

Occurrence of toxigenic *Escherichia coli* in raw milk cheese in Brazil

[*Ocorrência de Escherichia coli toxigênica em queijo-de-minas frescal no Brasil*]

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ABSTRACT

The occurrence of toxigenic *Escherichia coli* in raw milk cheese was surveyed in Middle Western Brazil. Fifty samples of cheese from different supermarkets were analyzed for *E.coli*. The isolates were serotyped and screened for the presence of verotoxigenic *E. coli* (VTEC) and enterotoxigenic *E. coli* (ETEC) by Polymerase Chain Reaction (PCR). The susceptibility to thirteen antimicrobial agents was evaluated by the disk diffusion method. *E.coli* were recovered from 48 (96.0%) of the samples. The serogroups identified were O125 (6.0%), O111 (4.0%), O55 (2.0%) and O119 (2.0%). Three (6.0%) and 1(2.0%) of the *E.coli* isolates were VTEC and ETEC, respectively. Most frequent resistance was observed to the following antimicrobials: cephalothin (60.0%), nalidixic acid (40.0%), doxycyclin (33.0%), tetracycline (31.0%) and ampicillin (29.0%).

Keywords: *Escherichia coli*, cheese, VTEC, ETEC, antimicrobial resistance

RESUMO

Pesquisou-se a ocorrência de *Escherichia coli* toxigênica, em queijo produzido com leite não pasteurizado, na Região Centro Oeste do Brasil. Foram utilizados 50 queijos adquiridos em diferentes supermercados. As amostras isoladas foram classificadas por sorogrupo, avaliadas em relação à sensibilidade para 13 agentes antimicrobianos e submetidas à reação em cadeia da polimerase para a presença de genes característicos de *E. coli* verotoxigênica (VTEC) e enterotoxigênica (ETEC). *E. coli* foi recuperada em 48(96,0%) dos queijos. Foram identificados os sorogrupos O125 (6,0%), O111 (4,0%), O55 (2,0%) e O119 (2,0%). Três (6,0%) amostras de *E. coli* foram classificadas como VTEC e uma (2,0%) como ETEC. Os maiores índices de resistência foram verificados para: cefalotina (60,0%), ácido nalidíxico (40,0%), doxiciclina (33,0%), tetraciclina (31,0%) e ampicilina (29,0%).

Palavras-chave: *Escherichia coli*, queijo, VTEC, ETEC, resistência antimicrobiana

INTRODUCTION

Minas Frescal cheese is one of the most popular cheeses produced in Brazil. This soft, white cheese is made of pasteurized or raw milk. It is characterized by a high water content (43.0%) and low pH (5.1-5.6) (Freitas et al., 1993). Microbiological standards for soft cheese

inspection are determined by the Brazilian National Public Health Agency (Portaria..., 1997). Recovery and counting of *Escherichia coli* is used as an index of recent fecal contamination and indicate that other microorganisms of fecal origin may be present (Portaria..., 1997).

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After an outbreak of foodborne disease caused by enteropathogenic *E. coli* (Marier et al., 1973), the presence of these microorganisms in cheese acquired additional significance. Toxigenic *E. coli* of bovine origin has been classified into three categories: enterotoxigenic *E. coli* (ETEC), verotoxigenic *E. coli* (VTEC), and necrotoxicogenic *E. coli* (NTEC) (Quinto and Cepeda, 1997). The first group constitutes one of the most important vectors of *E. coli* diarrhea, and is considered the major cause of diarrhea in children in developing countries; it is also the most frequently etiological agent responsible for travellers' diarrhea (Nataro and Kaper, 1998). ETEC causes diarrhea by adhering to the intestinal mucosa by their unique colonization factors, producing either heat-labile enterotoxins (LT-I and LT-II), heat-stable enterotoxins (STa and STb), or both (Nataro and Kaper, 1998).

Verocytotoxin (VT)-producing *E. coli* (VTEC) are a well recognized cause of severe disease in human beings, such as haemolytic uraemic syndrome (HUS). While numerous outbreaks have been related to *E. coli* serotype O157:H7, several other VTEC serotypes have been associated with human diseases (Paton and Paton, 1998). Outbreaks and sporadic cases of illnesses were also traced to consumption of VTEC-contaminated cheese (Deschenes et al., 1996).

