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

Luis Fernando dos Santos,1 Kinue Irino,2 Tânia Mara Ibelli Vaz2 and Beatriz Ernestina Cabilio Guth1

1Department of Microbiology, Immunology and Parasitology, Universidade Federal de São Paulo, São Paulo, Brazil
2Section of Bacteriology, Adolfo Lutz Institute, São Paulo, Brazil

*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*, *espI* 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 uremic 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.

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 uremic 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, Stx2d, has been further subdivided into two distinct toxins, Stx2d-EH250 (Piérand et al., 1998) and Stx2d-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/SEROTIPOSHUM.htm).
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)* | stx genotype† | PCR for virulence markers‡ | Hybridization with subAB probe§ | PFGE pattern|| | PFGE (sub) cluster |
|------------------|----------------|-----------------------------|-----------------------------|----------------|------------------|-----------------|
|                  | ehxA subAB epeA espP cdt-V astA espI iha saa |                  |                  |                  |                  |                  |
| Ec 258/01 (C)    | 2, 2dact      | + + + + + + − − + + + + | + 1 A1               |                  |                  |                  |
| Ec 472/01 (C)    | 2, 2dact      | + + + + + − + + + + + | + 1 A1               |                  |                  |                  |
| Ec 585/05 (C)    | 2             | + + + + − − − − + + + | + 2 A1               |                  |                  |                  |
| Ec 227/01 (C)    | 2, 2dact      | + + + + − − − − + + + | + 3 A1               |                  |                  |                  |
| Ec 858/05 (GO)   | 2dact         | + + + − − − − − − − + | + 4 A1               |                  |                  |                  |
| Ec 503/05 (GO)   | 2dact         | + + + − − − − − − − + | + 5 A2               |                  |                  |                  |
| Ec 648/05 (C)    | 2dact         | + + + − − − − − − − + | + 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, 2dact      | + + + + − − − − − + + | + 8 A2               |                  |                  |                  |
| Gc 138 (C)       | 2             | + + + + − − − − + + + | + 9 A3               |                  |                  |                  |
| Gc 20 (C)        | 2             | + + + + − − − − + + + | + 10 A3              |                  |                  |                  |
| Ec 784 (M)       | 2             | + + + + − − − − − − + | + 11 A3              |                  |                  |                  |
| Ec 689/04 (C)    | 2dact         | + + + + − − − − − + + | + 12 A4              |                  |                  |                  |
| Ec 678/04 (C)    | 2dact         | + + + + − − − − − + + | + 12 A4              |                  |                  |                  |
| Ec 684/04 (C)    | 2, 2dact      | + + + + − − − − − + + | + 13 A4              |                  |                  |                  |
| Ec 62/03 (C)     | 2, 2dact      | + + + + − − − − − + + | + 14 A4              |                  |                  |                  |
| Ec 301/02 (C)    | 2dact         | + + + + − − − − − + + | + 15 A5              |                  |                  |                  |
| Ec 254/01 (C)    | 2, 2dact      | + + + + − − − − − + + | + 16 A5              |                  |                  |                  |
| Ec 264/89 (HD)   | −             | − − − − − − − − − + + | − 17 A6              |                  |                  |                  |
| Ec 265/89 (HD)   | −             | − − − − − − − − − − + | − 18 A6              |                  |                  |                  |
| Ec 36/91 (HD)    | −             | − − − − − − − − − − + | − 19 A6              |                  |                  |                  |
| G102 (C)         | 1, 2, 2dact   | − − − − − − − − − + + | NT 20 B              |                  |                  |                  |
| G254/2 (C)       | 1, 2dact      | − − − − − − − − − + + | NT 20 B              |                  |                  |                  |
| G261/1 (C)       | 1, 2dact      | − − − − − − − − − + + | NT 20 B              |                  |                  |                  |
| G226/1 (C)       | 1, 2dact      | − − − − − − − − − + + | NT 20 B              |                  |                  |                  |
| G397/02 (C)      | 1, 2dact      | − − − − − − − − − + + | NT 20 B              |                  |                  |                  |
| Ec 727/05 (C)    | 1, 2dact      | − − − − − − − − − + + | NT 22 B              |                  |                  |                  |
| Ec 182/04 (BU)   | 1, 2dact      | − − − − − − − − − + + | NT 23 B              |                  |                  |                  |
| Ec 41/03 (C)     | 1, 2dact      | − − − − − − − − − + + | NT 24 B              |                  |                  |                  |
| Ec 670/05 (BU)   | 2dact         | − − − − − − − − − + + | NT 25 B              |                  |                  |                  |
| Ec 624/05 (C)    | 1, 2dact      | − − − − − − − − − + + | NT 26 B              |                  |                  |                  |
| Ec 596/05 (C)    | 1, 2dact      | − − − − − − − − − + + | NT 27 B              |                  |                  |                  |
| Ec 267/01 (C)    | 1, 2          | + + + − − − − − − − − | + 28 C               |                  |                  |                  |
| G2/18 (C)        | 2dact         | + + + − − − − − − − − | + NT                 |                  |                  |                  |
| Ec 719/05 (C)    | 1, 2, 2dact   | + + + − − − − − − − − | NT NT                |                  |                  |                  |
| Total (%)        | 23            | 23                        | 22                    | 23              | 7               | 11              | 13              | 27              | 23              |

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

†2dact, 2d-activatable.

‡All the strains were positive for lpfA113 and negative for eae, toxB, cnf1, cnf2, 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, cytotoxicity 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 stx1, stx2, and were STEC. Strains were maintained in tryptic soy broth with 15 % glycerol at 70 °C.

Detection of the stx2d-activatable and stx2d-EH250 genes and other virulence determinants (toxins, putative adhesins and auto-transporter proteases). To test for the presence of the stx2d activatable allele (stx2d-activatable), a recently described PCR strategy (Zheng et al., 2008) was employed. Gene stx2d-EH250 was investigated according to the PCR-RFLP methodology of Pierard 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 stx1, stx2, ehxA, eae, toxB, saa, lpfA113 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 gene, 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 XbaI endonuclease (Invitrogen) for 18 h, PFGE was performed on a CHEF-DR III 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 stx2d-activatable genotype

The Stx2d-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 stx2d-activatable in combination with stx1, stx2, or stx2d. (Table 1). None of the strains possessed stx2d-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.
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 stx2d-activatable either alone or in association with stx1. To our knowledge, this is the first description of activatable Stx2 (Stx2d-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 stx2d-activatable are likely to be more pathogenic. A link between Stx2d-activatable-producing strains and severe disease, mostly HUS, has already been demonstrated (Bielaszewska et al., 2006).

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

Associations of several virulence genes which are implicated in adhesion and toxin production were found. High frequencies of lpfA113, 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).
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 stx2, stx2d-activatable and astA, and this result differed from previous studies in which this gene was only associated with stx2d-E11250 (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-STECC 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 stx2. 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 diarrhea, 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.

ACKNOWLEDGEMENTS

The authors gratefully acknowledge the assistance of Sylvia P. Cardoso Leão for dendrogram analysis. This study was supported by grants from Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP), process number 05/04634-8, Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq - Brasil) and Programa de Apoio a Núcleos de Excelência PRONEX MCT/CNPq/FAPERJ. L.F. dos S. receives a research fellowship from FAPESP, process number 06/06289-0.

REFERENCES


