Distribution and molecular analysis of Subtilase cytotoxin gene (*subAB*) variants in Shiga toxin-producing *Escherichia coli* (STEC) isolated from different sources in Iran

Running tittle: *subAB* in STEC

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Abstract

Subtilase is a potent cytotoxin that was first described in O113:H21 strain in Australia as a plasmid-encoded cytotoxin (*subAB1*). Subsequently, chromosomal variants including *subAB2-1*, *subAB2-2*, and *subAB2-3* were described. In the present study a collection of 101 archived STEC strains isolated from various sources in Iran (2009-2016) were analyzed for the detection of different genes encoding the subtilase variants, plasmidic and chromosomal virulence genes, together with the phylogroup and serogroups. Overall, 57 isolates (56.4%) carried at least one variant of *subAB.* Most strains from small ruminants including 93% of sheep and 96% of caprine isolates carried at least one chromosomally encoded variant (*subAB-2-1* and/or *subAb2-2*). In contrast, 12 cattle isolates (24%) only harbored the plasmid encoded variant (*subAB1*). STEC strains from other sources including deer, pony and humans were positive for *subAB-2-1* and/or *subAb2-2.* Concerning the virulence markers, some strains showed an association with hosts the bacteria were isolated from. In particular, *tia* was associated with sheep, goats and pony isolates and *astA* gene was present in deer, pony and goats and *terD* was only found in deer and pony isolates. Only cattle STEC carried *espP* and *epeA,* the important markers of pO113 plasmid. Some genes were widespread among strain of various sources like *ehly*, *iha* and lpf^{O113} and some genes were not detected such as *efa1*, *toxB* and *katP*. Most strains belonged to phylogenetic group B1 (89.47%), but five strains from cattle, deer, pony and a goat were assigned to A phylogroup. Most cattle strains belonged to O113, while O5 was just detected in ovine isolates, and O128 and O113 were present in caprine strains. In conclusion, the present study reveals the presence of potentially pathogenic genotypes among LEE-negative isolates and some host specificity related to subtilase variants and other virulence markers that may aid in source tracking of STEC during outbreak investigations.

Keywords

Subtilase variants, LEE-negative, STEC, animals, source tracking

1. Introduction

Shiga toxin- producing *E. coli* (STEC) is considered as one of the most important food-borne pathogens worldwide causing diseases in humans with different severity such as diarrhea, hemorrhagic colitis (HC), and some fatal conditions such as hemolytic uremic syndrome (HUS).

The early reported large outbreaks were due to O157:H7 *E. coli*, which harbor sets of important virulence markers beside the Shiga toxin (s) coding genes, such as locus of entrocyte effacement (LEE) and Enterohemorrhagic *E. coli* hemolysin (*ehly*) (Gyles 2006). Later on, it become evident that STEC belonging to other serogroups and showing different genotypes, can also cause severe infections and outbreaks. For example, some strains isolated from HUS are negative for the LEE locus and belong to diverse serogroups such as O55, O73, O91, O104, O113, O128, O145, O163, O178 (Buvens, Lauwers, & Piérard, 2010; Mellmann et al., 2008). Most of our knowledge on pathogenesis of STEC infection derives from the study of the strains belonging to five serogroups such as O157, O111, O103, O145 and O26, described as belonging to the seropathotypes A and B (Karmali et al., 2003), however, more recent studies are highlighting the particular importance of LEE-negative strains and emerging seropathotypes (Grande et al., 2016; Gyles, 2007; Newton, 2009). Notably, one of the largest and most severe HUS outbreaks was attributed to O104: H4, a hybrid LEE-negative Stx2-producing strain with enteroaggregative genomic backbone (Helalat, Rezatofighi, Ardakani, Santos, & Badouei, 2020).

Among the virulence factors of highly pathogenic LEE-negative STEC, subtilase cytotoxin was described to be one of the major player as recent studies suggest (Bondì et al., 2017; Seyahian et al., 2017). Subtilase is a potent AB5 toxin showing high cytotoxicity to Vero cells and lethality when injected intraperitoneally to mice (Paton, Srimanote, Talbot, Wang, & Paton, 2004). Wild type SubAB encoding strain provoked cytotoxic effect almost similar to the highly pathogenic O157:H7 strain

(EDL933) (Hauser et al., 2016). Additionally, besides damaging renal epithelial cells, in a mice experimental model it also induces multi-organ systemic response very similar to HUS pathogenesis (Seyahian et al., 2017).