CNF-producing *E. coli*, known as necrotizing *E. coli* (NTEC) have been isolated from animals with enteritis (De Rycke et al., 1987), however they have been rarely found in Brazil (Salvadori et al., 2003).

Cattle are an important reservoir of toxigenic *E. coli*, and have been implicated as a source of *E. coli* that infect and cause disease in human beings (Hornitzky et al, 2002). The aims of the present study were to determine the presence of ETEC and VTEC in soft cheese made of raw milk in Middle Western Brazil, and also to verify the resistance of *E. coli* strains to antimicrobial agents.

MATERIALS AND METHODS

Fifty samples of soft cheese made of raw milk were purchased from Araguaina (Tocantins State, Brazil) city supermarkets. All samples

were kept under refrigeration in plastic bags; information about dates of production and of assigned shelf lives were not presented. Cheese samples were collected over a period of six months between February and July 2003, and were analyzed on the day of acquisition. Samples were transported under refrigeration (4-6°C) in thermal boxes containing ice packs and were tested immediately after collection.

A 25g portion of each cheese was blended with 225ml of nutrient broth¹ for two min at normal speed, using a Stomacher lab blender and incubated at 37°C for 24h. An 1ml sample of the nutrient broth culture was mixed with 9ml of MacConkey broth² and further incubated at 37°C for 24h. One loop of each tube was streaked on MacConkey agar. Four colonies from each plate with typical *E. coli* morphology were selected and examined by biochemical tests, including hydrogen sulphide, citrate, urease and indole (Koneman et al., 1997). Hundred and sixty eight *E. coli* strains were isolated from fifty cheese samples, and one isolate from each cheese was used for further characterization. A total of forty eight samples were studied for the determination of O antigens, all *E. coli* colonies were tested for slide agglutination with commercial polyvalent and monovalent antisera³.

The antimicrobial susceptibility patterns of *E. coli* strains isolated from cheese was done by the disk diffusion method using commercial disks⁴, according to the guidelines of the National Committee for Clinical Laboratory Standards (Performance..., 2002), testing the susceptibility against 13 antimicrobial drugs. Antimicrobial agents tested, loaded on the disks (concentration expressed in µg) were the following: ampicillin (AMP-10); amikacin (AMI-30); nalidixic acid (NAL- 30); cephalothin (CFL- 30); cefotaxime (CTX-30); cefazolin (CFZ- 30); doxycyclin (DOX-30); gentamicin (GEN-10); neomycin (NEO-30); netilmicin (NET- 30); tetracycline (TET-30); tobramycin (TOB-10); trimethoprim (TRI-5).

Bacterial strains were overnight grown in nutrient broth⁵ at 37°C. A 100µl sample of the culture was

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centrifuged and the pellet resuspended in distilled water. After boiling the suspension for 10min, the supernatant was used as a template for PCR. The base sequences, annealing temperatures and predicted sizes of the amplified products for the

specific oligonucleotide primers used in this study are shown in Table 1. The amplified product was visualized by ethidium bromide staining after gel electrophoresis of 10µl of the final reaction mixture in 1.5% agarose.

Table 1. Sequences and predicted lengths of PCR amplification products of the oligonucleotide primers used

Primer	Oligonucleotide sequence (5' - 3')	Annealing		
		Product size (bp)	Temperature(°C)	Reference
VT1	CAGTTAATGTGGTGGCGAAG CTGCTAATAGTCTGCGCATC	894	55	Orden et al. (1998)
VT2	CTTCGGTATCCTATTCCCGG GGATGCATCTCTGGTCATTG	478	55	Orden et al. (1998)
LT-I	TGGATTCATCATGCACCACAAGG CCATTTCTTTTTGCCTGCCATC	360	63	Pass et al. (2000)
LT-II	AGATATAATGATGGATATGTATC TAACCCTCGAAATAAATCTC	300	52	Penteado et al. (2002)
STa	TCTGTATTATCTTTCCCCTC ATAACATCCAGCACAGGC	186	43	Schultz et al. (1994)
STb	CCCTCAGGATGCTAAACCAG TTAATAGCACCCGGTACAAGC	166	43	Schultz et al. 1994

