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Original

Analysis of virulence genes in *Escherichia coli* isolated from grated cheese

Pesquisa de genes de virulência em Escherichia coli isolada de queijo ralado

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Abstract

This research aimed to verify the presence of virulence genes in strains of *Escherichia coli* isolated from grated cheese sold in farmers' markets of Cuiabá-MT, Brazil. Forty samples of this food were submitted for microbiological analysis and 22 (55%) tested positive for *E. coli*. Next, 64 strains of *E. coli* were isolated from the positive samples and screened by the polymerase chain reaction (PCR) for the presence of the genes encoding the following virulence factors: *stx1* and *stx2* (verotoxin types 1 and 2), *eae* (intimin), *lt1* (heat-labile toxin type 1), *st1* (heat-stable toxin type 1), *cnf1* and *cnf2* (cytotoxic necrozing factor types 1 and 2), and *cdtB* (cytolethal distending toxin). All the isolates were negative for the genes *stx1, stx2, eae, lt1, st1, cnf1,* and *cdtB,* and five strains (7.81%) were positive for *cnf2*. A low prevalence of *E. coli* positive for virulence factors associated with the pathogenesis of diarrhoea was observed in this study. However, the presence of CNF-2 producing strains and the possibility of occurrence and scattering of other virulence factors that were not surveyed in the work indicate the risk related to the consumption of grated cheese from farmers' markets.

Keywords: *Escherichia coli; virulence genes; grated cheese; Cuiabá - MT.*

Resumo

Este trabalho teve como objetivo verificar a presença de genes de virulência em linhagens de *Escherichia coli* isoladas de queijo ralado comercializado em feiras livres de Cuiabá-MT. Quarenta amostras desse alimento foram submetidas a provas microbiológicas, sendo 22 (55%) positivas para *E. coli*. Em seguida, 64 isolados de *E. coli* provenientes das amostras positivas foram testados, por meio da reação da polimerase em cadeia (PCR), para a presença dos genes que codificam os seguintes fatores de virulência: *stx1*e *stx2* (verotoxina tipo 1 e 2), *eae* (intimina), *lt1* (toxina termolábil tipo 1), *st1* (toxina termoestável tipo 1), *cnf1* e *cnf2* (fator de necrose citotóxico tipo 1 e 2) e *cdtB* (toxina citoletal distensora). Todos os isolados analisados foram negativos para os genes *vt1, vt2, eae, lt1, st1, cnf1, cdtB e* cinco (7,81%) foram positivos para o gene *cnf2*. Observou-se uma baixa prevalência de *E. coli* portadora de fatores de virulência associados à patogênese da diarréia. Entretanto, a presença de linhagens produtoras de CNF-2 e a possibilidade de ocorrência e dispersão de outros fatores de virulência não pesquisados neste estudo apontam para o risco vinculado ao consumo de queijo ralado de origem informal.

Palavras-chave: *Escherichia coli; genes de virulência; queijo ralado; Cuiabá - MT.*

1 Introduction

According to the Brazilian legislation, grated cheese is the product obtained by grating from one or up to four types of cheese suitable for human consumption (BRASIL, 1997). The usage of inappropriate raw material associated with hygiene failures during food manufacturing, storage, and commercialization can result in risk of infection and intoxication to customers (ZAFFARI et al., 2007). However, the sale of grated cheese produced from homemade raw-milk cheese is very common in Brazilian farmers' markets.

The contamination of cheeses with coliforms has been largely reported in Brazil (BRANT et al., 2007; ZAFFARI; MELLO; COSTA, 2007; LOGUERCIO; ALEIXO, 2001). The coliforms colonize the intestinal tract of warm-blooded animals, and *Escherichia coli* is the most frequent member of this group and some strains are pathogenic for humans and animals. (LOGUERCIO; ALEIXO, 2001).

Diarrhoeagenic *E. coli* can be grouped into five categories: enterotoxigenic (ETEC), enteropathogenic (EPEC), enterohemorrhagic (EHEC), enteroinvasive (EIEC), and enteroaggregative (EAggEC) (GONZALEZ et al., 2000).

