IDENTIFICATION OF THE SUBTILASE CYTOTOXIN GENE AMONG SHIGATOXIGENIC ESCHERICHIA COLI ISOLATED FROM DIFFERENT SOURCES

JACEK OSEK
Department of Hygiene of Food of Animal Origin,
National Veterinary Research Institute,
24-100 Pulawy, Poland
e-mail: josek@piwet.pulawy.pl

Received for publication November 07, 2005.

Abstract

The presence of the recently described subtilase cytotoxin gene (subAB) among shigatoxigenic E. coli (STEC) strains isolated from humans, cattle, pigs, and food was tested using PCR with primers flanking the conserved region of the subA coding sequence. Moreover, the Shiga toxin and several other putative marker genes were analysed among subA-positive isolates. It was found that 4 out of the 65 (6.1%) STEC isolates tested were able to produce the subtilase cytotoxin. These strains were isolated from humans with haemorrhagic colitis (HC) (3 strains) and from cattle (1 isolate) and belonged to different serotypes (O55:H9, O62:H-, O113:H20, O157:NM). None of the subA-positive isolate had the eaeA gene responsible for the production of adhesive protein intimin. The results suggest that subAB cytotoxin may be a new virulence toxin marker among STEC strains of human and animal origin.

Key words: shigatoxigenic Escherichia coli, subtilase cytotoxin, Shiga toxin, virulence markers, PCR.

Shigatoxigenic Escherichia coli (STEC) is an important cause of gastrointestinal disease in humans, particularly since these infections often may result in severe complicating problems that include haemorrhagic colitis (HC) and the life-threatening haemolytic uraemic syndrome (HUS) (1, 4, 11, 18). The STEC family is very diverse and strains belonging to a broad range of serotypes have been associated with human diseases (11, 18). Among them, E. coli O157:H7 is the most common serotype connected with sporadic cases and large outbreaks of diseases in many countries (1, 18).

Cattle are the major reservoir of E. coli O157:H7 as well as other serogroups of STEC. In addition, STEC has been isolated from pigs, sheep, goats, horses, dogs, deer and seagulls (1, 5, 7, 8, 11). The colonization of the intestinal tract of these animals with STEC is generally asymptomatic and transient (less than 1 month) but can persist in herds over extended periods.

The pathogenicity of STEC is mainly mediated by Shiga toxin (Stx), encoded by bacteriophage genes, and the products of the locus of enterocyte effacement pathogenicity island (LEE), with the eaeA gene that encodes the intimin protein involved in the intimate adhesion of bacteria to enterocytes (4). Another important virulence marker of Stx-positive E. coli is the plasmid-encoded enterohaemolysin (Hly), which is closely associated with O157:H7 serotype (11). The main virulence property of STEC is, however, the production of Shiga toxin that has been divided into two major subclasses, Stx1 and Stx2 (1, 11). Although Stx1 is relatively homogenous, at least five main subtypes of Stx2 have been identified: Stx2, Stx2c, Stx2d, Stx2e, Stx2f, and Stx2g (5, 11). Recently, Paton et al. (12) reported that some STEC strains produced a new cytotoxin, which was lethal for mice and also resulted in extensive microvascular damage and necrosis in multiple organs, including the brain, kidneys and liver. The new toxin has been named subtilase cytotoxin because its 35-kDa A subunit is a subtilase-like serine protease related to the similar product of Bacillus anthracis. The toxin (SubAB) was detected in the LEE-negative O113:H21 STEC strain which was responsible for a small outbreak of HUS in Australia in 1998 (14). The genes encoding the cytotoxin are located on a large plasmid designated pO113 (12).

The first step in the pathogenesis of STEC infection is the colonization of the intestinal mucosa to prevent the elimination of bacteria by peristalsis. This process is probably mediated by several putative adherence factors described both in LEE-positive and LEE-negative STEC strains. Tarr et al. (19) identified a chromosomally encoded 67-kDa adherence-conferring protein (Iha) that is similar to Vibrio cholerae IrgA. Nicholls et al. (6) described a very large adhesin Efa1 (EHEC factor for adherence) which mediated the binding of bacteria to CHO cells in vitro. Furthermore, Tatsuno et al. (20) found the toxB gene localized on plasmid pO157, required for full expression of adhesive properties of some of O157:H7 E. coli isolates.
Recently, Torres et al. (23), Toma et al. (22), and Tatarczak et al. (21) described novel putative virulence genes \( \text{lpfA}_{\text{O157:F5}}\), and \( \text{lpfA}_{\text{O157:F5}} \) that were associated with O157 serogroup. Moreover, there are other putative adhesin genes found in LEE-negative STEC isolates recovered from humans. However, their role in pathogenesis of infections is not well understood. These include the \( \text{saa} \) (an autoagglutinating adhesin) marker (13) as well as the \( \text{lpfA}_{\text{O113}} \) gene responsible for expression of long polar fimbriae in strains of serotype O113:H21 (2).

