Identification and characterization of atypical enteropathogenic and Shiga toxin-producing *Escherichia coli* isolated from ground beef and poultry breast purchased in Botucatu, Brazil

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Abstract

Atypical enteropathogenic (serotypes O4:H16, O8:H25, O68:H2, O105:H7, and OR:H25) and Shigatoxigenic (ONT:H46) *Escherichia coli* were isolated from samples of ground beef and poultry breast purchased in Botucatu, Brazil. Phenotypic and molecular characterization indicated the potential of these isolates to adhere to host epithelial cells and cause damage.

Keywords EPEC · STEC · Pathogenesis · Genotyping · Ground beef · Poultry breast and diarrhea

On the basis of virulence features, diarrheagenic *Escherichia coli* (DEC) are divided into six distinct pathotypes: enterotoxigenic *E. coli* (ETEC), enteroinvasive *E. coli* (EIEC), enteroaggregative *E. coli* (EAEC), Shiga toxin-producing *E. coli* (STEC), enteropathogenic *E. coli* (EPEC), and diffusely adherent *E. coli* (DAEC) [1]. EPEC is sub-grouped into typical (tEPEC) and atypical (aEPEC) according to the occurrence of the virulence plasmid pEAF (EPEC adherence factor plasmid) only in tEPEC [2, 3]. pEAF harbors the *bfp* operon, which encodes the bundle-forming pilus (BFP); BFP mediates the localized adherence (LA) pattern, characterized by the presence of compact bacterial microcolonies on the epithelial cell surface [4–6].

STEC is characterized by the production of potent cytotoxins, Stx1 and/or Stx2, which inhibit protein synthesis in eukaryotic cells [7]. In the early 1980s, STEC O157:H7 was implicated as the cause of two different outbreaks of hemorrhagic colitis [8]. However, it has become evident that non-O157 STEC, particularly those belonging to serogroups O26, O45, O103, O111, O121, and O145, can cause similar illnesses as STEC O157:H7 [9]. A large outbreak of gastroenteritis and HUS (hemolytic uremic syndrome), caused by a hybrid EAEC/STEC (serotype O104:H4), occurred in Germany, during 3 months of 2011. In this outbreak, 3816 patients were reported with diarrhea, of which 845 (22%) developed HUS. In addition, 54 deaths were registered, thus indicating the high virulence of this hybrid EAEC/STEC pathogen [10, 11].

EPEC and some STEC isolates, known as enterohemorrhagic *E. coli* (EHEC), can also produce attaching and effacing (AE) lesions, which are characterized by intimate bacterial attachment, localized destruction of brush border microvilli, and accumulation of F-actin filaments underneath adherent bacteria resulting in pedestal-like structures on the surface of infected epithelial cells [1]. The proteins necessary for AE lesion formation are encoded by genes located in a 35.5-kb chromosomal pathogenicity island (PAI) termed locus of enterocyte effacement (LEE) [12]. The *eae* (*E. coli* attaching and effacing) gene, located within the LEE region, encodes an adhesive protein known as intimin, which is required for intimate adhesion and cytoskeletal reorganization in AE lesion formation [13].

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In this study, we checked for EPEC and STEC pathotypes in 203 food samples (100 ground beef and 103 poultry breast) purchased in Botucatu, São Paulo State, Brazil. Additionally, the EPEC and STEC isolates were phenotypically and molecularly characterized, mainly emphasizing their ability to interact with host epithelial cells and cause damage.

We analyzed 100 samples of ground beef (bought from May 2013 to April 2014) and 103 samples of poultry breast (33 samples bought in 2013 and 70 samples from January 2017 to May 2017). The samples were bought from 56 different retailers located in Botucatu, São Paulo State, Brazil, and were transported to the laboratory, within 2 h after being acquired, in isothermal refrigerated boxes. In the laboratory, 25 g of the samples were homogenized with 225 ml of buffered peptone water (1%), using a Stomacher Lab Blender 400 for 30 s, and incubated for approximately 18 h at 37 °C. One loopful of the culture was streaked onto Chromocult Coliform Agar (Merck, Darmstadt, Germany) and incubated for 18 h at 37 °C. After incubation, five to ten well-isolated colonies, presumably *E. coli* due to taking on dark blue color, were selected and confirmed to be *E. coli* by using standard biochemical techniques [14, 15].

A total of 929 *E. coli* isolates were obtained from 86.7% of the food samples analyzed (Table 1). On the basis of the detection of the virulence factor-encoding genes used for EPEC (*eae* and *bfpB*) and STEC (*eae*, *stx1*, and *stx2*) identification, performed as previously described [16], six isolates were classified as aEPEC and one as STEC (Table 2). Of note, none of the 929 *E. coli* isolates harbored the *aatA* and/or *aggR* genes, thus indicating the absence of the EAEC pathotype among the meat samples analyzed (data not shown). The isolation of STEC and aEPEC from meat products observed in this study corroborates reports of previous studies performed in Brazilian cities, including São Paulo, Rio de Janeiro, Ribeirão Preto, Campinas, and São José do Rio Preto [17–20]. Regarding the classification of the *E. coli* isolates in the different phylo-groups, using a quadruplex PCR method [21], we found that five aEPEC isolates were assigned to phylo-group A, while the aEPEC serotype O105:H7 and STEC ONT:H46 were assigned to phylo-group B1 (Table 2).