Subtilase, was first described in 2004 in O113:H21 strain (98NK2) isolated during a HUS outbreak in southern Australia (Paton et al., 2004). This novel toxin was first described to be encoded by a operon comprising two components of *subA* and *subB* co-transcribed from genes located on pO113 transmissible megaplasmid which subsequently named *subAB1* (Orden et al., 2011; Paton et al., 2004). Other studies demonstrated the presence of chromosomally encoded variants in small ruminants and other STEC strains and named *subAB2-1* and *subAB2-2* (Funk, Stoeber, Hauser, & Schmidt, 2013; Michelacci et al., 2013). The *subAB2-1* is carried on a pathogenicity island SE-PAI and in most instances was linked to *tia* gene which encodes invasion protein first reported in enterotoxigenic *E. coli*. The *subAB2-2* is adjacent to outer membrane efflux protein locus (OEP); moreover, a novel variant was also discovered as *subAB2-3* in deer STEC (Strain 48) in 2014 (Funk et al., 2013; Michelacci et al., 2013; Nüesch-Inderbinen et al., 2015).

Many studies in Iran showed that non-O157 STEC strains are widely distributed in food producing animals. We recently demonstrated the virulence properties of non-O157 STEC in cattle and small ruminants in Iran (Jajarmi, Askari Badouei, Imani Fooladi, Ghanbarpour, & Ahmadi, 2018; Jajarmi, Imani Fooladi, Badouei, & Ahmadi, 2017). As our data showed so far, the prevalence of LEE-negative non-O157 strains are quite high; therefore, we aimed to investigate the most important virulence determinants in such strains for the first time. For this purpose, we examined the presence of *subAB* genes in a collection of STEC strains isolated from different sources during 2007 to 2016 then we determined the allelic variants, virulence determinants, serogroups and phylogroups of the subtilase-producing STEC in Iran.

2. Materials and Methods

2.1. E. coli strains

A total of 101 STEC strains isolated from different sources in three veterinary institutions in Iran during the period from 2007 to 2016 were selected for this study. To test the purity of the isolates, they were sub-cultured on MacConkey agar and a single colony was used in subsequent analysis. The presence of Shiga toxin genes (*stx*), was confirmed using a multiplex-PCR targeting *stx1, stx2*, *eae*, and *ehly* as described previously (Paton & Paton, 1998). The isolates were obtained from cattle (n=50), goats (n=25), sheep (n=15), wild captive animals (n=8) and humans (n=3) as shown in Table 1.

2.2. PCR detection of subAB genes and determination of the allelic variants

The STEC isolates were first subjected to a PCR assay recognizing different chromosomal and/or plasmid encoded Subtilase variants. Then, the *subAB*+ isolates were analyzed by PCR to discriminate allelic variants of the Subtilase gene. The *subAB1* and *subAB2-2* variants were detected as described by Funk et al. (2013), and the *subAB2-1* was identified as described by Michelacci et al. (2013). For the detection of the novel *subAB2-3* variant, a pair of primers was designed according to the published sequence of this variant (accession no. JPQG00000000); primers were also tested *in silico* against the deposited sequences containing this variant (http://insilico.ehu.es/PCR/). The primers were SubB2-3 (5'-AACGCCTGAAAACATGCCAT-3'), and JD73R (5'-CGCTATTCTCGCAGGTACAG-3') amplifying a 2037 bp fragment of the novel variant and the adjacent hypothetical gene. The condition for amplification of *subAb2-3* consisted of 94 °C (60s), 55 °C (60s), and 72 °C (120s) and repeated for 35 cycles.

2.3. Virulence genes and genetic determinants

All *subAB*+ strains were subjected to PCR analysis for various virulence genes. The presence of some plasmid encoded genes such as *saa*, *espP*, *epeA*, *toxB*, and *katP* were investigated as described previously (Askari Badouei, Morabito, Najafifar, & Mazandarani, 2016). Presence of other chromosomally encoded virulence/genetic determinants including *astA* , *cdt*, *iha*, *efa1*, *lpf O113* and *terD* were also tested by PCR as described before (Askari Badouei et al., 2016; Nicholls, Grant, & Robins-Browne, 2000; Schmidt et al., 2001).