The aim of the present study was to test the prevalence of the subtilase cytotoxin gene in Shiga toxin-producing \( \text{E. coli} \) strains isolated from different sources and establish a correlation between this gene marker and other STEC-associated virulence factors.

**Material and Methods**

**Bacterial strains.** A total of 65 STEC strains isolated from humans (\( n = 39 \)), cattle (\( n = 11 \)), pigs (\( n = 6 \)), and food (\( n = 6 \)) were used in this study. Several of these strains were previously tested for the presence of \( \text{stx} \) and \( \text{eaeA} \) genes (7-10) as well as for several putative adhesive factor genes (21). The following positive and negative reference \( \text{E. coli} \) strains were also included: EDL933 (O157:H7, \( \text{eaeA} \), \( \text{stx1} \), \( \text{stx2} \), \( \text{rFB} \), \( \text{fbIC} \), \( \text{iha} \), \( \text{toxB} \), \( \text{efa1} \), \( \text{lpfA}_{\text{O157:F5}} \), \( \text{lpfA}_{\text{O157:F5}} \)). \( \text{E. coli} \) HB101 with the pWSK29 plasmid (\( \text{iha} \), \( \text{toxB} \), \( \text{ef1} \), \( \text{lpfA}_{\text{O157:F5}} \), \( \text{lpfA}_{\text{O157:F5}} \)).

**Detection of Shiga toxin, intimin, and putative adhesive factor genes.** The Shiga toxins (\( \text{stx1} \), \( \text{stx2} \) and subtypes), intimin (\( \text{eaeA} \)), and several putative adhesive factor genes (i.e. \( \text{toxB} \), \( \text{lpfA}_{\text{O113}} \), \( \text{lpfA}_{\text{O157:F5}} \), \( \text{lpfA}_{\text{O157:F5}} \)), \( \text{E. coli} \) \( \text{HB101 with the pWSK29 plasmid} \) (\( \text{lpfA}_{\text{O113}} \)) and C600 (K-12). For the \( \text{saa} \) and \( \text{subA} \) adhesive and cytotoxin factor genes, respectively, the isolated DNA preparation kindly supplied by James Paton (Department of Molecular Biosciences, Adelaide University, Australia) was used.

**Identification of subA subtilase cytotoxin gene.** Bacteria were grown on Luria-Bertani (LB) agar at 37°C for 18 h and one individual colony of each \( \text{E. coli} \) isolate was suspended in 50 µl of sterile, DNase- and RNase-free deionised water (ICN Biomedicals, Costa Mesa, USA). The suspensions were heated at 99°C for 5 min (Thermomixer, Eppendorf, Germany) and centrifuged at 13 000 x g for 1 min. The supernatant (5 µl) was subsequently used as a source of DNA template. PCR amplifications were performed in 50 µl mixtures consisting of: 5 µl of PCR buffer (10x concentrated), 5 µl of each deoxynucleotide (dNTPs, final concentration 200 µM), 6 µl of 25 µM MgCl\(_2\) (final concentration 3 µM), 1.0 µl of each primer (F: TATGGCTTCCCTCATTTGCC; and R: TATAGGCTTGGCTTCTGACG; final concentration 0.2 µM) (14), 1 U of \( \text{Taq} \) DNA polymerase (Fermentas, Vilnius, Lithuania) and water. The PCR program consisted of initial DNA denaturation at 94°C for 5 min, followed by 30 cycles at 94°C for 1 min, 55°C for 1 min and extension at 72°C for 2 min. The final extension step was run at 72°C for 5 min. The amplified product (10 µl of the final PCR reaction mixture) was visualized by gel electrophoresis in 1.5% agarose gel in TAE buffer at 100 V. After staining with ethidium bromide (5 µg/ml) for 2 min and washing in distilled water, the gel was photographed under UV light using the Gel Doc 2000 documentation system (Bio-Rad, Hercules, USA). The size of the PCR amplicon was compared to the 100 bp DNA marker (Fermentas).

**Results**

PCR identification of the \( \text{subA} \) gene revealed that among the 65 STEC strains analysed, 4 isolates (6.1%) were positive for this virulence marker as determined by the presence of the 556 bp amplicon (Fig. 1, lanes 1-4). The remaining 61 strains did not generate a PCR amplicon of 556 bp or any other size (Fig. 1, lane 5). It was noted that the \( \text{subA} \)-positive \( \text{E. coli} \) were isolated from humans with HC (3 strains) and from cattle (1 isolate). The characteristics of the \( \text{subA} \)-positive strains are presented in Table 1. The human isolates were of O62:H-, O113:H21, and O157:NM serotypes. All of them had the genes encoding the Stx2 toxin, either alone or with the \( \text{stx1c} \) gene. One bovine STEC strain was of O55:H9 serotype and produced Stx1 Shiga toxin only. Interestingly, none of the \( \text{subA} \)-positive strains, regardless of the origin, was \( \text{eaeA} \) (intimin)-positive although they had several other putative adhesive factor genes. All the isolates were positive for the \( \text{iha} \) gene marker responsible for the expression of 67-kDa adherence-conferring protein (Iha). Moreover, three of the strains analysed had the \( \text{toxB} \) gene encoding some adhesive properties of STEC. Furthermore, two isolates, O113:H21 and O55:H9, recovered from humans and cattle, respectively, possessed the \( \text{saa} \) gene marker which is responsible for expressing the relatively rare autoagglutinating adhesin described by Paton et al. (13).

