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Research Paper

PCR and ELISA (VIDAS ECO O157®) *Escherichia coli* O157:H7 identification in *Minas Frescal* cheese commercialized in Goiânia, GO

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Abstract

Escherichia coli O157:H7 has been incriminated in food poisoning outbreaks and sporadic cases of hemorrhagic colitis and hemolytic uremic syndrome in many countries. Considering the high susceptibility of Minas Frescal cheese to contamination by *E. coli* O157:H7, the aim of this study was to determine the occurrence of this pathogen through PCR (Polymerase Chain Reaction) and ELISA (VIDAS ECO O157®, bioMérieux, Lyon, France) test. Thirty cheese samples manufactured by artisan farmhouse producers were collected from open-air markets in Goiânia and thirty from industries under Federal Inspection located in Goiás State which trade their products in supermarkets in Goiânia. *E. coli* O157:H7 was detected in 6.67% samples collected in open air markets using ELISA, and 23,33% with PCR. The pathogen was not detected in samples from industries under Federal Inspection.

Key words: Minas Frescal cheese, STEC/VTEC , PCR, VIDAS ECO O157®.

Introduction

Food-borne diseases are of major importance due to public health concerns and damages to consumers. *Escherichia coli* O157:H7 was first linked to food-borne diseases in the United States of America after an outbreak by ingestion of undercooked hamburgers at a fast food restaurant in 1982 (Riley, *et al.*, 1983). Since then *Escherichia coli* O157:H7 has become a worldwide threat to public health and is one of today's most troubling food-borne pathogens. The main *E. coli* O157:H7 reservoir is the gastrointestinal tract of cattle, being a source of potential contamination during milking procedures (Hussein and Sakuma, 2005). *E. coli* O157: H7 was detected in 6.8% of feces samples from cattle and 0.2% of dairy cows in Argentina (Padola, *et al.*, 2004; Fernández, *et al.*, 2010), while in the United States, LeJeune *et al.* (2006) isolated this serotype in 0.6% of dairy cows from Ohio State. Undercooked beef, unpasteurized milk and use of water contaminated by cattle manure have been associated to human outbreaks of hemorrhagic colitis and hemolytic uremic syndrome (Hussein and Sakuma, 2005; Sandrini, *et al.*, 2007; Karmali, *et al.*, 2009).

Verocytotoxin-producing *E. coli* O157 (VTEC/STEC) comprise over 400 serotypes (Scheutz, *et al.*, 2005). SerotypeO157:H7 is prevalent in many world regions (Armstrong, *et al.*, 2006) and considered the most dangerous vegetative pathogen associated with raw milk and raw milk cheeses (Baylis, 2009). Murphy *et al.* (2007) detected five verocytotoxigenic *E. coli* O157 isolates in a surveillance of dairy production holdings supplying raw milk to farmhouse cheese makers in Ireland. Paneto *et al.* (2007) analyzed 50 samples of cheese produced from raw milk obtained from Araguaina (Tocantins State, Brazil) supermarkets and determined STEC occurrence in 6%. In a review conducted by Baylis (2009) numerous cases of food poisoning by *E. coli* O157:H7 due to unpasteurized milk products consumption were reported.

In Brazil, no *E.coli* O157:H7 food-borne outbreak was reported so far although this serotype has been isolated

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from sporadic cases of human diarrhea (Irino, *et al.*, 2002) and cattle feces (Riley, *et al.*, 1983; Cerqueira, *et al.*, 1999; Sandrini, *et al.*, 2007; Stella *et al.*, 2008).

Raw milk cheese may be a source of *E.coli* as the pathogen may survive the manufacturing process. Brazilian government legislation provides for the use of pasteurized milk in cheese production but farm cheese craft manufacturers still use raw milk. The classic example in Brazil is *Minas Frescal* cheese where a production of 28,8 metric tons was reported in 2004 (Brasil, 2004). As a fresh product susceptible to microbiological changes it is frequently involved in outbreaks of food poisoning.

This study was developed in order to verify the occurrence of *E.coli* O157:H7 in cheese commercialized in Goiania, by PCR and ELISA (VIDAS ECO O157 commercial kit ®).

Materials and Methods

From January to March 2009 60 samples of *Minas Frescal* cheese were collected, 30 of which came from open-air street markets (farmers market) from Goiânia city, Goiás State and 30 from milk and dairy plants under Federal Inspection (IF), located in the State of Goias.

The occurrence of *E.coli* O157:H7 in *Minas Frescal* cheese was determined by the commercial kit VIDAS® ECO O157 (bioMérieux, Lyon, France) and a polymerase chain reaction (PCR) protocol. Analytical procedures were directed primarily to the presence of somatic antigen "O157" and then positive samples were processed for the detection of flagellar antigen "H7".

