

Molecular epidemiology of sequence type 33 of Shiga toxin-producing *Escherichia coli* O91:H14 isolates from human patients and retail meats in Korea

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Sequence type (ST) 33 of Shiga toxin-producing *Escherichia coli* (STEC) strain O91:H14 has been proposed as a potential domestic clone of STEC in Korea because of its high prevalence among human patients with mild diarrhea or asymptomatic carriers. Herein, the clonal diversity of 17 STEC O91:H14 isolates of ST33 during 2003 to 2014 was analyzed by pulsed-field gel electrophoresis, including 14 isolates from human patients and 3 from retail meats. Their virulence characteristics, acid resistance, and antimicrobial susceptibility were also determined. Our results showed that all isolates were clustered mainly into three different pulsotypes and were likely low pathogenic without antimicrobial resistance.

Keywords: Shiga toxin; *Escherichia coli*; Multilocus sequence type; O91; Korea

Since its first outbreak in 1982, Shiga toxin-producing *Escherichia coli* (STEC) has caused many illnesses in humans, including hemorrhagic colitis (HC), hemolytic uremic syndrome (HUS), and thrombotic thrombocytopenic purpura [13]. Although *E. coli* strains of all known O serogroups appear to be associated with the production of Shiga toxins (Stxs), the single serovar O157:H7 (hereafter referred to as O157 STEC) is known to be responsible for many severe epidemic outbreaks in humans.

In addition to O157 STEC, epidemiological surveillance data suggest that four non-O157 STEC serovars, namely O26, O103, O111, and O145, are frequently associated with human outbreaks worldwide, and they have been designated the “big four” non-O157 STEC [3]. Using multilocus sequence typing, interestingly, a recent study demonstrated that the majority (~93.6%) of those big four non-O157 STEC isolates can be clustered into unique sequence type complexes (STCs) including STC10, STC20, STC29, and STC32, which implies that they are potentially evolved from some shared ancestors or common lineages of *E. coli* [5]. For example, approximately

86.9% of STEC O26 and O111 isolates were assigned to STC29 comprising 15 sequence types (STs) that included ST16, ST21, and ST29 [5]. Most STEC O103 isolates were assigned to STC20 comprising ST17 and ST386, while STEC O145 isolates were assigned to STC32 comprising ST32 and ST137 [11]. Therefore, it was proposed that highly virulent serovars of non-O157 STEC might share similar phylogenetic lineages.

In our attempt to define a pathogenic clone(s) of STEC in Korea, it was demonstrated that the STEC O91:H14 serovar belonged to the ST33 lineage and was highly prevalent among human patients and retail meat samples collected during 2003 to 2014 [8]. Although the isolates do not belong to the big four non-O157 STEC, the same serotypes have been reported among the most common STEC isolates from diarrheal patients without HUS in Germany [11]. Moreover, it has also been reported that ST33 of STEC O91:H14 is a major causative agent of diarrhea in human patients in Japan [10].

In this study, it was hypothesized that STEC O91:H14 isolates with ST33 are phylogenetically related and might be a

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potential domestic clone of STEC in Korea. To that end, a total of 17 STEC O91:H14 belonging to the ST33 lineage were collected from human patients (n = 14) or retail meat samples (n = 3) during 2003 to 2014 (Table 1) and analyzed using standard pulsed-field gel electrophoresis (PFGE). Their virulence potentials were also evaluated by measuring (i) the

ability of *E. coli* to produce one or more Stxs, (ii) the presence of previously characterized STEC virulence genes including Stx subtypes, (iii) glutamate-induced acid resistance, and (iv) antimicrobial susceptibility.

As shown in Fig. 1, PFGE profiles with > 80% similarity were analyzed as previously described [8]. The results showed

Table 1. Prevalence of virulence genes among the 17 STEC O91:H14 isolates belonging to ST33 and isolated in Korea during 2003 to 2014

STEC isolates (No. of isolates)	Source (sampling year)*	Presence of the following virulence genes					
		<i>subAB</i>	<i>eae</i>	<i>saa</i>	<i>iha</i>	<i>ehxA</i>	<i>toxB</i>
H2003 (1)	Diarrheal patient (2003)	+	-	+	+	+	-
H2004 (1)	Asymptomatic carrier (2004)	+	-	+	+	-	-
H2007 (1)	Diarrheal patient (2007)	+	-	+	+	+	-
B2007-75 (1)	Retail beef (2007)	+	-	+	+	+	-
B2007-76 (1)	Retail beef (2007)	+	-	+	+	+	-
P2010-29 (1)	Retail pork (2010)	-	-	-	-	+	-
H2012-1 to -4 (4)	Diarrheal patients (2012)	+	-	+	+	+	-
H2013-1 to -6 (6)	Diarrheal patients (2013)	+	-	+	+	+	-
H2014 (1)	Diarrheal patient (2014)	+	-	+	+	+	-
Prevalence (positive/total)		16/17	0/17	16/17	16/17	16/17	0/17