O:H serotyping, performed with absorbed somatic (O1–O188) and flagellar (H1–H56) antisera produced at Adolfo Lutz Institute [22], demonstrated that the six aEPEC isolates belonged to five distinct serotypes, namely O4:H16, O68:H12, O105:H7, OR:H25 (1 isolate each), and O8:H25 (2 isolates), whereas the STEC isolate was ONT:H46 (Table 2). According to the literature, aEPEC O105:H7 was previously isolated from a diarrheic child in an epidemiological study conducted in Northeast Brazil [23], while aEPEC O4:H16 was obtained from a non-diarrheic child living in the city of Rio de Janeiro [24].

STEC ONT:H46 was previously isolated from beef and dairy cattle in different Brazilian states [25], as well as, from a patient with diarrheal disease during the year 2015 in Brazil [22]. For comparative purpose, three STEC ONT:H46, one from a diarrheic patient [22] and two from cattle [25], were evaluated in order to investigate if these isolates share virulence markers and clonal relatedness with the STEC ONT:H46 (20-1) obtained from ground beef in this study. The four STEC ONT:H46 isolates sharing an identical virulence-encoding gene profile (*stx2*, *iha*, *saa*, *ehxA*, and *espP*) were assigned in the phylo-group B1 and presented more than 80% similarity in pulsed-field gel electrophoresis (PFGE) analyze (Fig. S1). Together, the previously mentioned results may suggest that STEC ONT:H46, isolated from distinct sources in Brazil, are phylogenetically related.

The aEPEC isolates serotypes O4:H16 and O68:H12 harbored intimin subtype β 1, while the aEPEC serotype O105:H7 harbored intimin-subtype θ (Table 2). The *eae* gene from the three other aEPEC isolates (45-3, 47-1, and 47-3) was non-typeable (NT) with the primers and PCR conditions tested [26]. Intimin subtypes β 1 and θ appear as the most common types identified in aEPEC isolates obtained worldwide [3, 27, 28].

Furthermore, the six aEPEC and one STEC isolates were tested regarding their ability to adhere to HeLa cells (adherence assay), and to induce F-actin polymerization underneath adherent bacteria (fluorescence actin staining-FAS test), as previously described [29, 30]. aEPEC 18-5 (O4:H16) adhered to HeLa cells, forming loose microcolonies on the cell surface and producing an adherence pattern termed localized

Table 1 Number of food samples analyzed and diarrheagenic *Escherichia coli* (DEC) isolates obtained in the present study

Food sample	No. of food samples positive for:		No. of isolates obtained:	
	<i>E. coli</i> (%)	DEC ^a (%)	<i>E. coli</i>	DEC ^a
Ground beef (<i>n</i> = 100)	76 (76.0)	3 (3.0)	471	3
Poultry breast (<i>n</i> = 103)	100 (97.1)	3 (2.9)	458	4 ^b
Total (<i>n</i> = 203)	176 (86.7)	6 (3.0)	929	7

^a Diarrheagenic *Escherichia coli*

^b Two DEC isolates (aEPEC of serotype O8:H25) were obtained from the same poultry breast sample

Table 2 Phenotypic and genetic features of atypical enteropathogenic (aEPEC) and Shiga toxin-producing (STEC) *Escherichia coli* isolates obtained from ground beef and poultry breast purchased in Botucatu, São Paulo, Brazil

Origin	Food sample	<i>E. coli</i> isolates	DEC pathotype ^a	Serotype ^b	DEC virulence markers			Additional virulence factor-encoding genes ^d			HeLa cells		Stx ^g	Phylo-group
					<i>eae</i> (intimin subtype) ^c			<i>hlyB</i>	<i>stx1</i>	<i>stx2</i>	Adherence pattern ^e	FAS ^f		
Ground beef	11	11-1	aEPEC	O105:H7	+	(θ)	—	—	—	<i>nleB</i> , <i>nleE</i>	UND	+	NT	B1
	18	18-5	aEPEC	O4:H16	+	(β1)	—	—	—	—	LAL	+	NT	A
	20	20-1	STEC	ONT:H46	—	—	—	+	—	<i>ihfA</i> , <i>saa</i> , <i>ehxA</i> , <i>espP</i>	UND	—	+	B1
Poultry breast	28	28-3	aEPEC	O68:H12	+	(β1)	—	—	—	—	UND	+	NT	A
	45	45-3	aEPEC	OR:H25	+	(NT)	—	—	—	—	UND	+	NT	A
	47	47-1	aEPEC	O8:H25	+	(NT)	—	—	—	—	UND	+	NT	A
	47	47-3	aEPEC	O8:H25	+	(NT)	—	—	—	—	UND	+	NT	A

