Phylogenetic Clades 6 and 8 of Enterohemorrhagic *Escherichia coli* O157:H7 With Particular *stx* Subtypes are More Frequently Found in Isolates From Hemolytic Uremic Syndrome Patients Than From Asymptomatic Carriers

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Background. Enterohemorrhagic *Escherichia coli* (EHEC) O157:H7 infection causes severe diseases such as bloody diarrhea and hemolytic uremic syndrome (HUS). Although EHEC O157:H7 strains have exhibited high genetic variability, their abilities to cause human diseases have not been fully examined.

Methods. Clade typing and *stx* subtyping of EHEC O157:H7 strains, which were isolated in Japan during 1999–2011 from 269 HUS patients and 387 asymptomatic carriers (ACs) and showed distinct pulsed-field gel electrophoresis patterns, were performed to determine relationships between specific lineages and clinical presentation.

Results. Clades 6 and 8 strains were more frequently found among the isolates from HUS cases than those from ACs ($P = .00062$ for clade 6, $P < .0001$ for clade 8). All clade 6 strains isolated from HUS patients harbored *stx2a* and/or *stx2c*, whereas all clade 8 strains harbored either *stx2a* or *stx2a/stx2c*. However, clade 7 strains were predominantly found among the AC isolates but less frequently found among the HUS isolates, suggesting a significant association between clade 7 and AC ($P < .0001$). Logistic regression analysis revealed that 0–9 year old age is a significant predictor of the association between clade 8 and HUS. We also found an intact *norV* gene, which encodes for a nitric oxide reductase that inhibits Shiga toxin activity under anaerobic condition, in all clades 1–3 isolates but not in clades 4–8 isolates.

Conclusions. Early detection of EHEC O157:H7 strains that belonged to clades 6/8 and harbored specific *stx* subtypes may be important for defining the risk of disease progression in EHEC-infected 0- to 9-year-old children.

Keywords. clade; EHEC; HUS; O157; *stx*.

Over the last 3 decades, enterohemorrhagic *Escherichia coli* (EHEC), one of the major categories of diarrheagenic *E coli*, has been frequently reported to cause severe diseases such as hemorrhagic colitis, hemolytic uremic syndrome (HUS), and encephalopathy [1, 2]. The serogroup O157 (serotype O157:H7/H−) was identified as the major EHEC responsible for sporadic cases and outbreaks in several countries [3–7]. In Japan, more than 3000 annual cases of EHEC infections (including asymptomatic carriers [ACs]) were reported during 2004–2012 [8]. Among the isolates collected during 2008–2012, serogroup O157 predominated (62.2%) followed by O26 (22.5%), O111 (3.25%), and O103 (3%) [8–12]. During this time period, serogroup O157 was found in more than 85% of total HUS cases [8–12].

Shiga toxin (Stx) is the most important and critical virulence factor of EHEC that is known to cause these severe diseases. There are 2 antigenically distinct types of Stx, Stx1 and Stx2. Several studies have suggested that, compared with strains harboring *stx1* gene, strains with *stx2* are more often associated with HUS [13–15]. These 2 genes have been further divided into several subtypes: subtypes *stx1a*, *stx1c*, and *stx1d* for the *stx1* gene, and subtypes *stx2a–g* for the *stx2* gene [16]. The subtypes *stx2a* and/or *stx2c* were more often found to be associated with HUS than the other *stx* types [17, 18].

Genomic diversity of serotype O157:H7 has been extensively studied by using several molecular subtyping methods including pulsed-field gel electrophoresis (PFGE) [19–21], multilocus variable tandem repeat analysis (MLVA) [22–24], octamer-based genome sequencing [25], lineage-specific polymorphism assay-6 (LSPA-6) [26], and single-nucleotide polymorphism (SNP) typing [27]. Although PFGE and MLVA have revealed considerable genetic diversity of O157:H7 strains and have been used for epidemiological analysis, they are not applicable to phylogenetic or population genetic analyses. Octamer-based genome sequencing analysis initially identified 2 distinct lineages of EHEC O157:H7 strains, designated as lineage I and lineage II [25]. Lineage-specific polymorphism assay analysis, which can subtype strains by the amplicon sizes of 6 polymerase chain reaction (PCR) products, showed that most of the strains belonging to lineage I were LSPA type 111111, whereas the strains belonging to lineage II were consisted of LSPA types 222222, 222211, 222212, and 222221 [26]. A microarray-based comparative genome hybridization analysis further identified another lineage, termed lineage I/II, which contains LSPA type 211111 [28]. An SNP-based subtyping method was also used to classify O157:H7 strains into 9 genetic clades (clades 1–9) [27]. The lineage I described above corresponded to clades 1, 2, 3, and 4 [29, 30]; lineage I/II corresponded to clades 6, 7 [30], and 8 [29–31]; and lineage II (LSPA type 222222 and 212111) corresponded to clade 7 and 9 [30].