The kit VIDAS ® ECO O157 (BioMérieux, Lyon, France) was used in accordance with manufacturer's instructions. To confirm a positive result as *E. coli* O157:H7 isolation of the microorganism and specific serological tests were performed following the AFNOR's protocol for general food products (Association Française de Normalisation - No. BIO-12/8-07/00).

Initially, 25 g of each individual sample were enriched in 225 mL of modified trypcase-soy broth (Difco, USA) supplemented with an aqueous solution of acriflavine-HCl (final concentration of 10 mg/L) and incubated at 41 °C for 6 to 7 h. Then, 1 mL of pre-enrichment broth was transferred to 9 mL of MacConkey broth (Difco, USA) with Cefixime (final concentration 0,05 mg/L) and potassium tellurite (final concentration 2.5 mg/L), incubating at 36 °C for 18 h. After incubation, 2 mL of broth were transferred into a new tube, and heated to 100°C for 15 min. Next, 0.5 mL of warmed broth were analyzed by the kit VIDAS® ECO O157 and the rest of the sample was kept refrigerated. In cases of positive results, the broth was plated on MacConkey sorbitol agar (BD, Heidelberg, Germany) and incubated at 35 °C ± 1 °C for 24-48 h. Subsequently, 5 to 10 colony forming units (CFU) suggestive of *E. coli* were selected, plated on CHROMagar O157 agar® (BD, Heidelberg, Germany) and incubated at 35 °C ± 1 °C for 24-48 h.

Typical CFUs were transferred to triple sugar iron agar tubes and after incubation yellow bezel gas production and no H_2S tubes were submitted to biochemical characterization. Results were considered *E. coli* when positive for indole production, methyl red reaction, decarboxylation of lysine and ornithine, and negative for Voges Proskauer reaction, citrate and sorbitol utilization. Isolates with typical biochemical pattern underwent serology by using anti-O157 and H7 antisera (Difco, USA).

At all steps *Escherichia coli* (EHEC) O157:H7 INCQS 00171 was used as positive control strain. Reference strains were acquired from the collection of the Laboratory for Reference Materials, National Institute for Quality Control in Health (INCQS) Oswaldo Cruz Foundation.

For the detection of *Escherichia coli* O157:H7 by PCR two sets of primers were used. RFB (Hu, *et al.*, 1999) primer pair are complementary to the gene region *rfbE* involved in the biosynthesis of O157 somatic antigen and Flic (Wang, *et al.*, 2002) primer pair are complementary to region of *fliC* encoding the H7 flagellar antigen.

An aliquot of 1.5 mL of MacConkey broth with Cefixime and Potassium Tellurite was centrifuged at 10.000 rpm for 10 min. Then, the supernatant was discarded and the pellet was re-suspended in 500 µL of Tris-EDTA (TE, 10 mm Tris, 1 mM EDTA, pH 8.0). Genomic DNA extraction was performed following High Pure PCR Template Preparation Kit (Roche®, Mannheim, Germany) manual instructions. DNA quality and concentration were estimated in 0.8% agarose gel electrophoresis by visual comparison with standard molecular weight DNA Low Mass Ladder (Invitrogen®, Brazil) and adjusted to 20 ng/mL.

The PCR reactions and amplification conditions followed primer's authors indications (Hu, *et al.*, 1999; Wang, *et al.*, 2002). As negative control for PCR reaction mixture, ultra-pure water was used replacing the DNA template and *E. coli* reference strain (EHEC) O157: H7 INCQS 00171 was used as positive control.

Results

Samples from free markets

Minas Frescal cheese from street fairs were positive for *E. coli* O157 in 83.33% samples (25/30) when analyzed by the kit VIDAS® ECO O157. However, when suspected microorganisms were submitted to isolation and serology, only 2/30 (6.67%) wereconfirmed as belonging to serotype O157:H7.

Concerning the detection of *E.coli* O157:H7 by PCR (Figures 1 and 2), 70% (21/30) samples from street markets were positive to RfbF/RfbR primers and 23.33% (7/30) to Flic-a/Flic-b primers.

