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Search for diarrheagenic *Escherichia coli* in raw kibbe samples reveals the presence of Shiga toxin-producing strains

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ABSTRACT

The aim of this study was to investigate the occurrence of diarrheagenic *Escherichia coli* (DEC) strains in raw kibbe samples. For this purpose, 70 samples of raw kibbe were collected from retail establishments and analyzed. Isolated bacterial strains that presented a biochemical profile of *E. coli* were screened by multiplex PCR for the genetic markers defining the main DEC pathotypes. Two strains belonging to O125:H19 and O149:H8 serotypes were positive for *stx* 1c genetic sequence and expressed the gene, being thus classified as Shiga-toxigenic *E. coli* (STEC). These strains were further characterized in respect to other virulence traits and susceptibility to antimicrobial agents. One of them presented the genes *ast*A and lpf_{O113} and the other harbored only lpf_{O113}. One of the strains was susceptible to all the antimicrobials tested but the other presented an intermediate profile of susceptibility to ampicillin. To the best of our knowledge this is probably the first description of O125:H19 and O149:H8 STEC serotypes in refrigerated raw kibbe and foods in general.

1. Introduction

Escherichia coli are a heterogeneous group of typically harmless bacteria. However, throughout the evolutionary process some clones of *E. coli* have become pathogenic to humans due to the acquisition of specific virulence genes (Nataro & Kaper, 1998). Pathogenic *E. coli* are capable of causing intestinal and extra-intestinal infections (Kaper, Nataro, & Mobley, 2004). Strains associated with intestinal infections are designated Diarrheagenic *E. coli* (DEC). Based on their virulence strategies and some epidemiological features, DEC strains are currently divided into six categories or pathotypes: enteropathogenic *E. coli* (EPEC), enteroinvasive *E. coli* (EIEC), enterotoxigenic *E. coli* (ETEC), enteroaggregative *E. coli* (EAEC), diffusely adherent *E. coli* (DAEC) and Shiga toxin-producing *E. coli* (STEC) (Croxen et al., 2013).

Of the currently known foodborne pathogens, STEC strains are regarded as one of the most important (Blanco et al., 2004; CDC, 2012) mainly due to their ability to cause an array of diseases ranging from uncomplicated diarrhea to hemorrhagic colitis (HC) and hemolytic uremic syndrome (HUS) – an extraintestinal pathology characterized by hemolytic anemia, thrombocytopenia, thrombotic thrombocytopenic purpura (TTP) and acute renal failure (Nataro & Kaper, 1998; Paton & Paton, 1998; Riley et al., 1983).

STEC may produce one or more types of so-called Shiga toxins (Stx), which are antigenically divided into Types 1 and 2 (Stx1 and Stx2) and each type can present a variable number of subtypes. These toxins have similar structures and immune reactivity with the toxin produced by *Shigella dysenteriae* serotype 1 (Melton-Celsa & O'Brien, 1998). In addition to Stx, other virulence factors are known and characterized in STEC, including the EHEC enterohemolysin (Ehx) (Beutin et al., 1989) and a specialized adhesin called intimin (Frankel, Phillips, Rosenshine, Kaper, & Knutton, 1998). Other toxins such as the cytolethal distending toxin V

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(Cdt-V) and the STEC subtilase toxin (SubAB) (Janka et al., 2003; Paton, Srimanote, Talbor, Wang, & Paton, 2004) and adhesins, such as, the long polar fimbriae (Lpf_{O113}), the Enterohemorrhagic *E. coli* (EHEC) factor for adherence (Efa), the STEC autoagglutinating adhesin (Saa), the Iron regulated homologue adhesin (Iha) and the protein ToxB (Doughty et al., 2002; Nicholls, Travis, & Robins-Browne, 2000; Paton, Srimanote, Woodrow, & Paton, 2001; Tarr et al., 2000; Tatsuno et al., 2001) may also be present in STEC strains.

It is estimated that STEC causes 265,000 cases of disease in the United States annually, with more than 3600 hospitalizations and 30 deaths (Scallan et al., 2011). In Brazil, most infections due to this pathogen are of a sporadic nature and generally occur, with or without bloody diarrhea, in children and HIV-positive patients. However, cases of HUS have also been reported in Brazil (Dos Santos, unpublished; Guth, Lopes, Vaz, & Irino, 2002; Souza, Carvalhaes, Nishimura, Andrade, & Guth, 2011).

Ruminant animals, especially cattle and sheep are considered to be a natural reservoir for STEC. Thus, through the consumption of foods of animal origin, in particular meat and milk, STEC enters the food chain and infects humans (Blanco et al., 2004). Recently, an increasing number of different kinds of foods have been associated with STEC transmission, including cheese, salami, unpasteurized juices, melon, different kinds of vegetables and even water (CDC, 1995; Gyles, 2007; Karmali, Gannon, & Sargeant, 2010; Sandrini, Pereira, Brod, Carvalho, & Aleixo, 2007).