A detailed epidemiological analysis demonstrated that patients with HUS were more likely to be infected with strains belonging to clade 8 than with strains belonging to other clades [27]. However, only 11 isolates from HUS patients were available for characterization in this study, and thus the association between clade membership and HUS requires confirmation, particularly for strains isolated from different geographic locations. Follow-up studies demonstrated that some of the clade 8 isolates showed elevated expression levels of *stx2* and locus of enterocyte effacement genes (LEE, which is responsible for the intimate adhesion of EHEC to the epithelial cells), as well as increased adhesion of these isolates to cultured epithelial cells relative to strains of clades 1–3. However, not all clade 8 isolates screened exhibited the same phenotype, suggesting that genetic variability exists within these clade 8 strains [32–34].

In the present study, we have determined the clade distribution and analyzed the *stx* subtypes of 656 EHEC O157:H7 isolates, which were collected in Japan during the years 1999–2011 for evaluating their public health significance.

METHODS

Escherichia coli O157:H7 Isolates Used in This Study

We collected more than 320 O157:H7 isolates from HUS patients during 1999–2011 in cooperation with local public health laboratories in Japan. Among these isolates, we chose a total of 296 isolates, consisting of all isolates from sporadic cases and 1 representative isolate from each outbreak. We also chose a total of 392 O157:H7 isolates from ACs including food handlers or workers in daycare centers, who were required by law to undergo periodic stool examination. Table 1 lists the ages of all HUS patients and ACs included in this study.

Table 1. Age-Wise Distribution in HUS Patients and AC

Age (years)	Strains Derived From:	
	HUS	AC
0–9	192	29 (137*)
10–19	20	24
20–64	27	312
>65	30	22
Total	269	387 (495*)

Abbreviations: AC, asymptomatic carrier; HUS, hemolytic uremic syndrome.
* Total number of test strains after addition of 108 strains (see text).

Because the above-described set of strains contained only 29 isolates from ACs 0 to 9 years old, we included another 108 O157:H7 strains, each one of which was isolated from a 0- to 9-year-old AC and exhibited a distinct PFGE pattern, for our analysis.

Pulsed-Field Gel Electrophoresis

Pulsed-field gel electrophoresis analysis was carried out as described previously [21]. Pulsed-field gel electrophoresis patterns were analyzed by BioNumerics software, version 6.6 (Applied Maths, Belgium).

Mismatch Amplification Mutation Assay-Polymerase Chain Reaction for Detecting Clade 8 Strains

Based on the available information that the clade 8-specific SNP found in the *ECs2357* gene (open reading frame number of *E coli* O157:H7 Sakai strain [accession no. BA000007.2]) was C539A [38], we developed a PCR assay, called the mismatch amplification mutation assay (MAMA)-PCR here, to specifically detect clade 8 isolates. For the PCR amplification, a common primer, 2357-F3 (5′-GAAGTGTGCGATCTGTCAGAA-3′), and 2 specific primers, 539A-R (5′-<u>AAGAGC</u>GTTTTCCAG TGG<u>CTCTT</u>-3′) and 539C-R (5′-<u>CAGAGC</u>GTTTTCCAGTG G<u>CTCTG</u>-3′), were designed to detect clade 8 and other clades, respectively. The 3′-end nucleotides of 539A-R and 539C-R primers (T and G, respectively) were complementary to A (clade 8) and C (other clades), respectively, at position 539, but a mismatched nucleotide was introduced at the penultimate base at the 3′ ends of both primers (designed T for C) to enhance the specificity. The underlined nucleotides in 539A-R and 539C-R primers, as shown above, indicate the hairpin regions, which could also increase the specificity. The condition to amplify the 222 base pair (bp)-long PCR product was as follows: 95°C for 2 minutes, followed by 25 cycles of 95°C for 30 seconds, 65°C for 30 seconds, and 72°C for 30 seconds, with a final extension at 72°C for 5 minutes. ExTaq polymerase (Takara, Japan) was used for the PCR amplification, and PCR was performed using a thermal cycler (T1 Thermocycler, Biometra, Germany).

