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Paper

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

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Abstract

Subtilase exhibits strong cytotoxicity 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. We aimed to investigate the presence of *subAB* genes in a collection of Shiga toxin-producing *Escherichia coli* (STEC) strains (n=101) isolated from different sources in Iran. A collection of 101 archived STEC strains isolated from cattle (n=50), goats (n=25), sheep (n=15), wild captive animals (n=8: persian fallow deer, n=3; caspian pony, n=1; *Macaca mulatta*, n=4), and humans (n=3) during 2007-2016 were analyzed for the detection of different genes encoding the Subtilase variants, plasmidic and chromosomal virulence genes, phylogroups 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*. Our results reveal the presence of potentially pathogenic genotypes among locus of enterocyte effacement (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

Introduction

Shiga toxin-producing *Escherichia coli* (STEC) are a significant global foodborne pathogen, responsible for a range of human diseases, including diarrhea, hemorrhagic colitis (HC), and potentially fatal conditions like hemolytic uremic syndrome (HUS). The early reported large outbreaks were caused by O157:H7 *E. coli*, which possess essential virulence markers in addition to the genes coding for Shiga toxins. These markers include the locus of enterocyte effacement (LEE) and Enterohemorrhagic *E. coli* hemolysin (*ehly*) 1. Subsequently, it becomes evident that STEC belonging to other serogroups and showing different genotypes, can also cause severe infections and outbreaks. For

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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 2. 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, which are categorized under seropathotypes A and B 3, however, more recent studies are highlighting the particular importance of LEE-negative strains and emerging seropathotypes 4 1. 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 5.

Among the virulence factors of highly pathogenic LEE-negative STEC, Subtilase cytotoxin has been described as a major contributor, as suggested by Zotta *et al.* 6. Subtilase is a powerful AB5 toxin, exhibiting high cytotoxicity to Vero cells and causing lethality when injected intraperitoneally into mice 7. Wild type SubAB encoding strain provoked cytotoxic effect almost similar to the highly pathogenic O157:H7 strain (EDL933) 8. Additionally, besides damaging renal epithelial cells, in a mice experimental model it also induces multi-organ systemic response very similar to HUS pathogenesis 6.

Subtilase, was first described in 2004 in O113:H21 strain (98NK2) isolated during a HUS outbreak in southern Australia 7. This novel toxin was first described to be encoded by a operon comprisingtwo components of *subA* and *subB* co-transcribed from genes located on pO113 transmissible megaplasmid which subsequently named *subAB1* 9 7. Other studies demonstrated the presence of chromosomally encoded variants in small ruminants and other STEC strains and named *subAB2-1* and *subAB2-2* 10 11. 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 10 11.

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 12 13. 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.

Materials and methods

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. Strains were obtained by fecal sampling and 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 14. The isolates were obtained from cattle (n=50), goats (n=25), sheep (n=15), wild captive animals (n=8: persian fallow deer, n=3; caspian pony, n=1; *Macacamulatta*, n=4), and humans (n=3) as shown in Table II.

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 10, and the*subAB2-1* was identified as described by11. 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.