2.4. Phylogenetic groups

All strains carrying *subAB* were subjected to the updated protocol for *E. coli* phylogenetic grouping. First, the strains were tested by a quadruplex-PCR, and if the strain was not assigned to a particular phylogroup, complementary PCRs were conducted as described before (Clermont, Christenson, Denamur, & Gordon, 2013).

2.5. Molecular serotyping

All strains were tested for eight pathogenic STEC serogroups including O26, O45, O103, O111, O113, O121, O145 and O157 using a multiplex-PCR as described previously (DebRoy, Roberts, Valadez, Dudley, & Cutter, 2011). If the strains were negative for the top eight serogroups, the isolates were additionally tested for some other prevalent serogroups mostly associated with LEE-negative and *subAB*- encoding strains including O5, O91, O104, O113, and O128. The primers and PCRs were used as described previously (Iguchi et al., 2015; Sánchez, Llorente, Echeita, & Herrera-León, 2015).

3. Results

3.1. Screening PCR and allelic variants of subAB

In total, 57 of the 101 STEC tested (56.4%), yielded the specific amplicon for *subAB*. All positive isolates were typically the LEE-negative strains (Table 1). Most STEC from small ruminants including 93% of strains from sheep and 96% from goats carried at least one chromosomally encoded *subAB* variant; in fact, with two exceptions all carried both *subAB2-1* and *subAB2-2*. In contrast, of 50 cattle STEC isolates, only 12 (24%) carried the plasmid encoded variant (*sub AB1*). As presented in Table 2, four strains from deer and pony and three from diarrheic children were positive for *subAB2-1* and/or *subAB2-2*. None of the studied isolates yielded the specific amplicon for *subAB2-3*.

3.2. Shiga toxin genes and virulence determinants

The isolates from small ruminant harbored *stx1*, alone or in combination with *stx2,* but all cattle isolates only harbored the *stx2* gene. Three human isolates possessed only the *stx1,* but most deer and pony strains harbored both *stx1* and *stx2* genes. As far as the additional virulence genes are concerned, *tia* was present in sheep, goats, deer, and pony isolates, but was not found in cattle or human strains. Interestingly, *terD* which encodes tellurite resistance was only found in deer and pony strains. Similarly, *astA* was detected in deer and pony strains and only in two goat isolates. Only one goat isolate belonging to O128 serogroup yielded the *cdt* amplicon. Among the plasmid-encoded virulence associated genes, *ehly* was present in most isolates (94.7%) regardless of the source, but the distribution of other virulence genes showed some correlations with the host. For instance, only cattle STEC carried *espP* and *epeA*, and none of the sheep and goat strains carried *saa*. None of the isolates carried *toxB* and *katP*, markers of the pO157 large virulence plasmid (Rump et al., 2015). The adhesion genes *iha* and lpf^{O113} were present in most isolates belonging to different sources, while all strains tested were negative for *efa1* (Table 3).

3.3. Phylogenetic groups and serogroups

Most strains belonged to phylogenetic group B1 (89.47%), while five strains from cattle, deer, pony and a goat were assigned to A phylogroup. Only one cattle isolate was designated as E phylogroup (Table 3). Among the tested serogroups, the most prevalent O-type was O113 (n=15), followed by O5 (n=7), and O128 (n=2). Interestingly, most cattle strain belonged to O113, while O5 was just detected in ovine isolates, and O128 and O113 were present in caprine strains (Table 3).

4. Discussion

Studies mostly conducted in the past decade unveiled that a subset of LEE- negative STEC can lead to sever conditions such as HUS in humans. The genetic lineages and evolution of such strains seem to be separated from the typical LEE-harboring strains. Accordingly, several specific virulence determinants including toxins, adhesins and invasion proteins have been discovered in the STEC strains lacking LEE pathogenicity island (Montero et al., 2017; Newton, 2009). Of the many definite or hypothetical virulence determinants present in these isolates, Subtilase-producing strains are believed to be one of the most important pathogenic lineages. With rare exceptions, *subAB* carriage seem to be almost exclusively associated with the STEC pathotype (Irino et al., 2010; Krause et al., 2019; Tozzoli et al., 2010). Subtilase not only acts as a potent toxin, but also occurs in different allelic variants in strains of different origins (Michelacci et al., 2013; Nüesch-Inderbinen et al., 2015; Orden et al., 2011). Recent findings suggested that different SubAB variants exhibit different binding capacity toward their target cells which may affect their cytotoxic behavior (Krause et al., 2019).