^a DEC: diarrheagenic *E. coli*; aEPEC: atypical enteropathogenic *E. coli*; STEC: Shiga toxin-producing *E. coli*^b ONT: somatic antigen O non-typeable with the antisera used (O1-O188); OR: rough isolates^c Intimin subtypes: β1: beta1, θ: theta, NT: non-typeable^d Additional virulence factor-encoding genes investigated: *ihfA*, *paa*, *saa*, *efa1/lifA*, *nleB*, *nleE*, *ehxA*, and *espP*^e Adherence patterns exhibited in HeLa cells (6 h assay) in the presence of D-mannose (2%): LAL (localized adherence-like) and UND (undefined adherence)^f FAS: fluorescent-actin staining test^g Shiga toxin (Stx) production (Vero cells toxicity assay). NT, not tested

adherence-like (LAL) (Table 2, Fig. 1). The LAL pattern was first described in aEPEC serogroup O55 [31] and is considered the most common adherence pattern among aEPEC isolates [23, 32]. On other hand, adherence of five aEPEC isolates (11-1, 28-3, 45-3, 47-1, and 47-3) and STEC ONT:H46 (20-1) showed few bacteria interacting with the epithelial cells, thus not resulting in any of the adherence patterns described in the literature, which was referred as undefined (UND) adherence (Table 2, Fig. 1). The report of the number of aEPEC isolates exhibiting a UND adherence to HeLa/HEp2 cells has significantly increased, as observed in studies performed in the last 10 years [23, 33].

The FAS test demonstrated that all six aEPEC isolates were able to induce F-actin polymerization at the site of attachment, independently of the adherence pattern exhibited by these isolates on HeLa cells, indirectly indicating the ability of these isolates to promote AE lesions in infected epithelial cells (Table 2, Fig. 1). The AE lesion is the main virulence attribute of typical and atypical EPEC isolates [1–3]. A study with adult volunteers revealed that the administration of an EPEC isogenic mutant strain, unable to induce AE lesion, caused diarrhea in only 36.6% of the subjects challenged, thus confirming the importance of this virulence property in the establishment of diarrheal disease [34]. We also demonstrated that sterile supernatant, prepared with STEC 20-1, was able to induce a characteristic cytotoxic effect in Vero cells monolayers, confirming the ability of this pathogen to produce and secrete Stx2 (Table 2). Vero cells toxicity assay was performed as previously described [35]. Therefore, we clearly verified that the aEPEC and STEC isolates obtained in this study retain the main virulence features associated with these pathogens for causing disease in humans.

With the objective to determine if the aEPEC and STEC isolates obtained in this study harbored genes encoding additional virulence factors associated with the pathogenicity of these DEC pathotypes, we tested for the presence of *ihfA*, *paa*, *saa*, *efa1/lifA*, *nleB*, *nleE*, *ehxA*, and *espP* genes using primers and PCR conditions as previously described [27]. Besides the LEE region, we found that aEPEC 11-1 (O105:H7) harbored the *nleB* and *nleE* genes (Table 2), which are located in a PAI known as OI-122, in contrast with the other five aEPEC isolates, which lacked all the additional virulence factor-encoding genes investigated here (Table 2). PAI OI-122 has been identified more frequently in aEPEC isolates obtained from diarrheic patients than from healthy subjects [36, 37], and more recently, this PAI was statistically associated with diarrheal episodes of higher severity [38].

STEC 20-1 (ONT:H46), which lacked the LEE region, harbored the *ihfA* and *saa* genes, encoding proteins associated with the adherence of STEC isolates to epithelial cells and the *ehxA* and *espP* genes from the O157-EHEC plasmid (pO157), encoding for a EHEC-hemolysin and a serine protease with adhesive capability, respectively (Table 2).

Fig. 1 Fluorescent actin staining (FAS) test performed with two representative isolates: **a, b** aEPEC 18-5 and **c, d** STEC 20-1. aEPEC 18-5 adheres to HeLa cells, forming loose microcolonies (**a**), characteristic of the LAL (localized adherence-like) pattern, while STEC 20-1 exhibits a UND (undefined) adherence (**c**). Note foci of intense fluorescence in panel B (FAS-positive), indicating F-actin accumulation beneath attached bacteria (arrows), while in panel D (FAS-negative) this feature is not observed. Host cells and adherent bacteria were visualized with DAPI (4',6-diamidino-2-phenylindole dihydrochloride) staining (**a** and **c**). Bar scale = 10 μ m

Despite the low rate of isolation, we observed that the aEPEC and STEC isolates obtained in this study harbored virulence markers associated with the ability of these pathogens to cause disease in human hosts. Moreover, some of these isolates belonged to serotypes previously obtained from human infections in Brazil, thus indicating the circulation of these pathogens in Brazilian settings. These findings should serve as an alert for food risk assessment, so that appropriate strategies can be adopted to guarantee the food quality before consumption.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflicts of interest.

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