Samples obtained from plants with Federal Inspection (IF)

When the kit VIDAS® ECO O157 was used as a diagnostic technique on samples from plants under IF, 66.67%

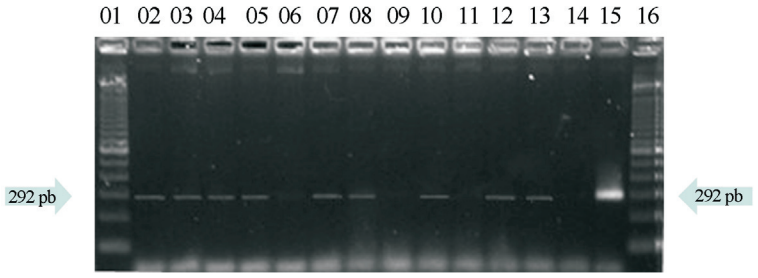

Figure 1 - Agarose gel electrophoresis of "O" antigen detection by PCR with primers RfbR/RfbR (292pb). Lines 1 and 16 denotes molecular weight (DNA *Ladder* 100 bp); line 15 display positive control (INCQS 00171); lines 2-5, 7, 8, 10, 12 and 13 positive samples; line 14 negative control; lines 6, 9 and 11 negative samples.

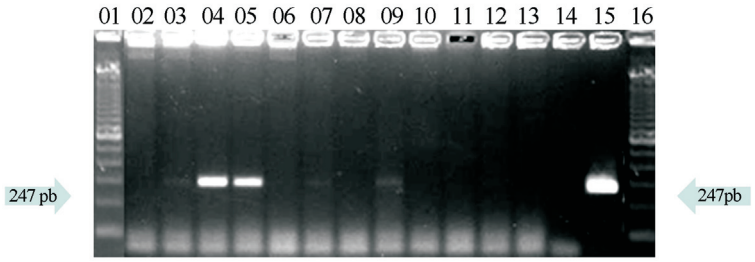

Figure 2 - Agarose gel electrophoresis of "H" antigen detection by PCR with primers FliC-a/FliC-b (247pb). Lines 1 and 16 denotes molecular weight marker (DNA *Ladder* 100 bp), line 15 positive control (INCQS 00171); lines 3, 4, 5, 7 and 9 positive samples; line 14 negative control; lines 2, 6, 8 and 10-13 negative samples.

(20/30) were positive, but none was confirmed by conventional bacteriological culture of the microorganism.

When the PCR technique was employed, the primer pair RfbF/RfbR detected 50% (15/30) positive for the antigen "O157", however, the pair of primers Flic-a/Flic-b did not detect any positive for the antigen "H7".

Discussion

Detection of *E.coli* O157:H7 in *Minas Frescal* cheese confirms the presence of this pathogen in dairy products marketed in Brazil, and this result is in accordance with previous reports from Cerqueira *et al.* (1999), Irino *et al.* (2005) and Sandrini *et al.* (2007). In spite of these findings, the pathogen was only confirmed in artisan farmhouse cheeses, produced with unpasteurized milk, generally under inappropriate hygienic conditions. Toxigenic *E. coli* in raw milk and cheese made from unpasteurised milk was also reported by Quinto and Cepeda (1997) in Spain.

Toxigenic *E. coli* O157:H7 in raw milk and cheese made from unpasteurised milk was also reported by De Reu *et al.* (2004), in Belgium, who isolated the pathogen in 0.7% samples of raw milk from farms that directly trade their products, Altalhi and Hassan (2009) found STEC in three isolates (9.1%) from raw milk in Saudi Arabia and by Zweifel *et al.* (2010) in Switzerland, who found *E. coli* STEC in 5.7% samples of cheese made with raw milk.

The absence of this pathogen in processed *Minas Frescal* cheese from creameries under IF is in accordance with the results obtained in Italy by Zago *et al.* (2007), and strengthens the use of Good Manufacturing Practices and the application of specific normatives, such as IN 51-Ministry of Agriculture (2002), enhancing the importance of control procedures along the entire "farm-to-fork" chain.

In contrast, the occurrence of *E. coli* O157:H7 in *Minas Frescal* cheese samples from free markets may be partly explained by the low quality of farm-elaborated milk products associated to the absence or inadequate monitoring of artisan cheesemakers. Raw milk has already been signaled as a significant source of food borne pathogens, including *E.coli* O157:H7 and there have been numerous food-poisoning outbreaks associated with direct consumption of raw milk or milk that has been inadequately heat-treated (Hussein and Sakuma, 2005; Baylis, 2009).

The isolation of *E.coli* O157 and specifically the serotype O157:H7 have confirmed that Brazilian dairy cattle are a reservoir of the pathogen. Results from this study strongly suggest the need for more research concerning this and other *E.coli* serotypes related to food safety and food borne infection in humans.

These results also call the attention from veterinary inspectors and health authorities regarding production and marketing of Minas Frescal cheese. Preparation of cheese without appropriate controls and good hygiene practices and sanitary procedures constitutes a risk to public health.

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