stx Subtyping and Detection of Internal Deletion in *norV*

Subtyping of *stx1* and *stx2* was performed as described previously [16]. To detect an internal deletion of 204 bp-long DNA in the *norV* gene, PCR amplification was performed using the following 2 primers: NORV-F1 (5′-ATGTCGAA TAACACCCGCAT-3′) and NORV-R1 (5′-GCCTTTTGCC GGATCGTAAA-3′). The condition used for the PCR amplification was as follows: 94°C for 2 minutes, followed by 25 cycles of 94°C for 30 seconds, 55°C for 30 seconds, and 72°C for 30 seconds, with a final extension at 72°C for 1 minute. Using this condition, we expected to obtain either a 540 bp DNA fragment (intact *norV*) or a 336 bp DNA fragment (*norV* containing an internal deletion of 204 bp).

Clade Typing

Clades 1–3 and 8 strains were subtyped by DNA sequencing based on their SNP profiles as described previously [27]. The remaining clades were identified on the basis of SNPs or combinations of SNPs specific for individual clade [27]. Minimal requirements for determining clades 4 or 5, 6, 7, and 9 were as follows: clade 9 was determined by using the SNP T281C of ECs0654; clade 7 was determined by using the SNP A247G of ECs0517 for strains not belonging either to clade 8 or to clade 9; clade 6 was determined by using the SNP A348C of ECs3942 for strains not belonging to clade 3; and clades 4 or 5 were determined by using the SNP G776A of ECs4380 for strains not belonging to clades 6–9.

Statistical Analysis

Statistical analysis was performed using χ^2 and Fisher's exact tests (EpiInfo version 7.1.1.14 or on-line calculator available at the online Website for statistical computation [http://vassarstats.net/odds2x2.html]). Logistic regression analysis was performed on the online calculator at http://statpages.org/logistic.html. Two-tailed *P* values < .01 were considered significant.

RESULTS

Pulsed-Field Gel Electrophoresis Analysis to Determine Genetic Relatedness of Tested Strains

We examined the phylogenetic relationship of EHEC O157:H7 isolates obtained from patients with HUS and ACs to determine whether specific lineages could be correlated with the strain source. For this purpose, we examined epidemiologically unlinked EHEC O157:H7 isolates, collected in Japan during 1999–2011, from 296 HUS patients and 392 ACs. We initially performed a cluster analysis of all strains using PFGE to determine how many of them shared the same PFGE patterns. Among all the isolates, 253 strains from HUS patients and 386 strains from ACs showed distinct PFGE patterns (results not shown). There were also multiple strains sharing the same PFGE pattern; thus, 46 strains from HUS patients showed 16 distinct PFGE patterns and 6 strains from ACs showed 1 PFGE pattern (results not shown). Therefore, from each group of strains with identical PFGE pattern, only 1 representative strain was chosen for clade typing.

Clade Typing of Isolates With Distinct Pulsed-Field Gel Electrophoresis Patterns

A total of 656 isolates (269 isolates from HUS patients and 387 isolates from ACs), all having distinct PFGE patterns as described above, were used for the clade typing (clades 1–9). Results shown in Table 2 suggest that clade 3 (*n* = 84, 31.2%) strains predominated among the HUS isolates, which was followed by clade 2 (*n* = 75, 27.9%) and clade 8 (*n* = 50, 18.6%)

Table 2. Distribution of Clade Type of O157:H7 Strains Isolated From HUS Patients and ACs

	Strains Derived From:		OR (95% CI), P Value for HUS
Clade	HUS (n = 269)	AC (n = 387)	
1	9	5	2.64 (0.88–7.98), .13
2	75	75	1.61 (1.11–2.32), .014
3	84	66	2.21 (1.53–3.2), <.0001
4/5	4	2	2.91 (0.53–16), .23*
6	24	10	3.69 (1.74–7.86), .00062
7	23	216	.074 (0.046–0.12), <.0001
8	50	13	6.57 (3.49–12.4), <.0001

Abbreviations: AC, asymptomatic carrier; CI, confidence interval; HUS, hemolytic uremic syndrome; OR, odds ratio.
* Fisher's exact test value (2-tailed).

