Distribution of IS629 and stx genotypes among enterohemorrhagic Escherichia coli O157 isolates in Yamaguchi Prefecture, Japan, 2004–2013

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Enterohemorrhagic Escherichia coli (EHEC), which produces Shiga toxin (Stx), is a human pathogen that causes hemorrhagic colitis, encephalopathy and hemolytic uremic syndrome (HUS) [1, 8]. In Japan, 3,768 cases of EHEC infection were reported in 2012. Among the EHEC isolates collected in 2012, the predominant serogroup was O157 (53%), followed by O26 (27%) and O103 (5%) [9].

Insertion sequences (IS), which are small mobile genetic elements, are widely distributed in bacterial genomes. IS629, a member of the IS3 family of insertion sequences, is prevalent in the O157 genome; for example, the genome of O157 strain Sakai contains 98 IS elements, and of these, 23 were identified as IS629 [2, 10]. O157 isolates show diverse patterns of IS629 insertion, and therefore, IS629 variability can be used for fingerprinting O157 isolates. Ooka et al. [12] developed a multiplex PCR tool for screening the distribution of IS629 in the O157 genome.

Several genomic subtyping tools have been developed for the EHEC O157 serogroup. Kulasekara et al. [6] showed that two forms of the anaerobic nitric oxide (NO) reductase gene, norV, are present in O157 isolates: an intact form and a form with a 204-bp deletion; the intact norV is a putative determinant of virulence in some O157 isolates, because NO inhibits Stx2 expression under anaerobic conditions [15]. In addition, Manning et al. [7] showed that single nucleotide polymorphisms (SNPs) could be used to classify O157 strains into nine clades and that HUS patients were significantly more likely to be infected with clade 8 strains. Further, a lineage-specific polymorphism assay can be used to classify O157 isolates (lineages I, II and I/II), and lineage I isolates are more commonly associated with human disease than lineage II isolates [5, 13, 16, 18].

Some IS elements are thought to play an important role in the diversification and evolution of bacteria, including those of EHEC O157 isolates [11, 13]. Yokoyama et al. [17] and Hirai et al. [3] showed a biased distribution of IS629 among different lineages or clades of O157 isolates. However, the isolates examined in these studies were collected from a limited number of geographical areas, and the distribution of IS629 among O157 isolates from Yamaguchi Prefecture, Japan, has not been investigated. In the present study, we investigated the distribution of IS629 as well as norV types in EHEC O157 isolates collected in Yamaguchi Prefecture between 2004 and 2013. We also examined the association between stx genotype and O157 phylogeny.

**MATERIALS AND METHODS**

**EHEC O157 isolates**: A total of 203 EHEC O157 isolates were used in the present study. The isolates were sent to our laboratory from hospitals and health care centers in Yamaguchi Prefecture, Japan, between 2004 and 2013, and originated from epidemiologically unrelated patients (189 isolates) and asymptomatic carriers (14 isolates). The clinical symptoms of the patients included watery diarrhea (n=165), abdominal pain (n=144), bloody diarrhea (n=115), fever (n=59) and vomiting (n=31). Five patients developed HUS. All isolates were negative for sorbitol fermentation, and 192 isolates were serotyped as O157:H7. The remaining isolates were non-motive and were therefore classified as O157:NM.
**DNA preparation:** To extract DNA for PCR analysis, individual isolates were cultured on Mueller-Hinton agar (Oxoid, Basingstoke, U.K.), and the DNA was extracted using a QIAamp DNA Blood mini kit (Qiagen, Hilden, Germany), according to the manufacturer’s instructions. Prepared DNA was stored at −20°C until use.

**IS629 typing:** Multiplex PCR using an O157 IS-printing system (Toyobo, Osaka, Japan), which can be used to detect all 32 IS629 loci, was used to type the isolates based on the number and location of the insertion sequences. The assay also screens for the presence of the four virulence genes, eae, hlyA, stx1 and stx2. The resulting banding patterns were analyzed using BioNumerics software v. 7.1 (Applied Maths, Sint-Martens-Latem, Belgium), which excluded bands corresponding to the 4 virulence genes. A simple matching coefficient and an unweighted pair group method with arithmetic mean (UPGMA) algorithm were used to generate a dendrogram. For the purposes of this study, isolates with >80% similarity were considered to belong to the same group.

**Detection of the intact and deletion variants of norV:** The 2 versions of norV, the intact version and the version containing a 204-bp deletion, were detected by PCR, as described previously [4].

**Detection of isolates belonging to clade 8:** We screened for the presence of clade 8 isolates using mismatch amplification mutation assay (MAMA) PCR, as described previously [4].

**stx genotyping:** The stx subtypes, stx1a, stx1c, stx1d and stx2a/stx2g, were detected by PCR, as described previously [14].

**RESULTS**

**IS629 distribution:** A total of 114 IS629 patterns were distinguished amongst the 203 O157 isolates. As shown in Fig. 1, the isolates could be divided into 8 groups (groups A–H). Group G was predominant (n=103), followed by group C (n=35). Isolates belonging to each of these 2 groups were identified in all years of the study (Table 1).

**Detection of norV types:** Intact norV was detected in 90 isolates (intact norV-type), and norV with the 204-bp deletion was detected in 113 isolates (deletion norV-type). All isolates belonging to groups A–F, and one isolate from group G, carried the intact norV (Fig. 1 and Table 1).

**Clade 8 isolates:** In total, 15 of the 203 O157 isolates were determined to belong to clade 8. All of these isolates contained an intact norV gene and were classified into IS group E.