![Fig. 1. Agarose gel electrophoresis of PCR products amplified from DNA of E. coli strains positive for the subA gene.](Image)

Lane M, 100 bp DNA marker; lane 1, \( \text{E. coli} \) O62:H (strain 415); lane 2, \( \text{E. coli} \) O157:NM (strain 463); lane 3, \( \text{E. coli} \) O113:H21 (strain 478); lane 4, \( \text{E. coli} \) O55:H9 (strain 449); lane 5, \( \text{E. coli} \) subA-negative; lane 6, \( \text{E. coli} \) DNA preparation positive for the \( \text{subA} \) gene (provided by J. Paton).
Table 1  
Genetic virulence markers of STEC strains positive for the *sua* gene of subtilase cytotoxin

<table>
<thead>
<tr>
<th>Strain number</th>
<th>Serotype</th>
<th>Source*</th>
<th>Virulence marker genes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td><strong>stx1</strong></td>
</tr>
<tr>
<td>415</td>
<td>O62:H-</td>
<td>H (HC)</td>
<td>-</td>
</tr>
<tr>
<td>463</td>
<td>O157:NM</td>
<td>H (HC)</td>
<td>+ (1c)</td>
</tr>
<tr>
<td>478</td>
<td>O113:H21</td>
<td>H (HC)</td>
<td>-</td>
</tr>
<tr>
<td>449</td>
<td>O55:H9</td>
<td>B</td>
<td>+</td>
</tr>
</tbody>
</table>

* H – human isolates; HC – haemorrhagic colitis; B – bovine isolate.
Discussion

The subA gene responsible for the production of subtilase cytotoxin was recently identified among some STEC human isolates responsible for outbreak of HUS (14). It was shown that the SubAB toxin is encoded by two closely linked genes (subA and subB) which are located on a large conjugative virulence plasmid pO113 (12). The primers used for the PCR detection of the cytotoxin were directed for two of three critical functional domains in the SubA coding sequence (14). The resulted amplicon had the size of 556 bp. Analysis of the 65 STEC strains isolated from different sources (humans, cattle, pigs, food) revealed that 4 isolates possessed the subA gene. Among them, there was the strain of O113:H21 serotype, i.e. the same serogroup originally described by Paton and Paton (14). These authors recently tested 44 STEC strains isolated from humans with HC or HUS and found 16 isolates (36.4%) positive for the subA gene. In the present study the number of such isolates of human origin was lower (3 strains out of 39 human strains analysed; 7.7%). Moreover, one strain of bovine origin was subA-positive and such animal isolates were not tested by Paton and Paton (14). Interestingly, all subA strains, both analysed by the mentioned authors as well as in the present study were negative for the eaeA gene, responsible for the expression of intimin protein, a marker which is crucial for intimate adhesion of STEC strains to the intestinal epithelium. Therefore, it may suggest that such eaeA-negative STEC isolates are able to express other surface adhesive factors such as those encoded by toxB or saa genes (13, 20).

Little is know about role of SubAB cytotoxin in the pathogenesis of STEC-induced syndromes in humans. Experiments performed with animals showed that intraperitoneal injection of purified subtilase toxin was fatal for mice and resulted in extensive microvascular thrombosis as well as necrosis in the brain, kidneys, and liver. Oral challenge of mice with E. coli K-12 with cloned subA and subB genes resulted in dramatic weight loss (12). Thus, these findings suggest that the toxin may contribute to the pathogenesis of human diseases. However, Paton and Paton (14) also demonstrated a strong association between the presence of subA and STEC carrying the stx2 gene only. Therefore, the role of subtilase cytotoxin alone in the pathogenesis of HC and HUS in humans needs to be further tested. Similar correlation between Shiga toxin 2 and SubAB cytotoxin was observed in the present study (3 human subA-positive isolates were also stx2-positive) although the small number of the SubAB-producing strains were found among STEC tested.

In the present study one bovine STEC isolate of O55:H9 serogroup was positive for the subA gene as demonstrated by the presence of the 556 bp PCR amplicon. This is the first information about the identification of this gene among shigatoxigenic E. coli isolated from animals. Since this isolate had the stx gene as well as several other putative gene markers it may be a potent human pathogen. The fact that subAB is carried on a mobile DNA element it shows the possibility of further transmission to other STEC serogroups. However, further studies are needed to analyse the prevalence of SubAB cytotoxin among shigatoxin-producing E. coli strains of animal origin as well as the role of the subAB gene and its product in the pathogenesis of STEC-mediated diseases.

References

Shiga-toxigenic *Escherichia coli* strains that are virulent for humans. Infect Immun 2001, **69**, 6999-7009.