Table 3. Association of *stx* Genotypes With Clinical Outcome

	Strains From:		OR (95% CI) P Value for HUS
stx Genotype	HUS (n = 269)	AC (n = 387)	
stx1a	1	3	.48 (0.049–4.62) .65*
stx2a	53	53	1.55 (1.02–2.35) .04
stx2c	10	131	.075 (0.04–0.15) <.0001
stx1a/stx2a	152	139	2.32 (1.69–3.19) <.0001
stx1a/stx2c	2	30	.06 (0.02–0.27) <.0001*
stx1a/stx2a/ stx2c	1	9	.16 (0.02–1.24) .05*
stx2a/stx2c	50	22	3.79 (2.23–6.43) <.0001

Abbreviations: AC, asymptomatic carrier; CI, confidence interval; HUS, hemolytic uremic syndrome; OR, odds ratio.
* Fisher's exact test value (2-tailed).

strains. In contrast, clade 7 (n = 216, 55.8%) strains predominated among the AC isolates, which was followed by clade 2 (n = 75, 19.4%) and clade 3 (n = 66, 17.1%) strains. We did not detect clade 9 strains in either group. The number of clade 8 strains found among the AC isolates was 13 (3.4%). Therefore, the frequency of clade 8 strains found among the HUS isolates was more than 5 times higher than those found among the AC isolates. This result suggests a statistically significant association between the clade 8 strains and HUS, and that this association was better than that with other clades (clades 1–7) combined (odds ratio [OR], 6.57; confidence interval [CI], 3.49–12.4; P < .0001). In addition to clade 8, statistically significant association was also observed between clade 3 (OR, 2.21; CI, 1.53–3.2; P < .0001) and clade 6 (OR, 3.69; CI, 1.74–7.86; P = .00062) strains and HUS (Table 2). Although the clade 8 strains were more likely to be isolated from HUS patients than the clade 3 (OR, 3.02; CI, 1.52–6.03; P = .0022) strains, no significant difference was observed between the clades 6 and 8 (OR, 1.6; CI, 0.62–4.17; P = .47) strains. Therefore, clades 6 and 8 strains were not very different with respect to their association with HUS. In contrast to these results, clade 7 strains were predominantly found among the AC isolates but were less frequently found among the HUS isolates (n = 23, 8.6%). In fact, the frequency of finding clade 7 strains among the AC isolates was 6 times higher than those among the HUS isolates. Thus, this result suggests a statistically significant association between the clade 7 strains and AC, and that this association was better than that with other clades (clades 1–6, 8) combined (OR, 0.074; CI, 0.046–0.12; P < .0001).

stx Subtypes and Their Association With Clade and Hemolytic Uremic Syndrome Cases

We next analyzed the *stx* subtypes of strains used for the clade analysis. As shown in Table 3, *stx1a/stx2a* was the most frequent *stx* subtype found among the isolates from both HUS patients

(152, 56.5%) and ACs (139, 35.9%). The *stx* subtypes *stx2c* and *stx1a/stx2c* were found more frequently among the AC isolates than among the HUS isolates (Table 3). This result is mainly due to the high prevalence of clade 7 among these *stx* subtypes (Table 4). The frequencies of finding *stx* subtypes *stx1a/stx2a* and *stx2a/stx2c* among the HUS isolates were higher than those from the AC isolates (Table 3). Because most of the strains harboring *stx1a/stx2a* belonged to clade 2 or clade 3 (275 of 291; 94.5%), higher association of these *stx* subtypes with HUS, rather than that with AC, was due to the high prevalence of their respective clades. The frequency of finding the *stx2a/stx2c* subtype was higher among the HUS isolates than among the AC isolates because most isolates harboring this *stx* subtype belonged to either clade 6 or to clade 8 (57 of 72; 79.2%). In contrast, the frequency of finding the *stx2a* subtype among the HUS and AC isolates were not significantly different (Table 3). However, 23 of 30 (76.7%) clade 8 strains were isolated from HUS patients, suggesting a statistically significant association