The *subAB+ E. coli* has been frequently isolated from food-producing animals including cattle, sheep, goats, deer and large game animals in different countries; here, we reported its carriage in equine for the first time.

Overall, very few studies explored all *subAB* types because different allelic variants have not been elucidated until recently. Nevertheless, many studies confirmed that the carriage rate and allelic

variants of *subAB* has been highly associated to the host species rather than the geographical origin of the strains. We similarly found that the *subAB1* mainly occurs in cattle and *subAB2* variants found in small ruminants, deer, horse and humans. We believe that such host specificity could be regarded as a primary tool for source tracking of disease epidemics due to LEE-negative STEC. In the present study, 24% of cattle, and 93 to 96% of sheep and goats carried variants of *subAB1* and *subAB2* (variants 1 and 2), respectively. Such carriage rate was strikingly similar to the other comprehensive research which found this gene in 25% of bovine and 91.9% of sheep and goats STEC in Spain (Orden et al., 2011). In Brazil, 21 out of 95 STEC collection strains (22%) were positive in *subA* PCR which mainly targets the *subAB1* (Velandia et al., 2011). Such surprising similarity in carriage of *subAB* may reflect the very old macro-evolutionary events in LEE- negative lineages which occurred in *E. coli* population within different hosts regardless of the geographical region. In other studies, the carriage rate of *subAB2-1* was 86% in sheep and 72% in cases of human diarrhea (Michelacci et al., 2013). In Spain, almost all caprine and ovine strains carried *subAB2-2,* but 61.4% and 64.3% carried *subAB2-1* respectively (Orden, Domínguez-Bernal, de la Fuente, & Carrión, 2016). In the present study, *subAB2-1/2-2* variants occurred together in most isolates of sheep and goat strains (Table 2). Previously, one of the highest carriage rates has been reported in wild ruminants including ibex (100%) and Chamois (92%), but the rate was also high in Red deer (52.6%) and Roe deer (26.6%). In the mentioned study, one strain from a Roe deer harbored a new *subAB2-3* in combination to *subAB2-1*, but 19 cattle isolates were negative for any *subAB2* variants (Nüesch-Inderbinen et al., 2015). In our study *subAB2* variants were present in all strains from captive wild ruminants but none included the new allelic type. As mentioned, the pathogenicity of the LEE- negative strains can be reinforced by possession of various virulence determinants, some of which seem to be almost restricted to this subset of STEC (Montero et al., 2017; Newton, 2009). We found that along with *subAB*, strains harbor potential adhesins and invasion proteins such as *iha*, *lpf* ^{O113}, and *tia* at high rates, and include other markers

such as *saa*, *espP*, *epeA*, and *astA* at lower frequencies. With these aforementioned markers, we also observed some host specificity. For example, the bovine strains mostly carried the combination of *stx2/ehly/iha/ lpf^{O113}/epeA/espP/saa*. This was not surprising as most of the cattle STEC belonged to O113 serogroup and many of such determinants are carried within pO113 mega plasmid (Ennis, McDowell, & Bolton, 2012). Interestingly, four other STEC O113 from deer, goats and pony belonged to A phylogroup and exhibited different profiles as they lacked *epeA* and *espP* but carried *stx1/stx2/ehly/tia/astA* and *saa* (in 3 out of 4 strains). This suggests the presence of different plasmids in different O113 lineages in *E. coli* residing in different hosts, or the possible presence of chromosomal variants of some important genes such as *ehly* and *saa* in *subAB2* carrying strains, which needs to be clarified in the future studies.

In conclusion, the present study showed for the first time the widespread presence of *subAB* variants in a large collection of STEC isolates in Iran. Our study clearly showed some host specific properties of *subAB*-harboring strains even within the same serogroup that makes typing of *subAB* variants a potential primary genetic tool that aids source tracking in outbreaks and epidemics due to LEE-negative STEC.

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Conflict of interest

The authors declare that they have no competing interests.

Ethical Statement

The study was conducted in compliance with ethical standards.

Data availability statement

All data is available upon direct request to the authors.

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