Set of virulence genes and genetic relatedness of O113:H21 *Escherichia coli* strains isolated from the animal reservoir and human infections in Brazil

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Escherichia coli strains of serotype O113:H21 are commonly described as belonging to a Shiga toxin (Stx)-producing *E. coli* (STEC) pathotype worldwide. Albeit this STEC serotype is frequently identified among cattle and other domestic animals, to the best of our knowledge no human infections associated with STEC O113:H21 have been registered in Brazil to date. Here, we report the virulence profile and genetic relatedness of a collection of O113:H21 *E. coli* strains mainly isolated from the animal reservoir aimed at determining their potential as human pathogens. The strains from the animal reservoir ($n=34$) were all classified as STEC, whereas the few isolates recovered so far from human diarrhoea ($n=3$) lacked *stx* genes. Among the STEC, the *stx*_{2d-activatable} gene was identified in 85 % of the strains that also carried *lpfA*_{O113}, *iha*, *saa*, *ehxA*, *subAB*, *astA*, *cdt-V*, *espP*, *espl* and *epeA*; the human strains harboured only *lpfA*_{O113}, *iha* and *astA*. All the strains except one, isolated from cattle, were genetically classified as phylogenetic group B1. High mass plasmids were observed in 25 isolates, but only in the STEC group were these plasmids confirmed as the STEC O113 megaplasmid (pO113). Many closely related subgroups (more than 80 % similarity) were identified by PFGE, with human isolates clustering in a subgroup separate from most of the animal isolates. In conclusion, potentially pathogenic O113:H21 STEC isolates carrying virulence markers in common with O113:H21 clones associated with haemolytic uraemic syndrome cases in other regions were demonstrated to occur in the natural reservoir in our settings, and therefore the risk represented by them to public health should be carefully monitored.

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INTRODUCTION

Among the human diarrhoeagenic *Escherichia coli* pathotypes, Shiga toxin-producing strains (STEC) are a major cause of infections and have been implicated in important morbidity and mortality worldwide (Griffin & Tauxe, 1991). STEC can produce a spectrum of diseases ranging from mild symptoms to severe clinical outcomes, such as haemolytic uraemic syndrome (HUS) (Paton & Paton, 1998).

Production of Shiga toxin (Stx) is believed to be the most important event towards HUS development (Karmali *et al.*, 1985). These cytotoxins can be of two main types: Stx₁ and Stx₂. Members of the Stx₁ group are antigenically similar, whereas Stx₂ toxins are quite heterogeneous and comprise several variants or subtypes (Paton & Paton, 1998). One of these subtypes, Stx_{2d}, has been further subdivided into two

distinct toxins, Stx_{2d-EH250} (Piérard *et al.*, 1998) and Stx_{2d-activatable} (Melton-Celsa *et al.*, 1996). The designation of the latter toxin derived from the observation that its cytotoxic titres are increased if the toxin is exposed to intestinal mucus prior to cell incubation. Evidence from animal studies also suggested a more virulent behaviour (Lindgren *et al.*, 1993).

In contrast to infections caused by other pathogenic *E. coli*, infections due to STEC have a proven zoonotic character (Chapman *et al.*, 2000). These bacteria are largely distributed among ruminant animals, and cattle can be considered the most important natural reservoir. STEC is transmitted from healthy excreting animals to humans via the food chain or by direct contact with the animals or their environment (Caprioli *et al.*, 2005).

It is known that about 400 different O:H serotypes of *E. coli* can harbour *stx* genes, and a considerable proportion of them have already been recovered from human hosts (<http://www.lugo.usc.es/ecoli/SEROTIPOSUM.htm>).

Abbreviations: EHEC, enterohaemorrhagic *Escherichia coli*; HUS, haemolytic uraemic syndrome; STEC, Shiga toxin-producing *E. coli*.