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Primer name	Sequence (5'-3' direction)	Amplicon size (in bp)	Reference
***Shiga toxin* genotypes**			
stx1-F	ATAAATCGCCATTCGTTGACTAC	180	Paton & Paton (1998)
stx1-R	AGAACGCCCACTGAGATCATC		
stx2-F	GGCACTGTCTCTCTGAAACTGCTCC	255	
stx2-R	TCGCCAGTTATCTGACATTCTG		
eae-F	GACCCGGCACAAGCATAAGC	384	
eae-R	CCACCTGCAGCAACAAGAGG		
ehxA-F	GGAACCGCTGAAAATGTAGG	534	
ehxA-R	ACTGGTCGTCTCCCTGTCC		
Subtilase variants			
RTsubAB-F	GCAGATAAATACCCTTCACTTG	232	Paton et al. (2004)
RTsubAB-R	ATCACCAGTCCACTCAGCC		
subAB1-F	CGTATCTGCGCCATATCCTG	1821	Funk et al. (2013)
subAB1-R	CTGTTCCGAGCAGCCATATC		
subAB2-1-F	CCCTGTAACATATTGACCAGCA	1060	Michelacci et al. (2013)
subAB2-1-R	ATCACCAGTCCACTCAGCC		
subAB2-2-F	TAATGTTTTTGAGACGGG	1170	Funk et al. (2013)
subAB2-2-R	AGGTCGGCTCAGTGTTC		
subB2-3-F	AACGCCTGAAAACATGCCAT	2037	This study
subAB2-3/JD73-R	CGCTATTCTCGCAGGTACAG		
Virulence genes			
saa-F	CGTGATGAACAGGCTATTGC	119	Paton & Paton (2002)
saa-R	ATGGACATGCCTGTGGCAAC		
espP-F	TTGCGAAAAATGGCGGAACTC	956	Wieler et al. (2000)
espP-R	CGGAGTCGTCAGTCAGTAGA		
epeA-F	CACCCTGTAGAATCTTA	1873	Leyton et al. (2003)
epeA-R	CTGAATAAATCCAGCCC		
toxB-F	ATACCTACCTGCTCTGGATTGA	602	Tarr et al. (2002)
toxB-R	TTCTTACCTGATCTGATGCAGC		
katP-F	GCGGAAGAGAAGATGACTGG	277	Amézquita-López et al. (2014)
katP-R	GCACCATGTGCTTTACCAAA		
astA-F	CCATCAACACAGTATATCCGA	111	Yamamoto & Nakazawa (1997)
astA-R	GGTCGCGAGTGACGGCTTTGT		
cdt-F	TAAATGGAATATAAATGTCCG	590	Hinenoya et al. (2014)
cdt-R	CGTTTCCTGCTACTGCATAATC		
iha-F	CAGTTCAGTTTCGCATTCACC	1305	Schmidt et al. (2001)
iha-R	GTATGGCTCTGATGCGATG		
efa1-F	AAGGTGTTACAGAGATTA	266	Nicholls et al. (2000)
efa1-R	TGAGGCGGCAGGATAGTT		
lpf O113-F	ATGAAGCGTAATATTATAG	573	Toma et al. (2006)
lpf O113-R	TTATTTCTTATATTCGAC		
terD-F	AGTAAAGCAGCTCCGTCAAT	434	Bielaszewska et al. (2005)
terD-R	CCGAACAGCATGGCAGTCT		
Phylogenetic groups			
chuA.1b	ATGGTACCGGACGAACCAAC	288	Clermont et al. (2013)
chuA.2	TGCCGCCAGTACCAAAGACA		
yjaA.1b	CAAACGTGAAGTGTCAGGAG	211	
yjaA.2b	AATGCGTTCCTCAACCTGTG		
TspE4C2.1b	CACTATTCGTAAGGTCATCC	152	
TspE4C2.2b	AGTTTATCGCTGCGGGTCGC		
AceK.f	AACGCTATTCGCCAGCTTGC	400	
ArpA1.r	TCTCCCCATACCGTACGCTA		
ArpAgpE.f	GATTCCATCTTGTCAAAATATGCC	301	
ArpAgpE.r	GAAAAGAAAAAGAATTCCCAAGAG		
trpAgpC.1	AGTTTTATGCCCAGTGCGAG	219	
trpAgpC.2	TCTGCGCCGGTCACGCCC		
Serogroups			
O26-F	CAATGGGCGGAAATTTTAGA	155	DebRoy et al. (2011)
O26-R	ATAATTTTCTCTGCCGTCGC		
O45-F	TGCAGTAACCTGCACGGGCG	138	
O45-R	AGCAGGCACAACAGCCACTACT		
O103-F	TTGGAGCGTTAACTGGACCT	321	
O103-R	GCTCCCGAGCACGTATAAAG		
O111-F	TGTTTCTTCGATGTTGCGAG	438	
O111-R	GCAAGGGACATAAGAAGCCA		
O113-F	TGCCATAATTCAGAGGGTGAC	514	
O113-R	AACAAAGCTAATTGTGGCCG		
O121-F	TCCAACAATTGGTCGTGAAA	628	
O121-R	AGAAAGTGTGAAATGCCCGT		
O145-F	TTCATTGTTTTGCTTGCTCG	750	
O145-R	GGCAAGCTTTGGAAATGAAA		
O157-F	TCGAGGTACCTGAATCTTTCCTTCTGT	894	Askari Badouei et al. (2022)
O157-R	ACCAGTCTTGGTGCTGCTCTGACA		
O5-F	CTTATCCGATTAATGGCTTC	144	Sánchez et al. (2015)
O5-R	TAGTCGATTTGCTTTTATGG		
O91-F	TTGCATCTGGCGCAATAAACACGG	616	
O91-R	ACACCATCCCAAATACCTGCTTGC		
O104-F	CGGTGTATTAAGAAGTGTTGTC	272	
O104-R	ATACTCCCCATAGAAACGC		
O128-F	TTTCGATCGTCTTGTTCAGG	193	
O128-R	CAATGGGCAATTAACACAGAG		

Table I. Primers used for identification of Shiga toxin genotypes, chromosomal/plasmid subtilase variants, virulence genes, phylogenetic groups, and serogroups in this study.

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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 15. 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 16 17 15.

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 18.

Molecular serogrouping

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 19 20. 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 21 22 20. All of the primers used in this study are presented in Table I.