Table 4. *stx* Genotype of Strains Belonging to Each Clade Group

	stx Genotype of Isolates From HUS/AC						
Clade	*stx1a*	*stx2a*	*stx2c*	*stx1a/ stx2a*	*stx1a/ stx2c*	*stx1a/ stx2a/ stx2c*	*stx2a/ stx2c*
1	0/0	1/0	0/0	8/5	0/0	0/0	0/0
2	1/1	11/3	0/0	62/69	0/0	1/2	0/0
3	0/2	2/2	0/0	82/62	0/0	0/0	0/0
4/5	0/0	3/0	0/2	0/0	0/0	0/0	1/0
6	0/0	2/1	3/1	0/0	0/3	0/0	19/5
7	0/0	11/40	7/128	0/3	2/27	0/7	3/11
8	0/0	23/7	0/0	0/0	0/0	0/0	27/6
Total	1/3	53/53	10/131	152/139	2/30	1/9	50/22

Abbreviations: AC, asymptomatic carrier; HUS, hemolytic uremic syndrome.
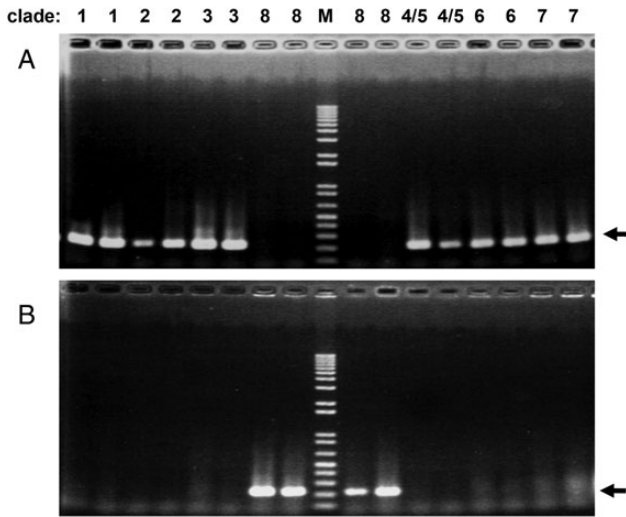
Fig. 1. Development of a clade 8-specific mismatch amplification mutation-polymerase chain reaction (PCR) assay. Lane M shows the DNA size markers (TrackIt 1 kb Plus DNA Ladder, Life Technologies) including DNA fragments sized 100, 200, 300, 400, 500, 650, 850, 1000 bp, and so on. The black arrows on the right side of both panels indicate positions of the PCR products. Two different strains from each clade were used. (A) Polymerase chain reaction performed using the 539C-R primer detected strains belonging to clades 1–7, but not strains belonging to clade 8. (B) Polymerase chain reaction performed using the 539A-R primer detected strains belonging to clade 8, but not strains belonging to clades 1–7.

between the clade 8 strains harboring only *stx2a* and HUS (OR, 4.37; CI, 1.14–16.69; *P* = .0012). Likewise, 40 of 51 (78.5%) clade 7 strains were isolated from ACs, suggesting a statistically significant association between the clade 7 strains harboring only *stx2a* and AC (OR, 11.75; CI, 4.72–29.3; *P* < .0001) (Table 4). Taken together, these results suggest that the clade typing rather than the *stx2a* genotype is a determinant for the association of strains with HUS or AC.

Development of Mismatch Amplification Mutation Assay-Polymerase Chain Reaction to Detect Further Clade 8 Strains

A previous study has developed a real-time PCR assay for detecting clade 8 strains [35]. In the present study, we have developed an alternative PCR-based assay, called MAMA-PCR, which allowed inexpensive detection of clade 8 strains without performing any DNA sequencing analysis. As shown in Figure 1, clade 8 was specifically detected by a specific primer set but not by the other primer set that can detect all other clades (clades 1–7). Using this assay, we were able to confirm all 63 clade 8 strains (listed in Table 2), which were subtyped by DNA sequencing (results not shown).

The 0- to 9-Year-Old Age Group Is a Significant Predictor for the Association Between Clade 8 and Hemolytic Uremic Syndrome

In Japan, incidence of EHEC infection was highest among the 0- to 4-year-old age group followed by those in the 5- to 9-year-

Table 5. Clade 8 Strains Isolated From Patients With HUS or ACs (Age-Wise Distribution)

Age (years)	Number of Clade 8/ Other Clades		OR (95% CI), *P* Value of Clade 8 for HUS
	HUS	AC	
0–9	42/150	6/131	6.11 (2.52–14.84), <.0001
>10	8/69	13/345	3.08 (1.23–7.7), .019*
Total	50/219	19/476	5.72 (3.29–9.93), <.0001

Abbreviations: AC, asymptomatic carrier; CI, confidence interval; HUS, hemolytic uremic syndrome; OR, odds ratio.