Although STEC O157:H7 is by far the most important serotype in human infections, some non-O157 strains also pose a substantial concern to public health, as they are able to

cause illnesses that are comparable in severity to O157-induced diseases (Johnson *et al.*, 2006; Tarr & Neill, 1996). This is the case for STEC O113:H21, a recognized serotype

Table 1. Virulence markers and genetic relatedness of O113:H21 *E. coli* strains isolated from the animal reservoir and human infections in Brazil

Strain (origin)*	<i>stx</i> genotype†	PCR for virulence markers‡									Hybridization with <i>subAB</i> probe§	PFGE pattern	PFGE (sub) cluster
		<i>ehxA</i>	<i>subAB</i>	<i>epeA</i>	<i>espP</i>	<i>cdt-V</i>	<i>astA</i>	<i>espI</i>	<i>iha</i>	<i>saa</i>			
Ec 258/01 (C)	2, 2d _{act}	+	+	+	+	+	–	–	+	+	+	1	A1
Ec 472/01 (C)	2, 2d _{act}	+	+	+	+	+	–	+	+	+	+		A1
Ec 585/05 (C)	2	+	+	+	+	–	–	–	–	+	+	2	A1
Ec 227/01 (C)	2, 2d _{act}	+	+	+	+	–	–	–	+	+	+	3	A1
Ec 858/05 (GO)	2d _{act}	+	+	+	+	–	–	–	–	+	+	4	A1
Ec 503/05 (GO)	2d _{act}	+	+	+	+	–	–	–	–	+	+	5	A2
Ec 648/05 (C)	2d _{act}	+	+	+	+	–	–	–	–	+	+		A2
Ec 226/04 (C)	2	+	+	+	+	–	–	–	–	+	–	6	A2
Ec 507/01 (C)	2	+	+	+	+	–	–	–	+	+	+	7	A2
G10/5 (C)	2	+	+	+	+	–	–	–	+	+	–		A2
Ec 253/02 (C)	2, 2d _{act}	+	+	+	+	–	–	–	+	+	+	8	A2
Gc 138 (C)	2	+	+	+	+	–	–	–	+	+	+	9	A3
Gc 20 (C)	2	+	+	+	+	–	–	–	+	+	+	10	A3
Ec 784 (M)	2	+	+	+	+	–	–	–	+	+	+	11	A3
Ec 689/04 (C)	2d _{act}	+	+	+	+	+	–	–	+	+	+	12	A4
Ec 678/04 (C)	2d _{act}	+	+	+	+	+	–	–	–	+	+		A4
Ec 684/04 (C)	2, 2d _{act}	+	+	+	+	+	–	–	–	+	+	13	A4
Ec 62/03 (C)	2, 2d _{act}	+	+	+	+	–	–	–	+	+	+	14	A4
Ec 301/02 (C)	2d _{act}	+	+	+	+	+	–	–	+	+	+	15	A5
Ec 254/01 (C)	2, 2d _{act}	+	+	+	+	+	–	–	+	+	+	16	A5
Ec 264/89 (HD)	–	–	–	–	–	–	+	–	+	–	–	17	A6
Ec 265/89 (HD)	–	–	–	–	–	–	+	–	–	–	–	18	A6
Ec 36/91 (HD)	–	–	–	–	–	–	+	–	–	–	–	19	A6
G102 (C)	1, 2, 2d _{act}	–	–	–	–	–	+	+	+	–	NT	20	B
G254/2 (C)	1, 2d _{act}	–	–	–	–	–	+	+	+	–	NT		B
G261/1 (C)	1, 2d _{act}	–	–	–	–	–	+	+	+	–	NT		B
G226/1 (C)	1, 2d _{act}	–	–	–	–	–	+	+	+	–	NT		B
G397/02 (C)	1, 2d _{act}	–	–	–	–	–	+	+	+	–	NT	21	B
Ec 727/05 (C)	1, 2d _{act}	–	–	–	–	–	+	+	+	–	NT	22	B
Ec 182/04 (BU)	1, 2d _{act}	–	–	–	–	–	+	+	+	–	NT	23	B
Ec 41/03 (C)	1, 2d _{act}	–	–	–	–	–	+	+	+	–	NT	24	B
Ec 670/05 (C)	2d _{act}	–	–	–	–	–	+	+	+	–	NT	25	B
Ec 624/05 (C)	1, 2d _{act}	–	–	–	–	–	+	+	+	–	NT	26	B
Ec 596/05 (C)	1, 2d _{act}	–	–	–	–	–	+	+	+	–	NT	27	B
Ec 267/01 (C)	1, 2	+	+	–	+	–	–	+	+	+	+	28	C
G2/18 (C)	2d _{act}	+	+	+	+	–	–	–	+	+	+	NT	–
Ec 719/05 (C)	1, 2, 2d _{act}	+	+	+	+	–	–	–	–	+	NT	NT	–
Total (%)		23 (62)	23 (62)	22 (59)	23 (62)	7 (19)	11 (30)	13 (35)	27 (73)	23 (62)			

