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Original Article

Virulence genes in *Escherichia coli* isolates from commercialized saltwater mussels *Mytella guyanensis* (Lamarck, 1819)

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Abstract

The isolation of *Escherichia coli* from food is a major concern. Pathogenic strains of these bacteria cause diseases which range from diarrhea to hemolytic-uremic syndrome. Therefore the virulence genes in *E. coli* isolates from the mussel (*Mytella guyanensis*) commercialized in Cachoeira, Bahia, Brazil were investigated. Samples were purchased from four vendors: two from supermarkets and two from fair outlets. They were conditioned into isothermal boxes with reusable ice and transported to the laboratory for analysis. *E. coli* strains were isolated in eosin methylene blue agar, preserved in brain-heart infusion medium with 15% glycerol and stored at -20 °C, after microbiological analysis. Virulence genes in the isolated strains were identified by specific primers, with Polymerase Chain Reaction. Twenty-four isolates were obtained, with a prevalence of *elt* gene, typical from enterotoxigenic infection, in 75% of the isolates. The *stx* and *bfpA* genes, prevalent in enterohemorragic and enteropathogenic *E. coli*, respectively, were not detected. The occurrence of *elt* virulence-related gene in the *E. coli* isolates of *Mytella guyanensis* reveals urgent improvement in food processing, including good handling practices, adequate storage and cooking before consumption, to ensure consumer's health.

Keywords: bivalve mollusk, thermotolerant coliform, PCR, *elt* gene.

Presença de genes de virulência em isolados de *Escherichia coli* provenientes de sururu *Mytella guyanensis* (Lamarck, 1819) comercializado

Resumo

O isolamento de *Escherichia coli* a partir de alimentos é uma grande preocupação, pois cepas patogênicas desta bactéria podem causar desde diarreia até síndrome hemolítico-urêmica. Diante do exposto, o objetivo do trabalho foi pesquisar genes de virulência em isolados de *Escherichia coli* provenientes do sururu *Mytella guyanensis* comercializado na cidade de Cachoeira, Bahia, Brasil. As amostras foram adquiridas de quatro comerciantes, sendo duas de mercados e duas em pontos de venda na feira livre da cidade de Cachoeira, acondicionadas em caixas isotérmicas com gelo reutilizável e transportadas até o laboratório para a análise. Após a análise microbiológica, as cepas de *Escherichia coli* foram isoladas em ágar Eosina Azul de Metileno e preservadas em caldo Brian Heart Infusion e glicerol a 15% e mantidas a - 20° C. A identificação dos genes de virulência nas cepas isoladas foi realizada utilizando *primers* específicos, por meio da Reação em Cadeia da Polimerase. Foram obtidos 24 isolados de *Escherichia coli*, destes a prevalência do gene *elt*, característico de *Escherichia coli* enterotoxigênica, foi de 75% dos isolados. Não houve a detecção dos genes *stx* e *bfpA* nos isolados, os quais são prevalentes nas cepas de *Escherichia coli* enterohemorrágica e *Escherichia coli* enteropatogênica, respectivamente. A presença do gene *elt* relacionado à virulência de *Escherichia coli* nos isolados de *Mytella guyanensis* revela a necessidade da melhoria no processamento, incluindo boas práticas de manipulação, armazenamento adequado e cocção previa ao consumo, visando a garantia da saúde do consumidor.

Palavras-chave: molusco bivalve, coliforme termotolerante, PCR, Gene *elt*.

1. Introduction

Escherichia coli may be a commensal bacterium or it may cause several infections in humans and animals (Backer, 2015). *E. coli* strains which cause intestine infections are called diarrheagenic, with six pathotypes: enterohemorrhagic *E. coli* (EHEC); enteropathogenic *E. coli* (EPEC); enterotoxigenic *E. coli* (ETEC); enteroinvasive

E. coli (EIEC); enteroaggregative *E. coli* (EAEC); diffused adherence *E. coli* (DAEC) (Croxen and Finlay, 2010).

Bacteria have different virulence factors to trigger infection, such as toxins and cell-adhesive mechanisms (Croxen and Finlay, 2010; Chandra et al., 2013). Highlighted toxins comprise Shiga (STX), produced by EHEC (Obrig, 2010) and codified by gene *stx* (Mauro and Koudelka, 2011); thermo-labile (LT) and thermo-stable (ST) toxins, produced by ETEC (Begum et al., 2014) and codified respectively by genes *elt* and *est* (Manzoor et al., 2015); and bacteria´s cell-adherence mechanisms associated with EPEC (Mainil and Daube, 2005), such as bundle-forming pilus (BFP), type IV, with main structural subunit codified by gene *bfpA* (Contreras et al., 2010; Teixeira et al., 2015).

