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Phylogenetic analysis and Shiga toxin production profiling of Shiga toxin-producing/enterohemorrhagic *Escherichia coli* clinical isolates

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ABSTRACT

Shiga toxin-producing *Escherichia coli* (STEC) can cause severe illnesses in humans such as hemorrhagic colitis and hemolytic-uremic syndrome. In this study, we carried out genotypic analysis of the Shiga toxin (*stx*) gene in 120 clinical isolates of STEC and enterohemorrhagic *E. coli* (EHEC) from patients in a southern district of Japan. We identified 88 stx_1^+ and 103 stx_2^+ strains. We further identified 12 stx_1^+ and stx_2^+ isolates expressing little or no Shiga toxin 1 (Stx_1) and/or 2 (Stx_2) by reversed passive latex agglutination (RPLA) and Vero cell toxicity assays. Among them, 1 strain could not produce Stx_1, 8 could not produce Stx_2, and 3 strains could produce neither. Two of the latter three strains were of the non-O157 serotype. Most of the Stx RPLA-negative strains belonged to the stx_1/stx_2 subtype (11/12, [91.7%]). Our quantitative reverse transcription PCR analysis indicated that the *stx* genes were not effectively transcribed in the RPLA-negative strains. This is the first report of the isolation of *stx*-positive strains showing Stx-negative phenotype from stx_1-bearing strains and non-O157 strains.

1. Introduction

Shiga toxin, which consists of 1 A subunit (33 kD) and 5 B, or binding, subunits (7.7 kD each), is a 70-kD exotoxin encoded by *Shigella dysenteriae* DNA while Shiga toxins 1 (Stx_1) and 2 (Stx_2) are encoded by bacteriophage DNA present in several *Escherichia coli* (*E. coli*) serotypes. Shiga toxin-producing *E. coli* (STEC) can cause severe illnesses in humans such as hemorrhagic colitis and hemolytic-uremic syndrome (HUS) [1]. Enterohemorrhagic *E. coli* (EHEC), a subset of STEC, carries the Shiga toxin (*stx*) gene and some other pathogenic factors such as the *eae* gene [2]. EHEC/STEC isolates belong to the O:H serotypes [3,4], and, among them, serotype O157:H7 is the most prevalent serotype associated with human infections in many countries and is also the most prominent serotype of the more than 30 serotypes associated with STEC/EHEC [5].

The ability to produce Stx_1 and/or Stx_2 is a critical determinant of whether a bacterium can cause the clinical syndrome associated with STEC. Stx_1 is relatively homogeneous and is encoded by genes designated stx_1, stx_{1c} and stx_{1d}. Stx_2, on the other hand, can be divided into several subtypes based on the heterogeneity of their amino acid sequences and immunological cross-reactivities [6]. The Stx_2 variants are encoded by genes designated stx_2, stx_{2c}, stx_{2d}, stx_{2e}, stx_{2f} and stx_{2g} [7,8]. *E. coli* serotypes that produce Stx_1 or Stx_2 can contaminate meat, milk, cheese, and other types of insufficiently cooked or pasteurized food while cattle serve as a major reservoir of *E. coli* O157:H7. Both the main toxin type and the toxin subtype play important roles in causing HUS [9–12]. For example, stx_{2d}-positive strains were shown to be less pathogenic for humans than other Stx_2-producing variants whereas stx_{2c}-positive EHEC strains can cause HUS [10,13]. Shiga toxin subtyping, thus, can be a useful tool for identifying virulent strains causing human infections and for predicting the potential health risks associated with particular *E. coli* serotypes [7,14].

Stx_1 and Stx_2 are carried in bacteriophages that are lysogenized in the *E. coli* chromosome [15]. Stx_1 and Stx_2 expression is under the regulatory control of the phage late genes and the toxins are produced during the phage lytic cycle [15]. Stx production is often measured by commercially available reverse passive latex agglutination (RPLA) assays, which are based on the reaction between Stx_1 or Stx_2 and anti-Stx_1 or anti-Stx_2 antibodies. The RPLA method is reliable in detecting Stx_1 and Stx_2 though Stx variants react with anti-Stx antibodies more weakly because of their structural differences [16]. One of the most popular genotyping methods is DNA fingerprinting using pulsed field gel electrophoresis (PFGE) [17]. However, the method may fail to yield DNA fingerprints of sufficient resolution, which could render difficult interpretation of the results. To obtain high resolution DNA fingerprints, Persson et al.

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carried out partial sequencing of *stx*$_2$ including the variable C-terminus of A subunit and most of B subunits [18].

Koitabashi et al. isolated *stx*$_2^+$ *E. coli* O157 strains from beef in Thailand, Malaysia, China and Japan, and from clinical specimens in Japan [19,20]. These strains, however, produced little or no Stx$_2$ as measured by the RPLA method, which probably resulted from low levels of transcription from the *stx*$_2$ gene due to the unique *Q* gene. Most of the *stx*$_2^+$ *E. coli* strains reported by Koitabashi et al. were from environmental sources with only 2 from clinical specimens, which highlights the fact that there have been few reports of *E. coli* clinical isolates that carry the *stx* genes but produce no Stx. In addition, the phylogenetic background for these strains has hitherto remained undefined. We identified 12 *stx*$_1^+$ and *stx*$_2^+$ EHEC clinical isolates which did not produce Stx$_1$ and/or Stx$_2$ as measured by RPLA and Vero cell toxicity assays. Here, we sought to analyze the virulence *stx* genes by single-nucleotide polymorphism (SNP) typing based on sequence analysis of the entire Stx$_1$ and Stx$_2$ coding sequences of clinical EHEC and STEC strains isolated in Japan. We also defined their phylogenetic relationships, and further examined their Stx production profiles. Additionally, we measured *stx*$_1$ and *stx*$_2$ mRNA levels by quantitative reverse transcription polymerase chain reaction (RT-PCR) to examine why certain *stx*$_1^+$ and *stx*$_2^+$ EHEC strains failed to produce Stx$_1$ and/or Stx$_2$.