*C, Dairy cattle or beef cattle; BU, buffalo; GO, goats; M, raw meat; HD, human diarrhoea.

†2d_{act}, *stx*_{2d-activatable}.

‡All the strains were positive for *lpfA*_{O113} and negative for *eae*, *tox**B*, *cnf**1*, *cnf**2*, *cif* and *katP*.

§NT, Not tested (due to the absence of large plasmid bands or DNA degradation during the extraction).

||NT, Not tested.

causing intestinal and extraintestinal infections in many countries (Blanco *et al.*, 2003; Hogan *et al.*, 2001). Although this serotype was mentioned in the first descriptions of STEC pathogens (Karmali *et al.*, 1985), its epidemiological importance remained largely underestimated until the late 1990s, when a small outbreak of HUS occurred in Australia (Paton *et al.*, 1999).

Normally, O113:H21 clinical isolates produce Stx₂, including the activatable form of this toxin, and lack the *eae* gene. This gene, which is present on a pathogenicity island termed *LEE*, is a defining feature of a subset of STEC serogroups such as O157, O111 and O26, known as enterohaemorrhagic *Escherichia coli* (EHEC). In the absence of *eae*, other determinants encoded on large virulence plasmids, such as the EHEC haemolysin (Ehx), a subtilase cytotoxin termed SubAB (Paton *et al.*, 2004), and a protein with putative adhesive functions, termed Saa (Paton *et al.*, 2001) (STEC autoagglutinating adhesin), have been implicated in the pathogenesis of HUS.

E. coli strains of serogroup O113 circulating in Brazil can be related to two distinct diarrhoeagenic pathotypes, enteroaggregative *E. coli* and STEC (Dos Santos *et al.*, 2007). The latter comprises only strains of serotype O113:H21, which is one of the most frequent serotypes recovered from cattle in our settings (Cerqueira *et al.*, 1997; Irino *et al.*, 2005). Nonetheless, cases of human infections involving STEC O113:H21 have not been registered so far in Brazil. However, a few O113:H21 *E. coli* (lacking *stx* genes) strains have been isolated from patients with diarrhoea in our country to date (Vaz *et al.*, 2004; L. F. Dos Santos, unpublished).

Taking into consideration the importance attributed to the O113:H21 serotype, especially in the context of STEC infections, the aim of the present study was to further characterize O113:H21 strains isolated from diverse sources in Brazil, and analyse their genetic relatedness.

METHODS

Bacterial strains. A total of 37 O113:H21 *E. coli* strains isolated during different surveys conducted in several Brazilian states from healthy food-producing animals (dairy or beef cattle $n=30$, buffalos $n=1$, goats $n=2$) (Cerqueira, 2000; Gonzales, 2003; Irino *et al.*, 2005; Leomil *et al.*, 2003; Pigatto *et al.*, 2008; Oliveira *et al.*, 2007, 2008), a meat sample ($n=1$) (Cerqueira *et al.*, 1997) and human stools ($n=3$) (Vaz *et al.*, 2004) were studied. These strains had their serotypes, cytotoxic activity and enterohaemolytic phenotype investigated and confirmed by standard procedures (Dos Santos *et al.*, 2007). The only three human strains were isolated from stools of patients with diarrhoea in São Paulo, and lacked *stx* genes, being herein designated non-STEC. All the other isolates carried *stx*, and were STEC. Strains were maintained in tryptic soy broth with 15% glycerol at -70°C .