Due to the harm done by pathogenic strains of *E. coli*, there is great concern with regard to strains of the microorganism in food, such as fish (Costa, 2013). According to Zhao et al. (2014), there is a high occurrence of diseases in developing countries caused by the consumption of contaminated food. The detection of contaminating agents is an important step for the development of prophylactic strategies. Polymerase Chain Reaction (PCR) is a commonly used method to detect human pathogens transmitted by the consumption of contaminated food. This highly sensitive technique is based on the amplification of specific regions of the DNA by specific primers.

Studies on the genotype characterization of bacterial isolates in commercialized mussels *Mytella guyanensis* are scarce, so this work aims to investigate virulence genes in *E. coli* isolates from the mussel (*M. guyanensis*) commercialized in the town of Cachoeira, Bahia, Brazil.

2. Material and Methods

2.1. Sampling

Samples of *M. guyanensis* were obtained from four sellers in the town of Cachoeira, Bahia, Brazil: two kilos of mussels were bought from two open stall outlets (1k each) and two kilos from supermarkets (1k each), between December 2015 and January 2016. Samples were conditioned in new plastic bags, identified and transported under refrigeration to the laboratory of the Research Nucleus in Food Safety and Nutrition of the Center of Health Sciences of the Federal University of Recôncavo of Bahia, in the town of Santo Antônio de Jesus, and immediately analyzed.

2.2. Isolation and purification of strains

E. coli populations were estimated by Petrifilm™ (3M) rapid counting method, with EC plates (AOAC 998.08). An amount of each sample (25 g) were added to a 225 mL of 0.9% NaCl saline solution homogenized in a stomacher for 30 seconds and diluted in series (10^{-2} and 10^{-3}). About 1 mL of the samples were placed on plates and incubated at 35 ± 1 °C for 24 ± 2h (Silva et al., 2007). Characteristical *E. coli* colonies (blue with gas bubbles) were removed by a platinum spatula, sprinkled with Eosin Methylene Blue agar (EMB) Kasvi® and incubated at 35±1° C for 24±2 h. A characteristic colony (black with brilliant metallic blue), isolated from each plate, was transferred to Brain Heart Infusion (BHI) broth Kasvi® and incubated at 35 ± 1 °C for 24 ± 2 h (Silva et al., 2007). Twenty-four isolates were kept by adding glycerol 15% and stored at -20° C (Silva et al., 2011).

2.3. DNA extraction and PCR technique

DNA extraction and PCR technique were performed according to a methodology adapted from Silva et al. (2011). Positive controls were used as standards: strain ATCC 11105 for *elt* gene, strain CDC EDL 933 for *stx* gene and strain CDC 086:H35 for *bfpA* gene. The standardization of the PCR tests for the genes under study was performed regarding to the amount of DNA in the reaction (data not shown) and the components used for the *stx* and *elt* genes were: milli-Q sterile water (15.2 uL), 10XPCR buffer (5 uL) in final concentration 2 X, 10 mM dNTP mix (0.5 uL) in final concentration 0.2 mM, 50 mM MgCl2 (1.5 uL) in final concentration 3 mM, direct initiator (0.8 uL) in final concentration 0.8 pmol, reverse initiator (0.8 uL) in final concentration 0.8 pmol, Taq DNA polymerase (0.2 uL) in final concentration 2 U, DNA mold (1uL). For the *bfpA* gene the following components were used: milli-Q sterile water (16.5 uL), 10XPCR buffer (2.5 uL) in final concentration 1 X, 10 mM dNTP mix (0.5 uL) in final concentration 0.2 mM, 50 mM MgCl2 (0.75 uL) in final concentration 1.5 mM, direct initiator (1 uL) in final concentration 1 pmol, reverse initiator (1 uL) in final concentration 1 pmol, Taq DNA polymerase (0.2 uL) in final concentration 2 U, DNA mold (3 uL). The total volume of components for the amplification of each gene was 25 uL. Amplification reactions were performed in thermal cycler Amplitherm® TX96 Plus, according to conditions in Table 1. PCR amplified products were loaded in a 2% agarose gel and placed in a horizontal electrophoresis system to visualize bands by employing 50 bp molecular weight as size marker. Electrophoresis run was performed in a digital source GSR® 200STD with parameters: 80 v, 80 min, 200 mA for genes *stx* and *elt* and 70 v, 100 min, 200 mA for gene *bfpA*. Loading Dye 6X Promega® plus SYBR Green Life Technologies® were used to visualize PCR products in ultraviolet Loccus L-PIX.