Results

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 II). 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 III, 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*.

Shiga toxin genes and virulence determinants

The isolatesfrom 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,* butmost 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 23. 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 IV).

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 IV). 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 IV).

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No. of Isolates	Virulence genes				No. of isolates (No. of *subAB*+)			
	stx1	*stx2*	*eae*	*ehly*	Sheep (n=15)	Goats (n=25)	Cattle (n=50)	Other species (n=11)
6	+					5 (3)	1	
7		+					7	
1	+	+					1	
23	+	+		+	3 (3)	9 (9)	8	3[b] (3)
17		+		+		1	15 (12)	1[c] (1)
26	+			+	11 (11)	11 (11)	1	3[d] (3)
13	+		+	+			13	
6		+	+	+			2	4[e]
1	+	+	+	+			1	
1	+		+				1	
28		+[a]	+					
Total Positive (%)					14 (93.3)	24 (92.0)	12 (24.0)	7 (63.6)

[a] *stx2f*+

[b] Persian fallow deer (n=2), Caspian pony (n=1)

[c] Persian fallow deer

[d] Diarrheic children

[e] *Macaca mulatta*

Table II. The E. coli isolates from different sources and distribution of major virulence genes with regard to subtilase possession.

Genetic traits		Hosts				Total
		Sheep	Goat	Cattle	Others[a]	
no. of *subAB*+ isolates/total		14/57	24/57	12/57	7/57; 3D, 3H, 1P	57
(%; 95%CI)		(24.5%; 14.1%-37.7%)	(42.1%; 29.1%-59.9%)	(21.1%; 11.3%-33.8%)	(12.2%; 5.1%-23.6%)	(100%; 93.7%-100%)
Subtilase profiles[b]; no. of positive isolates/total of a specific host (%; 95%CI)						
	subAB1	-	-	12/12 (100%; 73.5%-100%)	-	12/57 (21%; 11.3%-33.8%)
	subAB2-1	-	1/24 (4.1%; 0.1%-21.1%)	-	2/7; 1D, 1P (28.5%; 3.6%-70.9%)	3/57 (5.2%; 1.1%-14.6%)
	subAB2-2	1/14 (7.1%; 0.1%-33.8%)	-	-	-	1/57 (1.7%; 0.04%-9.3%)
	subAB2-1/2-2	13/14 (92.8%; 66.1%-99.8%)	23/24 (95.8%; 78.8%-99.8%)	-	5/7; 3H, 2D (71.4%; 29.04%-96.3%)	41/57 (71.9%; 58.4%-83%)
Shiga toxin profiles; no. of positive isolates/total of a specific host (%; 95%CI)						
	stx1	11/24 (78.5%; 49.2%-95.3%)	14/24 (58.3%; 36.6%-77.8%)	-	3/7; 3H (42.8%; 9.9%-81.5%)	28/57 (49.1%; 35.6%-62.7%)
	stx1/stx2	3/24 (21.4%; 4.6%-50.8%)	9/24 (37.5%; 18.8%-59.4%)	-	3/7; 2D, 1P (42.8%; 9.9%-81.5%)	15/57 (26.3%; 15.5%-39.6%)
	stx2	-	1/24 (4.1%; 0.1%-21.1%)	12/12 (100%; 73.5%-100%)	1/7; 1D (14.2%; 0.3%-57.8%)	14/57 (24.5%; 14.1%-37.7%)
Sero-group; no. of positive isolates/total of a specific host (%; 95%CI)						
	O5	7/14 (50%; 23%-76.9%)	-	-	-	7/57 (12.2%; 5%-23.6%)
	O113	-	2/24 (8.3%; 1%-27%)	10/12 (83.3%; 51.5%-97.9%)	3/7; 2D, 1H (42.8%; 9.9%-81.5%)	15/57 (26.3%; 15.5%-39.6%)
	O128	-	7/24 (8.3%; 1%-27%)	-	-	2/57 (3.5%; 0.4%-12.1%)
	Unknown	7/14 (50%; 23%-76.9%)	20/24 (83.3%; 62.6%-95.2%)	2/12 (16.6%; 2.1%-48.4%)	4/7; 3H, 1D (57.1%; 18.4%-90.1%)	33/57 (57.8%; 44%-70.8%)
Phylo- group; no. of positive isolates/total of a specific host (%; 95%CI)						
	A	-	1/24 (4.1%; 0.1%-21.1%)	1/12 (8.3%; 0.2%-38.4%)	3/7; 2D, 1P (42.8%; 9.9%-81.5%)	5/57 (8.7%; 2.9%-19.3%)
	B1	14/14 (100%; 76.8%-100%)	23/24 (95.8%; 78.8%-99.8%)	10/12 (83.3%; 51.5%-97.9%)	4/7; 3H, 1D (57.1%; 18.4%-90.1%)	51/57 (89.4%; 78.4%-96%)
	E	-	-	1/12 (8.3%; 0.2%-38.4%)	-	1/57 (1.7%; 0.04%-9.3%)

[a]Other sources are indicated as letters, H (Humans), D (Deer), P (Pony) [b]Subtilase AB2-3 was negative in all isolates

Table III. Distribution of Subtilase variants, Shiga toxin gene(s), O-serogroups and phylogenetic groups among isolates from various sources.