Detection of the *stx*_{2d}-activatable and *stx*_{2d}-EH250 genes and other virulence determinants (toxins, putative adhesins and auto-transporter proteases). To test for the presence of the *stx*₂ activatable allele (*stx*_{2d}-activatable), a recently described PCR strategy (Zheng *et al.*, 2008) was employed. Gene *stx*_{2d}-EH250 was investigated according to the PCR-RFLP methodology of Piérard *et al.* (1998). The presence of

several other virulence-associated genes was determined by PCR using the *Taq* platinum Green PCR mastermix system (Promega). Assays were carried out in total reaction volumes of 25 μl . We sought the sequences related to SubAB, CDT-V, CNF₁, CNF₂, Cif, EAST-1, KatP, EspP, EspI and EpeA. Cycling conditions and the specific primers employed in the assays were as previously reported (Abe *et al.*, 2008; Beutin *et al.*, 2005; Blanco *et al.*, 1996; Brunder *et al.*, 1999; Cergole-Novella *et al.*, 2007; Leyton *et al.*, 2003; Schmidt *et al.*, 2001). The presence of genes encoding *stx*₁, *stx*₂, *ehxA*, *eae*, *tox*B, *saa*, *lpf*_{O113} and *iha* had been previously investigated (Dos Santos *et al.*, 2007).

Phylogenetic classification. Strains were phylogenetically classified as proposed by Clermont *et al.* (2000).

Plasmid profiles and confirmation of the STEC O113 megaplasmid (pO113). The occurrence of large plasmids was analysed in agarose gels. After extraction of plasmid DNA by the alkaline lysis method (Birnboim & Doly, 1979), plasmid masses were estimated by using the GelCompar II system. *E. coli* strain R861, with known molecular size plasmid bands, was used as the marker for the analysis. The STEC O113 megaplasmid (pO113) was confirmed by Southern blotting and probing of the extracted plasmid DNA with a peroxidase-labelled DNA fragment of the *subAB* operon, which was obtained by PCR amplification of the respective genes using primers SubAF and SubBR (Paton *et al.*, 2004).

PFGE. We followed the method described by Gautom (1997), with some modifications. After digestion of the agarose-embedded DNA with *Xba*I endonuclease (Invitrogen) for 18 h, PFGE was performed on a CHEF-DRIII PFGE apparatus (Bio-Rad) by using an initial pulse time of 5 s and a final pulse time of 35 s over a period of 20 h. Band patterns were analysed by using the GelCompar II system, and the similarity between the restriction patterns was evaluated by using the Dice coefficient similarity (tolerance of 1%). Strains were considered to have the same PFGE pattern when all bands were identical.

RESULTS AND DISCUSSION

Phenotypic characterization of the isolates

Twenty-four of the 37 strains included in this study had been previously characterized in relation to their serotypes, cytotoxicity to Vero and HeLa cells and expression of EHEC enterohaemolysin (Ehx) (Dos Santos *et al.*, 2007). It was demonstrated that all these strains belonged to the O113:H21 serotype and that 21 and 14 of them, all from non-human sources, were able to produce cytotoxin and enterohaemolysin, respectively. Thirteen strains were later isolated from more recent surveys, and then added to this study; they also had their O113 and H21 antigens confirmed, and were cytotoxic to Vero and HeLa cells. Moreover, nine of them were enterohaemolytic.

Search for the *stx*_{2d}-activatable genotype

The *Stx*_{2d}-activatable sequence was identified in 28 (82%) of the 34 *stx*-containing strains. Eleven strains (32%) had this gene as their unique *stx* allele. The other 17 strains possessed *stx*_{2d}-activatable in combination with *stx*₁, *stx*₂ and/or *stx*_{2c} (Table 1). None of the strains possessed *stx*_{2d}-EH250.

Screening for the presence of several virulence-associated genes

The occurrence and distribution of several other virulence markers is also shown in Table 1. The most prevalent gene was *lpfA*_{O113}, which occurred in all strains, followed by *iha*, present in 73 % (27 of 37) of the strains. The genes *ehxA*, *subAB*, *espP* and *saa* were found equally in 62 % of the strains, and the sequences related to *epeA*, *espI*, *astA* and *cdt-V* could be identified in 59 %, 35 %, 30 % and 19 % of the strains, respectively. The *eae*, *toxB*, *cnf1*, *cnf2*, *cif* and *katP* related sequences were not detected.