STEC, Shiga toxin-producing *Escherichia coli*; ST, sequence type; *subAB*, subtilase cytotoxin; *eae*, intimin; *saa*, STEC agglutinating adhesin; *iha*, iron-regulated gene A homolog adhesin; *ehxA*, enterohemorrhagic *E. coli* hemolysin; *toxB*, *E. coli* putative adhesin encoded in pO157. *Diarrhea represents watery diarrhea without hemorrhage.

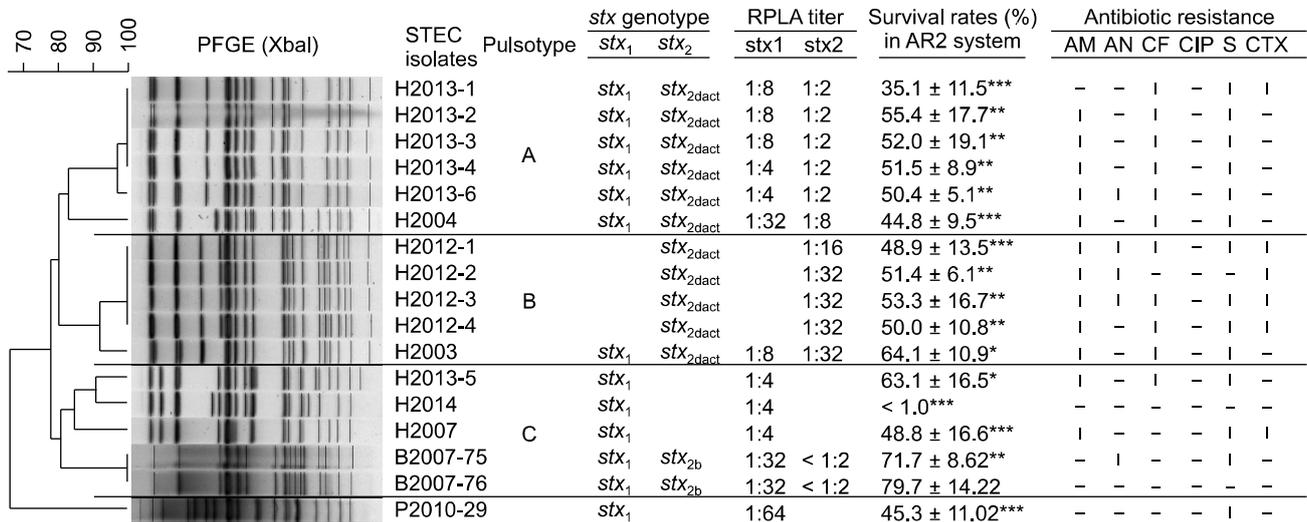


Fig. 1. Pulsed-field gel electrophoresis (PFGE) profiles and virulence characteristics of the 17 Shiga toxin-producing *Escherichia coli* (STEC) O91:H14 isolates with sequence type 33. The data, including multilocus sequence types, *stx* genotypes, reverse passive latex agglutination (RPLA) titers of Shiga toxins (Stxs), glutamate-induced acid resistance, and antibiotic resistance phenotypes, were combined and presented with the pulsotypes. The STEC isolates were divided into three pulsotypes (A to C) based on > 80% similarity of PFGE profiles. The RPLA titers of Stxs and the acid survival rates (AR2) were quantitated and compared with those of EDL933, which produced both Stx₁ and Stx₂ (1:16 and ≥ 1:128 RPLA titers, respectively) as well as surviving well in EG media (pH 2.5) with 1.5 mM glutamate. AM, ampicillin; AN, amikacin; CF, cephalothin; CIP, ciprofloxacin; S, streptomycin; CTX, cefotaxime; I, intermediate resistance. **p* < 0.05, significantly different from EDL933; ***p* < 0.01, significantly different from EDL933; ****p* < 0.001, significantly different from EDL933.

that 16 of the 17 STEC O91:H14 isolates were classified mainly into three pulsotypes: the first included six isolates from human patients, which possessed both *stx*₁ and *stx*_{2dact} genes (designated pulsotype A); the second contained five isolates from human patients, which harbored only the *stx*_{2dact} gene, except one patient (H2003) who had both *stx*₁ and *stx*_{2dact} genes (pulsotype B); and the third comprised three isolates from human patients that had only the *stx*₁ gene and two from retail beef samples harboring both *stx*₁ and *stx*_{2b} genes (pulsotype C). One isolate from pork (P2010-29) containing only the *stx*₁ gene was included as an outgroup with a distinct PFGE profile from the others (Fig. 1).