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Virulence gene profile											Sero-group	*subAB*			Source	Phylo-group	Total
stx1	*stx2*	*ehly*	*tia*	*saa*	*espP*	*epeA*	*terD*	*astA*	*Lpf*$_{O113}$	*iha*		*1*	*02-gen*	*02-feb*			
+									+	+	O128		+	+	goat	B1	2[b]
+		+							+	+	ND[a]		+	+	sheep	B1	2
+		+							+	+	O5		+	+	sheep	B1	2
+		+	+						+	+	O5		+	+	sheep	B1	2
+		+	+						+	+	ND		+	+	sheep	B1	4
+		+	+						+	+	ND		+	+	goat	B1	6
+		+	+						+		ND		+	+	goat	B1	5
+		+	+						+		O5		+	+	sheep	B1	1
+		+	+	+					+	+	ND		+	+	human	B1	3
+			+						+	+	ND		+		goat	B1	1
+	+	+							+	+	O5		+	+	sheep	B1	2
+	+	+									ND			+	sheep	B1	1
+	+	+	+						+		ND		+	+	goat	B1	1
+	+	+	+						+	+	ND		+	+	goat	B1	7
+	+	+	+					+			O113		+	+	goat	A	1
+	+	+	+	+			+	+	+	+	O113		+	+	deer	A	1
+	+	+	+	+			+	+	+	+	O113		+		deer	A	1
+	+	+	+	+			+	+	+	+	O113		+		pony	A	1
	+	+	+						+	+	ND		+	+	deer	B1	1
	+	+						+			O113		+	+	goat	B1	1
	+	+		+	+				+		ND	+			cattle	A	1
	+	+		+	+					+	ND	+			cattle	E	1
	+	+		+	+	+			+	+	O113	+			cattle	B1	10

[a] Not-Defined

[b] One strain yielded a specific amplicon for *cdt* gene

Table IV. Virulence gene combinations, subtilase variants, serogroups and phylo-groups of Shiga toxin-producing E. coli strains isolated from different reservoirs.

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 24 25. 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 26 27. Subtilase not only acts as a potent toxin, but also occurs in different allelic variants in strains of different origins 28 11 9. Recent findings suggested that different *subAB* variants exhibit different binding capacity toward their target cells which may affect their cytotoxic behavior 26.

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 are 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 9. In Brazil, 21 out of 95 STEC collection strains (22%) were positive in *subA* PCR which mainly targets the *subAB1* 29. 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 11. In Spain, almost all caprine and ovine strains carried *subAB2-2,* but 61.4% and 64.3% carried *subAB2-1* respectively 30. In the present study, *subAB2-1/2-2* variants occurred together in most isolates of sheep and goat strains (Table III). 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 28. 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

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determinants, some of which seem to be almost restricted to this subset of STEC 25 24. 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 31. 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.

Conclusions

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.

Abbreviations

astA: EAEC heat-stable enterotoxin, *cdt*: cytolethal distending toxin, *eae*: *E. coli* attaching and effacing, *efa*: Enterohemorrhagic *Escherichia coli* factor for adherence, *ehly*: enterohemolysin, *epeA*: autotransporter protease, *espP*: Extracellular serine protease plasmid-encoded EspP, HC: hemorrhagic colitis, HUS: hemolytic uremic syndrome, *Iha*: bifunctional enterobactin receptor/adhesin protein, *katP*:catalase-peroxidase, LEE: locus of enterocyte effacement, *lpf O113*: long polar fimbria major subunit O113, OEP: outer membrane efflux protein locus, PCR: polymerase chain reaction, pO113: plasmid O113, *saa*: Shiga toxin-producing *Escherichia coli* autoagglutinating adhesion, SE-PAI: Subtilase-encoding pathogenicity island, STEC: Shiga toxin-producing *Escherichia coli*, *stx*: Shiga toxin, *subAB*: Subtilase, *terD*: tellurium resistance membrane protein TerD, *tia*: adhesion,*toxB*: putative cytotoxin B.

Ethics approval and consent to participate

All methods were carried out in accordance with relevant guidelines and regulations presented by Iran National Committee for Ethics in Biomedical Research.

Competing Interest

The authors declare that they have no competing interests.

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