The first group constitutes one of the most important vectors of *E. coli* diarrhoea. ETEC causes diarrhoea by adhering to the intestinal mucosa through its unique colonization factors and produces either heat-labile enterotoxins (LT-I and LT-II) or heat-stable enterotoxins (STa and STb), or both (PANETO et al., 2007). Virulent EPEC often exhibit a typical adherence phenotype to epithelial cells (localized adherence – LA) and induce attaching-effacing (A/E) lesions on gut enterocytes. The virulence genes *eaf* and *eae* are associated with these features (GONZALEZ et al., 2000). Shiga toxin-producing *E. coli* strains (STEC) are defined by the production of Shiga toxin (Stx) or possession of the Stx encoding gene subtype *stx1* and *stx2*, or both *stx1* and *stx2*. Highly human-virulent STEC strains are also called enterohemorrhagic *E. coli* (EHEC) strains (BEUTIN et al., 2007).

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There is a close association between the development of human diseases and the presence of additional virulence markers. One of those markers is the *eae* gene, which encodes an outer membrane protein required for intimate attachment to the host intestinal mucosa (COBBOLD; DESMARCHELIER, 2001). The necrotoxigenic *E. coli* (NTEC) produce the Cytotoxic Necrozing Factor (CNF) type 1 and 2 and have been implicated in opportunistic diarrhoeal diseases in humans (QUINTO; CEPADA, 1997). Cytolethal distending toxin (CDT) is a potent bacterial exotoxin that has dramatic effects on target cells in culture. Previous studies suggest that CDT also may be a virulence factor in vivo (CLARK et al., 2002).

Soft and semi-soft cheeses have been previously associated with disease outbreaks involving pathogenic strains of *E. coli* (PANETO et al., 2007; QUINTO; CEPEDA, 1997). However, little information about the presence of these microorganisms in grated cheese is available. Given these considerations, this study aimed to verify the prevalence of genes encoding virulence factors in strains of *E. coli* isolated from grated cheese commercialized in farmers' markets.

2 Materials and methods

Forty samples of grated cheese were collected from farmers' markets located in Cuiabá-MT, Brazil. The samples were kept in sterile plastic bags and carried under refrigeration to the Laboratory of Veterinary Microbiology of the College of Agronomic Sciences and Veterinary Medicine, Federal University of Mato Grosso – UFMT.

A 25g portion of each sample was blended with 225 mL of peptone water and incubated at 37 °C for 24 hours. Afterwards, 10 µl of the culture were streaked onto MacConkey agar (Oxoid, United Kingdom) plates and incubated at 37 °C for 24 hours. Three colonies from each plate with typical *E. coli* morphology were selected and examined by the EPM-medium (TOLEDO; FONTES; TRABULSI, 1982a), MILi (TOLEDO; FONTES; TRABULSI, 1982b), and citrate test (HOLT; KRIEG, 1984) to confirm the identification.

The strains confirmed as *E. coli* were screened for the presence of virulence genes by the PCR technique.

For DNA extraction, one loop of bacterial growth obtained by incubation on Trypticase Soy Agar (Oxoid, United Kingdom) at 37 °C for 24 hours was suspended in 100 µl of sterile UHQ water, boiled for 10 minutes, and centrifuged at 10.000 × g for 2 minutes. The supernatant was used as the template in the PCR assays.

The PCR was carried out using oligonucleotide primers for the following genes: *stx1*and *stx2* (verotoxin types 1 and 2), *eae* (intimin), *lt1* (heat-labile toxin type 1), *st1* (heat-stable toxin type 1), *cnf1* e *cnf2* (cytotoxic necrozing factor types 1 and 2), and *cdtB* (cytolethal distending toxin).

The PCR mixture (30 µl) was prepared using 3 µL of 10X PCR buffer (Fermentas, United States), 2.4 µL of 25 mM MgCl2 (Fermentas, United States), 0.24 µL of 25 mM dNTP mixture (Fermentas, United States), 0.3 µL of Taq DNA polymerase - (5U/µL Fermentas, United States), sterile UHQ water (qsp), 7 µL of template DNA, and forward and reverse primers (1 µL each). The concentration of the primers, annealing temperatures, and predicted sizes of the amplified products are shown in Table 1.

Amplifications were executed in a thermal cycler (GeneAmp® PCR System 9700, Applied Biosystems, United States) according to the following conditions: initial denaturation at 94 °C for 10 minutes, 30 cycles (94 °C for 1 minute, annealing temperature according to Table 1 for 1 minute, 72 °C for 2'), and final extension at 72 °C for 2 minutes. *E. coli* K12C600 was used as a negative control of the reactions, and the following strains were used as positive controls: H30 (O26:H11 *stx1*) J2 (O157:H- *stx2* and *eae*), H100407 (*lt1* and *st1*), MR48 (*cnf-1)*, B26a (*cnf2*), and CLDT7(2) (*cdtB)*.PCR products were analysed by agarose gel electrophoresis and observed under UV light at a wavelength of 420nm after ethidium bromide staining.