Table 1. Sequence of primers, size of amplified fragment and PCR conditions.

Gene/serotype	Primer sequence 5'- 3'	Fragment size (bp)	Anealing (°C)
stx/EHEC	TTT ACG ATA GAC TTC TCG AC CAC ATA TAA ATT ATT TCG CTC	227	48
elt/ETEC	GGC GAC AGA TTA TAC CGT GC CCG AAT TCT GTT ATA TAT GTC	696	56
bfpA/EPEC	AAT GGT GCT TGC GCT TGC TGC GCC GCT TTA TCC AAC CTG GTA	330	56

Source: Silva et al. (2011).

3. Results

The results revealed amplification of *elt* gene, typical of ETEC, in 75% of *E. coli* isolates, of which 50% were originating from mussel samples collected in supermarkets and 50% were originating from mussel samples collected in open fair stalls. No amplification of *stx* and *bfpA* gene, respectively typical of EHEC and EPEC, were observed.

4. Discussion

Species for biomonitoring are generally good coliform accumulators (Farrapeira et al., 2010). This indicates the importance of these microorganisms as indicators of contamination, which also extends to food. The presence of *E. coli*, a thermotolerant coliform in fish, is related to their hygienic-sanitary quality, since this bacteria indicates fecal contamination (Dutta et al., 2015). Pathogenic strains of this bacteria may occur in food, as demonstrated with gene *elt* in this work.

Kambire et al. (2017) conducted studies to detect *E. coli* pathotypes in samples of water, sediment, fish and crabs, and pinpointed ETEC as the most prevalent pathotype in water, sediment and crab samples. According to Anand et al. (2016), ETEC is the pathotype that causes diarrhea due to the release of potent enterotoxins, one of which is codified by gene *elt*. Shahrokhi et al. (2011) demonstrated that the production of LT and ST in ETEC strains is similar, although some strains may produce only a single enterotoxin, that result may explain the absence of amplifications of the *stx* gene.

The production of LT helps in the colonization of the host's cells and also LT uses cell components to produce its toxic effect. LT interactions with the host cell produce the constitutive activation of adenylate cyclase and the production of cyclic monophosphate adenosine (AMPc). The component´s intracellular elevation activates the AMPc-dependent protein cinase A. The phosphoryl chloride is dominium R of the regular of the transmembrane conductance of cytic fibrose. The admission of chloride and water efflux in the intestinal lumen causes aqueous diarrhea (Johnson et al., 2009; Dubreuil et al., 2016). Therefore the research of this pathotype is extremely important in commercialization of mussels in natura.

Pathogenic strains of *E. coli* are a great concern for public health authorities due to their high dissemination capacity in different sources. Food is actually an important vector of this microorganism (Croxen et al., 2013). *M. guyanensis* is a filtering organism and can accumulate microorganisms and substances harmful to health (Carvalho et al., 2007). This is a major risk for consumption of raw or partially cooked foods as is usually consumed *M. guyanensis* (Santiago et al., 2013). Since LT of ETEC is inactivated by heating to 60 °C for 10 min (Takeda, 2011), the consumption of cooked food is highly relevant. According to Santos et al. (2014), treatment is a must to warrant sanitary conditions of the final product. Carvalho et al. (2016) showed that the detection of pathogenic bacteria in fish is crucial for the development of strategies that guarantee their quality for consumers and it may be extended do other marine source of food.

Genes related to *E. coli* virulence in isolates of *M. guyanensis* reveal the need of improvement during processing, including good handling practices, proper storage and cooking prior to consumption. These factors guarantee consumer's health. In fact, cooking maintains the microbiological quality of food and inactivates ETEC thermal labile toxin.

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