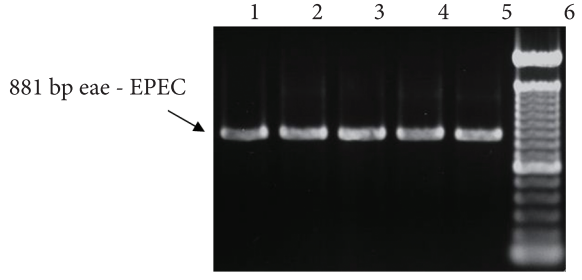


Figure 1. 2% Agarose Gel Electrophoresis. Visualization of amplified genes used as positive controls in multiplex PCR.



1-5 positive strains (MTD1, MTD2, MTD3, MTD4, MTD5) respectively;
6 - molecular weight (100 bp DNA ladder)

Figure 2. 2% Agarose Gel Electrophoresis. Amplification of *eae* gene for positive strains.

In 1988, Nascimento, Sabioni and Pimenta (1988), analyzing several Minas soft cheese in Ouro Preto-MG, Brazil, advised against the presence of EPEC strains in this food. Even though, improvements regarding food security have been observed since then, EPEC has been isolated from this kind of cheese, as confirmed by the present study. Preliminary data were reported by Gonzalez et al. (2000), Araújo et al. (2002) and Schrade and Yager (2001) and others.

All 5 isolated EPEC strains were isolated from a handmade cheese sample corresponding to 12.5% of the total of handmade analyzed samples. Although handmade cheese production is considered an important economic activity, Bramley and McKinnon (1990) reported the hazard of product contamination by pathogenic microorganisms affecting both product quality and consumer health (FURTADO, 1991).

Domestic animals from different species have been investigated as natural reservoirs of pathogenic *E. coli* strains. Several studies have highlighted the importance of cattle, swine, and ovine, which are considered the most common species involved in this kind of transmission (GLADYS; SONJA; LOTHAR, 2005). China et al. (1999) and Dias (2007) reported the presence of AEEC (attaching and effacing *E. coli*, atypical EPEC strains, isolated mainly from animals) strains with pathogenic profile in healthy bovines, which may be an important issue of health security since these animals are used for meat and milk production. According to Dias (2007), atypical EPEC strains show a compatible virulence profile in animals and humans because of the similarity of phenotypic and genotypic markers detected.

Similar results were reported by Ahmed, Ahmed and Moustafa (1988) and Najand and Ghanbarpour (2006), who noted the presence of EPEC in handmade cheese samples. Other pathogenic *E. coli* strains have also been reported in this product, such as ETEC and VETEC (PANETO et al., 2007), EAEC (SCAVIA et al., 2008), and STEC (STEPHAN et al., 2008).

Our results are in agreement with those of research reports worldwide, such as Araújo et al. (2002), Najand and Ghanbarpour (2006) and Planzer Junior et al. (2009), emphasizing the danger that cheese production from unpasteurized milk presents.

Among the isolated EPEC strains, 40% showed ampicillin resistance and 40% intermediary resistance profile to ampicillin-sulbactam association. Although multi-resistance *E. coli* cases have been reported (BACCARO et al., 2002; GUERRA et al., 2003; DIAS et al., 2010), such feature was not observed in this study.

High resistance to ampicillin was described by Baccaro et al. (2002) and Blanco et al. (2006); however, Dias et al. (2010) found only 2.27% of resistance to this antimicrobial. The *E. coli* strains isolated by Paneto et al. (2007) in Minas soft cheese showed higher cephalothin resistance (69%) and only 29% to ampicillin. In this study, the resistance to cephalothin was not observed. To Baccaro et al. (2002), resistance to the association trimethoprim-sulfamethoxazole

Table 2. Antimicrobial susceptibility, ESBL presence and motility test of isolated EPEC.

Antimicrobial MIC (mcg.ml^{-1})	**Strains**				
	MTD1	MTD2	MTD3	MTD4	MTD5
Amikacin (2)	S	S	S	S	S
Ampicilin (32)	R	S	R	S	S
Ampicilin-Sulbactan (16)	I	S	I	S	S
Azetreonam (8)	S	S	S	S	S
Cefepime (4)	S	S	S	S	S
Cefotaxime (4)	S	S	S	S	S
Cefoxitin (2)	S	S	S	S	S
Ceftazidime (8)	S	S	S	S	S
Cefalotin (8)	S	S	S	S	S
Ciprofloxacin (0.5)	S	S	S	S	S
Gentamicin (0.5)	S	S	S	S	S
Imipenem (4)	S	S	S	S	S
Meropenem (2)	S	S	S	S	S
Piperacilin-Tazobactam (8)	S	S	S	S	S
Trimethoprim-Sulfamethoxazole (10)	S	S	S	S	S
ESBL	negative	negative	negative	negative	negative
Motility	positive	positive	positive	positive	positive

(87.4%) was similar to ampicillin (86.8%). Costa et al. (2006) also observed a high resistance (73.5%) to the association trimethoprim-sulfamethoxazole, which was not observed in this study since 100% of the strains were sensible to these agents.

Some authors have associated beta-lactamase production with an increase in antimicrobial resistance (FERREIRA et al., 2006). The selective pressure exerted by antimicrobials such as beta-lactams compounds is related to the selection of resistant strains including extended spectrum beta-lactamase producing enterobacteria (ESBL) (CHOI et al., 2008). Emery and Weymouth (1997) demonstrated the existence of ESBL producing *E. coli*.

The extended spectrum beta-lactamase enzymes are capable to inactivate drugs such as ceftazidime and cefotaxime. In this study, it was observed 100% of sensibility to these antimicrobials, an expected result since ESBL strains were found to be negative.

The ampicillin-sulbactam association has been considered a potent inhibitor of beta-lactamases, which can inactivate this resistance (FERREIRA et al., 2006). In this study, 40% of the isolates showed intermediate resistance profile to these antimicrobials. However, resistant strains to this association have been observed, as showed by Nascimento et al. (2009), who found resistance to ampicillin-sulbactam of 75.6%. The results of antimicrobial susceptibility evaluation are described in Table 2.

Dias et al. (2010) reported that *E. coli* strains show high variability and high capacity to acquire resistance genes according to Gonçalves (2009). This association of factors can explain the divergences observed in the study of this microorganism.

4 Conclusions

All isolates analyzed were phenotypically classified as *Escherichia coli*. The presence of EPEC strains in Minas soft cheese represents an important risk factor to public health; the quality of raw material and hygienic-sanitary aspects are essential for product safety.

It was noted that the use of multiplex PCR optimized in this study is an effective tool to identify pathogenic *E. coli* strains in Minas soft cheese.

The ingestion of products contaminated with EPEC strains, such as those identified in this study, may cause the colonization of intestinal tract by antimicrobial resistant strains.

The results found are alarming since the analyzed product is ready for consumption and frequently used in hospital diets; therefore, the Minas soft cheese must be innocuous, and should not pose potential health risk.

The commercialization of milk and its derivatives without any kind of inspection, a typical countryside tradition in the past, is nowadays present in the small commerce and markets in Rio de Janeiro. Although there is no legal authorization for this commercialization, people who consume these products regularly get sick frequently. This may suggest that integrated actions among research, inspection, and extension centers associated with effective public policies could make milk and dairy production a viable, safe, and sustainable activity.

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