3 Results and discussion

Among the 40 samples analyzed, 22 (55%) were positive for *E. coli* and 64 strains of this microorganism were isolated from those samples. Pimentel (2002) evaluated the microbiological quality of 18 different brands of grated cheese produced under Brazilian Federal Inspection (SIF) and observed that all samples analyzed were negative for coliforms. According to the authors, this result could be correlated to the low water activity, low moisture level, and the presence of preservatives in grated cheese.

The higher level of contamination by *E. coli* reported in this study can be related to the source of the samples since informal food is more susceptive to contamination during its manufacture, commercialization, and storage. A previous research stated that 93.3% of raw cheese samples from free markets of Cuiabá - MT had levels of faecal coliforms higher than the limit established by the Brazilian legislation (LOGUERCIO; ALEIXO, 2001). Similar results were observed in other Brazilian regions (BRANT et al., 2007; ZAFFARI; MELLO; COSTA, 2007). The high prevalence

Table 1. Concentration, annealing temperatures of primers, and predicted sizes of the amplified products used in the PCR.

Gene	Concentration (ng.µl^{-1})	Annealing temperature (°C)	Predicted size (pb)	Reference
stx-1	90	53	364	Salvadori et al. (2003)
stx-2	90	53	386	Salvadori et al. (2003)
eae	60	63	384	Salvadori et al. (2003)
lt1	90	48	480	Salvadori et al. (2003)
st1	60	60	244	Salvadori et al. (2003)
cnf1	90	43	543	Salvadori et al. (2003)
cnf2	90	43	543	Salvadori et al. (2003)
cdtB	60	63	384	Silva e Leite (2002)

of soft cheeses contaminated by *E. coli* is a public health threat to their customers and a concern for grated cheese production.

Although the dehydration process carried out in the production of grated cheese reduce the susceptibility to bacterial growth, some micro-organisms can tolerate it. Le Magrex-Debar et al. (2000) investigated the recovery of bacteria stressed with dehydration increased in hypersalted mediums and affirmed that *E. coli* seemed protected from dehydration stress. Enteropathogenic *E. coli* have the ability to grow during the manufacture of cheese and to survive during the ripening of this product (QUINTO; CEPEDA, 1997). Therefore, the presence of *E coli* in grated cheese represents health hazard for the customers0, although the production process can reduce the microorganism charge.

Our analysis of the genes encoding important virulence markers of *E. coli* showed that none of the isolates carried the genes *vt1, vt2, eae, lt1, st1, cnf1,* and *cdtB* and only five (7.81%) strains were carriers of *cnf2*. The low prevalence of pathogenic *E. coli* in cheese, in spite of the large contamination by faecal coliforms, has been previously stated (PANETO et al., 2007; GONZALEZ et al., 2000); it was suggested that pathogenic *E. coli* could have been present as a small fraction of the coliform population and could have been overgrown by other strains (CONEDERA et al., 2004). This statement might explain the low percentage of strains carriers of virulence genes observed in this work.

According to Paneto et al. (2007), CNF-producing *E. coli* (NTEC) have been rarely found in Brazil. However, 7.81% of the strains analyzed in this study were carriers of the *cnf2* gene. NTEC strains have been associated with outbreaks and sporadic cases of diarrhoea in newborn children and adults (QUINTO; CEPEDA, 1997).

CNF2-producing strains have been isolated from calves with diarrhoea, septicaemic lambs, healthy cows and calves (BLANCO et al., 1998), and bovine clinical mastitis (RIBEIRO et al., 2002).

Therefore, the presence of CNF2-positive *E. coli* observed in this study could be related to the use of milk contaminated during milking process or originated from mammary quarters afflicted by mastitis in the production of the raw-milk cheeses subsequently employed in grated cheese manufacturing. This finding represents health hazard for customers and substantiates the need of implementation of hygienic measures in the production of this food.

4 Conclusions

Our study demonstrated low prevalence of virulence genes, which are associated with the pathogenesis of diarrhoea, in E. coli strains isolated from grated cheese. The presence of CNF-2 gene and the possibility of occurrence of other virulence factors that were not analysed in this study suggest risks of contamination in grated cheese sold in farmer's markets.

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