Based on the distribution of the genes investigated, several distinct virulence genotypes or profiles were identified in this study. Among the STEC, the set *ehxA*, *subAB*, *epeA*, *espP*, *lpfA*_{O113}, *iha* and *saa*, associated or not with *cdt-V*, was the most prevalent and could be detected in 38 % (13 of 34) of the isolates. The second most prevalent profile was formed by *espI*, *lpfA*_{O113} and *iha*, associated or not with *astA*, and was found in 32 % of the *stx*⁺ studied strains (11 of 34).

Plasmid profile characterization

Twenty-five of the 37 strains harboured at least one large-sized plasmid band compatible with the already described STEC virulence plasmid (>70 MDa). The three non-STEC human isolates also presented high mass plasmid bands, although they were negative by PCR for all the plasmid genes investigated. To confirm that the larger bands visualized in the agarose gels were related to the STEC O113:H21 megaplasmid (pO113), Southern blot hybridization analysis with a 1.8 kb probe prepared by PCR amplification of the entire *subAB* operon was performed (Fig. 1). Hybridization was positive in 20 strains, all of them belonging to the STEC group (Table 1). No reactive

fragments were observed in two STEC strains and in the three non-STEC human strains, even under low-stringency conditions.

Phylogenetic classification and PFGE analysis

Phylogenetic grouping classified all strains except one in the B1 group. The STEC strain Ec 267/01 isolated from dairy cattle was classified as group D. PFGE analysis allowed the definition of banding patterns in 35 of the 37 isolates. Two strains could not be analysed because of DNA degradation during extraction (Table 1). Twenty-eight PFGE restriction types were identified, forming three distinct clusters that we designated A, B and C (Table 1, Fig. 2). Cluster A was subdivided into six subgroups, A1–A6, and harboured the majority of the strains, including the strains from human sources that were all clustered in subgroup A6. The strains in cluster A showed degrees of similarity that ranged from 78 % to 100 %. Clusters B and C grouped only the animal STEC isolates. In cluster B (composed of 11 strains), four strains presented a single PFGE type (100 % similarity), and two other strains (G397/02 and Ec 727/05) showed more than 90 % similarity with this specific PFGE type. Cluster C was formed by only one strain (Ec 267/01) with the lowest similarity in relation to the others; this strain was the only one phylogenetically classified as D. This isolate was reconfirmed as being O113 by tube agglutination with specific O113 antiserum.

The further characterization of O113:H21 STEC strains from several food-producing animals isolated in Brazil showed that they carried virulence profiles that are very similar to the profiles normally presented by highly virulent O113:H21 STEC strains linked to HUS (Newton *et al.*, 2009). STEC O113:H21 is considered one of the relevant non-O157 HUS-causing STEC serotypes and belongs to the

Fig. 1. Plasmid profiles and Southern hybridization of O113:H21 *E. coli* plasmids with a probe for the *subAB* operon, a marker for the large plasmid (pO113) of highly virulent STEC strains. M, Plasmid DNA marker (*E. coli* R861). (a) Agarose gel showing the plasmid profiles of human non-STEC (lanes 1–3) and STEC (lanes 4–11) O113:H21 strains. (b) Results of the Southern hybridization for the same strains. Negative results were given by the three human isolates and one STEC strain (arrows). The STEC strain giving a negative result lacked large plasmids [lane 5 in (a)] and all the pO113 markers sought by PCR. The band sizes hybridizing with the *subAB* probe [corresponding to the higher bands visualized in (a)] are as follows: lane 4, 121.1 MDa; lane 6, 112 MDa; lane 7, 112 MDa; lane 8, 112 MDa; lane 9, 142.2 MDa; lane 10, 121 MDa; lane 11, 121 MDa.

Fig. 2. Dendrogram showing the relationships among O113:H21 STEC and human non-STEC strains based on macrorestriction analysis of genomic DNA with *Xba*I.