2. Results

2.1. Stx genotype

We genotyped 120 clinical EHEC/STEC isolates using specific primer pairs that bind to the 5′ and 3′ ends of *stx*$_1$ or *stx*$_2$ and found that serotype O157 was the most prevalent serotype (88/120, [73.3%]) while O26 and OUT were also frequently isolated from the patients (12/120, [10%] and 10/120, [8.3%], respectively) (Table 1). Among 88 O157 strains, 1 (1.1%), and 24 (27.3%) strains carried only *stx*$_1$ and *stx*$_2$, respectively. Additionally, 62 (70.5%) strains carried both *stx*$_1$ and *stx*$_2$. Of 32 non-O157 strains, 7 (21.2%) and 14 (42.4%) strains were positive for *stx*$_2$ and *stx*$_1$ alone, respectively. Furthermore, 12 (36.4%) strains were positive for both *stx*$_1$ and *stx*$_2$. We failed to amplify the *stx*$_2$ gene in 1 O157 strain by the sequencing primer sets and, therefore, the sequence information for this particular strain is still not available (Tables 1 and 2). We further sequenced 88 *stx*$_1^+$ strains and 103 *stx*$_2^+$ strains and subjected these sequences to multiple sequence alignment analyses. In addition, we constructed phylogenetic trees for both *stx*$_1$ and *stx*$_2$ by the neighbor-joining method (Figs. 1 and 2). The phylogenetic tree of *stx*$_1$ (Fig. 1) indicated that these isolates could be classified into 5 groups on the basis of their nucleotide sequence variations. The phylogenetic tree of *stx*$_2$ (Fig. 2) suggested that the isolates could be classified into 6 groups. We aligned the DNA sequences of *stx*$_1$ and *stx*$_2$ of the clinical isolates in the present study and compared them with the *stx*$_1$ and *stx*$_2$ variants that were reported in the literature [14,22]. The results showed that all *stx*$_1^+$ strains belonged to the *stx*$_1$ genotype. All but 2 *stx*$_2^+$ strains belonged to the *stx*$_2$ genotype, and the 2 non-*stx*$_2$ strains were of the genotype of *stx*$_{2c}$ and *stx*$_{2d}$, respectively (Figs. 1 and 2, Table 2). We also identified 6 new *stx*$_1$ variants (strains 127, 152, 108, 176, 120 and 115) and 11 new *stx*$_2$ variants (strains 109, 111, 92, 27, 176, 131, 130, 169, 125, 168 and 112) and their sequences were deposited in the GenBank under accession numbers from EU754724 to EU754740.

2.2. Genotype profiles and pathogenicity characteristics

As O157 is considered highly pathogenic for humans compared with other serotypes and given the important role of Stx in the pathogenesis of STEC/EHEC, we determined the production of Stx by O157 strains using RPLA. We also examined the production of 2 non-O157 strains (strains 130 and 176) whose *stx* genes could not be amplified by the primer sets stx$_1$F/stx$_1$R and stx$_2$F/stx$_2$R. The results showed that the majority of the strains produced Stx$_1$ with high titers (≥1:128, 62/66, [93.9%]) (Table 1). Our RPLA assays also

Table 1
Serotypes, Stx production, and genotypes of 120 EHEC/STEC clinical isolates.

Serotype	Pathotype	Number of isolates	*stx* genotype[n]	Stx$_1$ titer	Stx$_2$ titer
Unknown[a]	EHEC	2	*stx*$_1$/*stx*$_2$(2)	<1:2(2)	<1:2(2)
O26:H11	EHEC	1	*stx*$_1$(1)		
O111:H-	EHEC	1	*stx*$_1$/*stx*$_2$(1)		
O114:H19	STEC	1	*stx*$_2$(1)		
O8	EHEC	1	*stx*$_2$(1)		
O26	EHEC	7	*stx*$_1$/*stx*$_2$ (7)		
		4	*stx*$_1$(4)		
O91	EHEC	2	*stx*$_1$(2)		
O111	EHEC	1	*stx*$_1$/*stx*$_2$(1)		
		1	*stx*$_1$(1)		
O152	EHEC	1	*stx*$_1$(1)		
OUT	EHEC	3	*stx*$_2$(3)		
		1	*stx*$_{2c}$(1)		
		1	*stx*$_{2d}$(1)		
		5	*stx*$_1$(5)		
O157:H-	EHEC	2	*stx*$_1$/*stx*$_2$(2)	≥1:128(2)	1:8(1), ≥1:128(1)
		1	*stx*$_2$(1)		≥1:128(1)
O157:H7	EHEC	5	*stx*$_1$/*stx*$_2$(5)	≥1:128(5)	1:16(1), 1:32(1), ≥1:128(3)
		1	*stx*$_1$/*stx2*[b] (1)	≥1:128(1)	<1:2(1)
		1	*stx*$_1$(1)	≥1:128(1)	
		3	*stx*$_2$(3)		≥1:128(3)
O157	EHEC	55	*stx*$_1$/*stx*$_2$(55)	≥1:128(53)	<1:2(7), 1:4(4), 1:8(1), 1:16(2), 1:32(1), ≥1:128(38)
				<1:2(2)	<1:2(1), ≥1:128(1)
		20	*stx*$_2$(20)		1:8(1), 1:32(2), 1:64(2), ≥1:128(15)

n: The number of isolates.
[a] The serotype of the strain was not identified in the present study.
[b] The genotype of the strain has not been identified in the present study.