Heterogeneity of PFGE patterns and *stx* genotypes within the ST33 lineage of STEC O91:H14 was previously observed in Japan [10]. Indeed, the 26 ST33 of STEC O91 isolates from human patients in Japan were assigned to two different PFGE clusters [10]. The first cluster contained sixteen isolates, which had only the *stx*₁ gene whereas the second had ten isolates that possessed both *stx*₁ and *stx*₂ (or *stx*_{2dact}) genes [10]. In Netherlands, the nineteen ST33 of STEC O91:H14 isolates carried diverse *Stx* subtype genes (*stx*₁, *stx*₂, and *stx*₁ + *stx*_{2b}) [6]. Based on these results, we speculate that ST33 of STEC O91:H14 has evolved in different ways in different countries and their genetic divergence may include variation of *stx* genotypes.

In general, STEC strains with the *stx*₂ gene are considered to cause more severe diseases than those carrying *stx*₁. Especially, *Stx*_{2dact}, which was identified in our pulsotype A and B clusters, was reported to be as toxic as *stx*₂ in a murine infection model [9]. Therefore, the production of *Stx*s was quantitated by using a commercially available reverse passive latex agglutination (RPLA) kit according to the manufacturer's instruction (VTEC-RPLA; Denka Seiken, Japan). As shown in Fig. 1, however, the RPLA assays revealed that all six STEC isolates belonging to the pulsotype A produced notably lower levels of *Stx*₂ (1:2–1:8) than that from EDL933, an O157 STEC isolate from a human outbreak in the USA (\geq 1:128). Likewise, the five isolates belonging to pulsotype B expressed low levels of *Stx*₂ (1:16–1:32), whereas the two isolates belonging to pulsotype C produced undetectable levels of *Stx*_{2b} (< 1:2).

As determined via polymerase chain reaction analyses, all 17 ST33 of STEC O91:H14 isolates lacked the *eae* gene encoding intimin at the locus of enterocyte effacement pathogenicity island, which is required for full virulence of STEC (Table 1). Instead of missing the *eae* gene, they carried one or more alternative virulence genes encoding SubAB (subtilase cytotoxin AB), Saa (STEC autoagglutinating adhesion), Iha (IgrA homolog adhesion), and EhxA (enterohemolysin A) [4,10] (Table 1). Interestingly, a significant reduction in acid resistance phenotypes, compared to EDL933, was observed in most STEC isolates, except B2007-76 (Fig. 1); however, the reduction's biological significance *in vivo* was not evaluated. In

this study, the glutamate-inducible acid resistance of STEC isolates was evaluated using EG media containing 1.5 mM glutamate (pH 2.5) as previously described [8].

Taken together, these results indicate that our 17 ST33 of STEC O91:H14 isolates might have low pathogenicity and be ineffective in causing severe diseases such as HC and HUS in humans. Supporting this notion, the 14 human isolates were obtained from asymptomatic carriers and/or patients with mild watery diarrhea (Table 1). Moreover, a close phylogenetic relationship between the STEC O91 isolates with ST33 lineage and their low pathogenic potentials have been reported in other countries. In Europe, for example, STEC O91 has been the most common O serovar of the *eae*-negative STEC isolates [1,12,14,15]. Among them, O91:H14 and O91:H21 were the dominant serotypes in human patients, and they were divided into two distinct ST lineages; ST33 and ST442 [1,11]. Although most ST33 of STEC O91:H14 isolates seem to be associated with mild diseases [2,6,10,11], ST442 of STEC O91:H21 isolates have been epidemiologically linked to more severe diseases such as HC and HUS [2,7,11].

It is noteworthy that our STEC O91:H14 isolates appear to have low resistance to the antimicrobials tested in this study. Using a standard disk diffusion method with interpretive criteria of the inhibitory zone diameters (mm) according to Clinical and Laboratory Standard Institute standards, we observed that all strains were susceptible or intermediate resistant to one or more antimicrobials, including ampicillin (10 µg; BD, USA), amikacin (30 µg; BD), cephalothin (30 µg; BD), ciprofloxacin (5 µg; BD), streptomycin (10 µg; BD), and cefotaxime (30 µg; BD) (Fig. 1).

In conclusion, we characterized the ST33 of STEC O91:H14 isolates, a potential domestic clone of STEC in Korea. Our results imply that a low-pathogenic clone of STEC O91:H14 isolates with ST33 lineage is likely present and has divergently evolved in Korea.

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Conflict of Interest

The authors declare no conflicts of interest.

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