RESULTS AND DISCUSSION

E. coli was isolated in 48 (96.0%) out of the 50 tested cheeses. Forty eight strains were isolated and 7(14.0%) were identified by the slide agglutination serological test using available polyvalent and monovalent antisera as pertaining to serogroups O125, O111, O55 and O119 (Table 2), all commonly involved in human diseases (Nataro and Kaper, 1998). Similar

results were reported by Araujo et al. (2002) who found that 97.0% of soft cheese samples from Rio de Janeiro, Brazil, contained *E. coli* of the same serogroups, among others. Serogroups O111 and O119 have been recognized as important pathogens in Brazil (Gomes et al, 1991) and also have been isolated as STEC strains from diarrheic and non-diarrheic calves in Brazil (Leomil et al., 2003; Irino et al, 2005).

Table 2. Serogroup and virulence factors of *Escherichia coli* isolates from cheese made of raw milk in Brazil (n=48)

<i>E. coli</i> serogroup	Nº of isolates (%)	Gene (nº of isolates / %)
O ₁₂₅	3 (6.0)	-
O ₁₁₁	2 (4.0)	-
O ₅₅	1 (2.0)	-
O ₁₁₉	1 (2.0)	-
NT	41 (86.0)	VT2 (2/4.0), VT1 (1/2.0), LT-II (1/2.0)

NT- not typeable strain using the EPEC commercial antisera.

PCR showed that 2 isolates (4.0%) carried the VT2 gene, and 1 isolate (2.0%) the VT1 gene (Table 2), a value much higher than 0.4% registered by Quinto and Cepeda (1997) in soft

cheese in Spain, but less than the 13.0% reported by Vernozzy-Rozand et al. (2005) in French cheese. PCR of heat labile (LT-I and LT-II) and heat stable (STa and STb) enterotoxins (Table 2)

showed that only one isolate carried the LT-II gene while the ST gene was not found. Frank et al. (1984) reported the presence of 3.2% of ETEC strains in milk and milk products. Soft and semi-soft cheese have been previously associated with disease outbreaks involving pathogenic strains of *E. coli* (MacDonald et al., 1985; Deschenes et al., 1996) which demonstrated that contamination occurs at some point during cheese production and processing. Experimental studies have already shown that *E. coli* O157 can survive during the manufacturing process of soft Hispanic-type cheese (Kasrazadeh et al., 1995). These findings indicate that food of animal origin may be a significant source of pathogenic species of *E. coli*.

Most frequent resistance was observed to the following antimicrobials: cephalothin (60.0%), nalidixic acid (40.0%), doxycyclin (33.0%), tetracycline (31.0%) and ampicillin (29.0%) (Table 3). Zhao et al. (2001) examined VTEC strains from humans, animals and food, and reported a high antimicrobial resistance to sulfamethoxazole (48.0%), streptomycin (43.0%), tetracycline (43.0%) and ampicillin (33.0%), and some of them were similar to those found in this study. Resistance to at least one of a series of tested antimicrobial agents was found in 83.0% of the examined isolates. Khan et al. (2002) reported resistance to one or more antimicrobials in 49.2% of the VTEC strains in India, moreover, some of those strains showed multidrug-resistance. The high level of resistance may be a consequence of the abusive use of antimicrobials in animal therapeutics as well as in food additives used to promote animal growth.

It is concluded that toxigenic *E. coli* may pass to the milk destined to manufacture cheese, surviving in soft cheese made of raw milk, confirming the results of other authors (Quinto and Cepeda, 1997; Araujo et al., 2002; Vernozy-Rozand et al., 2005). They represent a health hazard and this suggests that soft cheese should be considered a vehicle for the transmission of potentially pathogenic bacteria.

Table 3. Antimicrobial susceptibility of 48 *Escherichia coli* isolates from cheese made of raw milk in Brazil

Antimicrobial agent	No	%
Cephalothin	29/48	60.0
Nalidixic acid	19/48	40.0
Doxycyclin	16/48	33.0
Tetracycline	15/48	31.0
Ampicillin	14/48	29.0
Neomycin	12/48	25.0
Trimethoprim	8/48	17.0
Cefazolin	6/48	13.0
Tobramycin	1/48	2.0
Gentamicin	0/48	0.0
Amikacin	0/48	0.0
Cefotaxime	0/48	0.0
Netilmicin	0/48	0.0

No= Number of isolates resistant/number of isolates;
%= percentage of resistant isolates.

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