* Fisher's exact probability test value (2-tailed).

old age group [8]. As shown in Table 1, 192 of 269 (71.4%) strains isolated from HUS patients were from children of ages between 0 and 9 years; in contrast, only 29 isolates were from ACs, which did not contain any clade 8 strain. Therefore, for further analyzing the frequency of occurrence of clade 8 strains among the 0- to 9-year-old age group, we included another 108 test strains, which were obtained from 0- to 9-year-old children without any symptoms, and each one of these strains exhibited different PFGE patterns (data not shown). Mismatch amplification mutation assay-PCR analysis (Table 5) demonstrated that 6 of 108 strains belonged to clade 8, suggesting that the association between clade 8 and HUS for the 0- to 9-year-old age group was still statistically significant (OR, 6.11; CI, 2.52–14.84; *P* < .0001). On the other hand, this association was not significant when strains were isolated from HUS patients or ACs who were over 10 years old (OR, 3.08; CI, 1.23–7.7; *P* = .019 [Fisher's exact test value]). Logistic regression analysis indicated that the 0- to 9-year-old age group was a significant predictor for the association between clade 8 and HUS; thus, crude and adjusted OR values for this age group were 5.72 and 4.44, and difference between them was more than 10%. However, no association was observed between gender and HUS status (OR, 1.02; CI, 0.74–1.41; *P* = 1). Gender was not a predictor for the association between clade 8 and HUS; in this case, the crude OR was equal to that of the adjusted OR (both were 6.3).

Clade Specific Deletion of *norV*

The *norV* gene, which encodes an anaerobic nitric oxide (NO) reductase, has been shown to be a putative virulence determinant in certain O157:H7 strains because NO inhibited Stx2 expression under anaerobic condition [36, 37]. In the reference strains EDL933 (belonging to clade 3) and Sakai (belonging to clade 1), the NO reductase activity was abolished because of an internal 204-bp deletion in the *norV* gene; however, in the spinach outbreak-derived clade 8 strain TW14359, the *norV* gene was found to be intact [38].

We designed specific PCR primers (see Methods) to examine the distribution of this 204-bp deletion in *norV* in each clade

group. We found that 84 of 145 strains (57.9%) isolated from HUS patients and 177 of 223 strains (79.4%) isolated from AC harbored the intact *norV* gene. Therefore, the presence of *norV* gene by itself does not cause HUS. We found that all tested isolates belonging to clade 1 ($n = 12$), clade 2 ($n = 47$), and clade 3 ($n = 48$) had the same *norV* deletion as that found in the Sakai and EDL933 strains [39]. On the other hand, all tested isolates belonging to clade 4 or 5 ($n = 4$), clade 6 ($n = 33$), clade 7 ($n = 173$), and clade 8 ($n = 51$) had intact *norV* gene. Therefore, presence of an intact *norV* is rather nonrandomly distributed across O157:H7 lineages (clades 1–3 vs clades 4–8).

DISCUSSION

A previous study, for which only 11 isolates from HUS patients were available, suggested that HUS patients were 7 times more likely to be infected with a clade 8 strain than with a strain belonging to other clades (clades 1–7) [27]. In the present study, clade typing of 656 epidemiologically independent O157:H7 isolates, collected from 269 HUS patients and 387 ACs in Japan during the years 1999–2011, revealed that the degree of association of clade 8 strains with HUS was statistically more significant than that with AC. Using the same strain set, we also found significant association between the strains belonging to clade 6 and HUS. To the best of our knowledge, this is the first report that uses a large set of O157 strains and demonstrates a significant association not only between clade 8 strains and HUS cases but also between clade 6 strains and HUS cases. In addition to these results, we also found statistically significant association between clade 7 strains and AC, which confirmed the earlier observation that the clade 7 strains caused less severe disease [27, 39].

To assess the potential contributions of high virulent lineages of EHEC O157 to HUS, it is highly desirable to have suitable methods available for their early detection. Therefore, detection of clade 6 and clade 8 strains by using a rapid and specific method, such as reverse transcription (RT)-PCR, is not only important to have but is also useful for clinical laboratories. Availability of such methods would also help in understanding the environmental reservoirs and sources of such high virulent strains. In the present study, we have developed MAMA-PCR, an inexpensive PCR-based method for detecting clade 8 strains, as an alternative to the RT-PCR-based detection method that was developed previously [35].