HUSEC collection proposed by Mellmann *et al.* (2008). Although non-O157 STEC strains are isolated from diarrhoeal disease and HUS in Brazil (Irino *et al.*, 2007; Souza *et al.*, 2007; Vaz *et al.*, 2004), to the best of our knowledge, human infections related to STEC O113:H21 have not been described so far in our settings, and the results presently obtained emphasize the importance of ruminant animals as natural reservoirs of potentially pathogenic clones in the context of STEC infections.

The majority of the strains studied were found to carry *stx*_{2d}-activatable either alone or in association with *stx*₁. To our knowledge, this is the first description of activatable *Stx*₂ (*Stx*_{2d}-activatable) in O113:H21 STEC strains circulating in Brazil. Oliveira *et al.* (2008) had previously reported the occurrence of this *stx* subtype in STEC from beef cattle, but those strains belonged to serotypes other than O113:H21. Previous data have suggested that strains

carrying *stx*_{2d}-activatable are likely to be more pathogenic. A link between *Stx*_{2d}-activatable-producing strains and severe disease, mostly HUS, has already been demonstrated (Bielaszewska *et al.*, 2006).

Associations of several virulence genes which are implicated in adhesion and toxin production were found. High frequencies of *lpfA*_{O113}, *iha* and *saa* are in accordance with published data (Tatarczak *et al.*, 2005; Toma *et al.*, 2004). Of particular interest was the observation that most of the strains harboured the novel subtilase toxin gene *subAB*. Currently there is little information regarding the distribution of *subAB* (Khaitan *et al.*, 2007), and the data presently obtained reinforce that this operon is widely distributed among O113:H21 STEC. In addition, almost 19% of the studied strains, all STEC, were positive for the *cdt-V* gene, which was previously described as a marker for isolates involved in HUS cases (Bielaszewska *et al.*, 2004). A

high prevalence of *espP*, *espI* and *epeA* genes, which belong to the SPATES (serine protease autotransporters in *Enterobacteriaceae*) family, was found in this study. This is one of the first reports on the occurrence of *epeA* after its initial description (Leyton *et al.*, 2003). The presence of *espI* was linked with *stx*₁, *stx*_{2d}-activatable and *astA*, and this result differed from previous studies in which this gene was only associated with *stx*_{2d}-EH250 (Schmidt *et al.*, 2001).

Sixty-seven per cent (25 of 37) of the strains were found to harbour at least one large plasmid. In the majority of the strains, this element could be characterized as being the pO113. Published observations for human isolates of several STEC serotypes correlated the possession of large plasmids with an enhanced capacity of causing severe disease (Newton *et al.*, 2009). The three non-STEC human isolates also presented high-mass plasmid bands, although they were negative by PCR for all the plasmid genes investigated, suggesting that these plasmids may carry unknown virulence genes.

PFGE was highly discriminatory, classifying the studied strains in three main groups. The majority of the strains were clustered in group A. However, group B was formed by isolates containing a distinct virulence genotype, which was formed by genes *astA* and *espI* always in association with *stx*₁. The absence of certain markers in this profile such as *ehxA* and *subAB* suggests that these strains may represent a subcategory of O113:H21 STEC with intermediate virulence ability.

The human isolates included in our study were recovered from cases of diarrhoea, suggesting a virulence potential. However, given the fact that we only had three isolates, comparisons and conclusions are more difficult to draw. Although belonging to the O113:H21 serotype, which is common among STEC isolates, they lacked *stx*-related genes upon isolation and presented a very limited set of virulence markers. Moreover, they were not genetically related to STEC strains, as indicated by plasmid profile and PFGE analysis. The possibility that they have lost *stx* genetic sequences exists (Karch *et al.*, 1992). Additional phylogenetic and *in vivo* studies are ongoing so that it will be possible to determine whether such strains are truly pathogenic and their relationships with the STEC pathotype. Lastly, the risk represented by animal O113:H21 STEC isolates to public health should be carefully monitored as such strains are widely present in the natural reservoir in our settings as in many other countries.

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