Among meat products, ground beef is the main ingredient in the preparation of kibbe, a dish from Lebanese cuisine appreciated worldwide. This kind of food can be consumed either cooked or raw. When raw, it has several intrinsic characteristics, such as water content, high levels of nutrients and a pH close to neutral, which favor the multiplication of different microorganisms, including DEC. Moreover, during its preparation, the handling process exposes the product to other sources of contamination thereby further increasing the risk of the transmission of enteropathogens (Nascimento, Valle, Boari, Alcântara, & Vieira, 2002).

Few studies have investigated the presence of DEC in raw kibbe samples (Cerqueira, Tibana, & Guth, 1997). So, the objective of this study was to investigate the possible occurrence of DEC strains in raw kibbe samples marketed in the northwestern region of São Paulo State, Brazil.

2. Material and methods

2.1. Raw kibbe samples: isolation and identification of E. coli

In the period between January 2010 and July 2011, one sample of 500 g of refrigerated raw kibbe was collected from different commercial establishments selected among the leading producers of raw kibbe in the city of São José do Rio Preto, which is located in the northwestern region of the São Paulo State, Brazil. Of the total samples (70), 32 were collected from supermarkets and 38 from butchers. These establishments were distributed in five distinct geographic regions of the city: central (14 establishments/samples), north (15 establishments/samples), south (14 establishments/samples), east (14 establishments/samples) and west (13 establishments/samples).

Right after the sampling procedure samples were transferred to a refrigerated box and maintained in this condition until the moment they were processed. The investigation of *E. coli* in general was performed by the Most Probable Number (MPN) technique as described in the Compendium of Methods for the Microbiological Examination of Foods (Kornacki & Johnson, 2001). After the isolation of thermotolerant coliforms, *E. coli* identification was achieved by standard presumptive biochemical tests (Kornacki & Johnson, 2001; Pessoa & Silva, 1974). All biochemically identified *E. coli* isolates were subcultured on Mac Conkey-Sorbitol Agar (SMAC) plates and sorbitol positive and/or negative colonies were further analyzed for virulence.

2.2. Virulence characterization of the isolated E. coli strains

2.2.1. Genotypic assays

DNA extraction: One to ten colonies of each raw kibbe sample positive for *E. coli* from SMAC plates (403 in total) were grown overnight in Luria-Bertani (LB) broth at 37 °C. After the incubation period, an aliquot of 100 uL of each bacterial inoculum were diluted in 900 μL of ultrapure sterile water and subjected to lysis at 100 °C in a dry bath. The resulting bacterial suspension was centrifuged at 10,000 × g for 5 min and the supernatant was collected and used directly in genotypic assays.

Investigation of markers specific for the known DEC categories: All the isolates identified as *E. coli* were initially subjected to multiplex polymerase chain reaction (PCR) in order to screen for genetic markers associated with DEC. Reactions were performed in a Gene Amp PCR System (Applied Biosystems) employing the following amplification conditions: 1 cycle at 50 °C (2 min), 1 cycle at 95 °C (5 min), 40 cycles at 95 °C (40 s), 58 °C (1 min) and 72 °C (2 min) and a final extension step at 72 °C for 7 min. The set of primers used in these reactions as well as amplicon sizes are described in Table 1.

Other putative virulence genes related to toxins, adhesins and autotransporter proteins: Additional virulence genes associated with DEC in general including the STEC pathotype and coding for toxins other than Stx, putative adhesins and autotransporter proteins were investigated by single PCR reactions. Sequences sought were: *ehxA*, sub_{AB}, *ast*A, Ipf_{O113}, *saa*, *iha*, *toxB* and *esp*P. The primers used in the reactions are described in Table 2.

Subtyping of stx genes: The subtyping of the *stx* genes was determined according to the recently proposed method of Scheutz et al. (2012).

2.2.2. Phenotypic assays

Serotyping: O:H serotypes were determined by using the method of Ewing (1986). Tube agglutination assays were performed employing absorbed antisera to somatic (O1 to O181) and flagelar (H1 to H56) antigens, prepared at the Instituto Adolfo Lutz, São Paulo, Brazil. In the case of the somatic antigen a strain was considered to belong to a given serogroup when it presented an agglutination title equal or higher than the title of the reference strain employed in the antisera production for the considered O group.

Vero cells cytotoxic assay: the ability to express *stx* genes was investigated in Vero cells cultivated *in vitro* similarly to Konowalchuk, Speirs, and Stavric (1977). Briefly, strains to be tested were cultivated at 37 °C under agitation in Penassay media for 48 h. After this period cultures were pelleted and the supernatants were sterilized by filtration and inoculated in the cells. An amount of 50 μl of the filtered supernatant was employed. Cells were observed daily for the characteristic cytotoxic effect of Shiga toxins, for a maximum of 96 h. Positive and negative strains for Stx production as well as filtered bacteria-free media were included in the assays as experimental controls. Assays were run in three independent times.

Enterohemolysin production: production of EHEC enterohemolysin (Ehx) was investigated using the method described by Beutin et al. (1989) in washed blood sheep agar plates. A drop of an overnight bacterial culture growth in Luria-Bertani broth was placed in the plate and incubated at 37 °C. A reading after 4 h of incubation was made for the investigation of α hemolysin production. The ascertainment of Ehx production was made after