Several previous studies showed that some of the clade 8 strains expressed elevated levels of several virulence-related genes and demonstrated enhanced ability to attach to epithelial cells compared with several other strains belonging to clades 1–3 [33–35]. However, further studies using a large set of strains, including the clade 6 strains, would be required to understand the molecular mechanism of lineage-specific variability in virulence of EHEC O157 strains.

Subtyping of *stx* gene in each clade revealed that all clade 6 strains isolated from HUS patients harbored *stx2a* and/or *stx2c*, whereas all clade 8 strains harbored either *stx2a* or *stx2a/stx2c*. In most clade 2 and clade 3 strains, the observed *stx* subtype was *stx1a/stx2a* (86.2% and 96%, respectively). Thus, there seems to be close association between these clades and *stx* subtypes of strains, which contribute to the observed high degree of association between the *stx* subtypes and HUS cases.

Clade 7 strains harbored several different *stx* subtypes, including *stx2a*, *stx2c*, *stx1a/stx2a*, *stx1a/stx2c*, *stx1a/stx2a/stx2c*, and *stx2a/stx2c*. Among these *stx* subtypes, the major *stx* subtypes found in clade 7 strains were *stx2c* and *stx1a/stx2c*. Thus, these subtypes showed higher degree of association with AC than with HUS.

Hemolytic uremic syndrome was more often found to be associated with the *stx* subtype *stx2a* than with any other *stx* subtypes [17, 18]. However, results shown in this study indicated that the frequency of finding the *stx2a* subtype among the HUS and AC isolates was comparable. On the other hand, our findings that the clade 7 strains harboring only the *stx* subtype *stx2a* showed higher association with ACs rather than with HUS cases ($P < .0001$) and the clade 6 or clade 8 strains harboring the *stx* subtype *stx2a* showed higher association with the HUS cases rather than with ACs ($P = .00079$), suggested that the clade typing is essential for determining the virulence potential of EHEC O157 strains harboring only the *stx* subtype *stx2a*.

In the present study, we also observed that the 0- to 9-year-old age group was a significant predictor for the association between clade 8 strains and HUS cases. In addition to the clade 8 strains, close association between the clade 6 strains and HUS was also observed for this age group. Thus, 12% (23 of 192) and 1.3% (1 of 77) of clade 6 isolates from HUS patients were actually obtained from the 0- to 9-year-old and >10-year-old age groups, respectively; in contrast, numbers of clade 6 strains isolated from ACs were comparable (4.4% vs 2.8%) for both age groups. Contrary to these observations, clade 7 strains isolated from patients with HUS were comparable in number (8.3% vs 9.1%) for both 0- to 9-year-old and >10-year-old age groups, whereas those isolated from ACs were 23.4% (32 of 137) and 58.1% (208 of 358) for the 0- to 9-year-old and >10-year-old age groups, respectively. Therefore, we found significant association between the clade 7 strains isolated from ACs and >10-year-old age group ($P < .0001$).

All of the clades 1–3 strains used in this study carried an internal 204 bp deletion in the *norV* gene, which encodes a putative virulence factor; however, all the tested strains belonging to clades 4–8 carried the intact *norV* gene. Consistent with these results, a previous study examining O157 strains isolated from geographically different places showed that all strains isolated in Argentina ($n = 57$) and Australia ($n = 58$) and harboring the intact *norV* gene belonged to clade 4, 6, 7, or 8 [40]. These results

suggest that the presence of intact *norV* does not have any association with HUS.

In conclusion, the present study using a large set of EHEC O157 strains isolated from HUS patients and ACs showed a significant association of clade 6 or 8 and clade 7 strains with HUS and ACs, respectively. All Clade 6 strains isolated from HUS patients harbored *stx2a* and/or *stx2c*, whereas clade 8 strains harbored either *stx2a* or *stx2a/stx2c* but not *stx2c*. Taken together, we believe that our observed results would prompt further investigations that would help us find the reservoirs and sources of these high virulent strains among geographically different samples and also help us develop molecular methods to rapidly and specifically identify clades with or without *stx* subtype.

Acknowledgments

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APPENDIX