**stx genotypes:** The stx1a, stx2a and stx2c subtypes were detected in 123, 154 and 68 isolates, respectively. The stx1a/stx2a genotype was predominant (n=105), followed by stx2c (n=32), stx2a (n=27), stx2a/stx2c (n=21), stx1a/stx2c (n=14) and stx1a (n=3). Only one isolate carried all three stx subtypes, stx1a, stx2a and stx2c (Table 2).

Among the seven stx genotypes detected, the stx2a genotype was common in both intact norV-type and deletion norV-type isolates. The stx2c, stx1a/stx2c, stx2a/stx2c and stx1a/stx2a/stx2c genotypes were common in the intact norV-type isolates, whereas the stx1a and stx1a/stx2a genotypes were associated with the deletion norV-type isolates.

Of the 15 clade 8 isolates, 7 showed the stx2a genotype, and 8 showed the stx2a/stx2c genotype.

**DISCUSSION**

Various IS629 patterns were observed among the O157 isolates collected in Yamaguchi Prefecture, Japan, between 2004 and 2013. The majority of the isolates collected over this 10-year period belonged to IS groups C and G, although isolates from 8 different groups were identified. As IS629 is thought to contribute to the diversity of O157 isolates, it is not surprising that isolates with distinct IS patterns are distributed in Yamaguchi Prefecture.

Recent studies demonstrated that the distribution of IS629 was biased among O157 clades or lineages [3, 17]. Iyoda et al. [4] also revealed that O157 clades were associated with certain norV types, with isolates belonging to clades 4–8 carrying an intact norV, whereas isolates belonging to clades 1–3 contained the deletion type. Our results showed that the 15 clade 8 isolates shared identical or highly similar IS patterns and belonged to a single IS group (group E). Moreover, with the exception of one isolate, the intact norV-type and deletion norV-type isolates were clearly divided into distinct IS groups (IS groups A–F and G–H, respectively). Therefore, our findings, along with those of previous studies [3, 4, 17], indicate that it is rare for isolates belonging to different clades to share similar IS profiles.

Kulasekara et al. [6] reported that most deletion norV-type isolates contained stx1a, whereas only 10% of intact norV-type isolates harbored stx1a. In the present study, 95.6% of deletion norV-type isolates and 16.7% of intact norV-type isolates carried stx1a, in accord with the previous study. Moreover, all isolates carrying stx2c also carried an intact norV. A previous study demonstrated that 98.8% of all stx2c-positive isolates carried an intact norV and belonged to clades 4–8 [4]. Thus, stx genotypes appear to be associated with specific O157 clades.

An additional association between stx genotypes and IS distribution was observed in this study. Among the seven stx genotypes identified, the predominant genotype, stx1a/stx2a, was only identified in isolates in IS groups G and H, whereas the 4 genotypes including the stx2c subtype were identified in IS groups A–F. Thus, particular stx genotypes may be associated with O157 isolates with particular IS629 distributions.

Five of the 203 isolates tested in the present study were obtained from HUS patients. These 5 isolates did not belong to clade 8 and were genotyped as stx1a/stx2a (n=3, deletion norV-type) and stx2a/stx2c (n=2, intact norV-type). Similar results were obtained by Iyoda et al. [4], who found that isolates with these 2 genotypes were significantly more likely to be associated with HUS patients than with asymptomatic carriers. Thus, the stx1a/stx2a and stx2a/stx2c genotypes may be associated with an increased risk of developing HUS. However, Manning et al. [7] also investigated the association between O157 clades, stx subtypes and HUS and...
Fig. 1. Cluster analysis of 203 enterohemorrhagic Escherichia coli O157 isolates based on IS-printing profiles. Isolates with >80% similarity were considered to belong to the same group. The hatched bar indicates the intact norV, and the open bar indicates the 204-bp-deletion norV. A “☆” indicates a HUS patient, and “★” indicates an asymptomatic carrier.
found that stx type alone cannot account for the variation in hospitalization and HUS rates by clade.

In the present study, the diversity of IS629 patterns and stx genotypes in O157 isolates collected in Yamaguchi Prefecture, Japan, were determined. The clade 8 isolates, which are considered to show high virulence, shared a particular IS629 distribution and stx genotype. Because analysis of patterns of IS629 distribution and stx genotype in O157 isolates can help to determine whether or not an isolate has high virulence in humans, it is important to identify the characteristics of O157 isolates to alert clinical laboratories to the risk of developing severe diseases, such as HUS.

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Table 1. Distribution of O157 isolates according to IS groups by year

| IS group | norV(a) | Clade(b) | n | 2004 | 2005 | 2006 | 2007 | 2008 | 2009 | 2010 | 2011 | 2012 | 2013 |
|----------|---------|----------|---|------|------|------|------|------|------|------|------|------|------|
| A        | Intact  | -        | 18|  5  |  4  |  2  |  2  |  2  |  2   |  2   |  1   |  1   |      |
| B        | Intact  | -        |  1|  1   |      |      |      |      |      |      |      |      |      |
| C        | Intact  | -        |  35|  4  |  1  |  1  |  5  |  7  |  5   |  3   |  3   |  3   |      |
| D        | Intact  | -        |  1|  1   |      |      |      |      |      |      |      |      |      |
| E        | Intact  | -        |  3|  3   |      |      |      |      |      |      |      |      |      |
|          | Clade 8 |          | 15|  3  |  1  |  1  |  1  |  1  |  3   |  4   |  1   |      |      |
| F        | Intact  | -        |  16|  2  |  1  |  1  |  4  |  3  |  1   |  3   |  1   |      |      |
| G        | Intact  | -        |  1|  1   |      |      |      |      |      |      |      |      |      |
| H        | Deletion|          | 102| 14  | 15  |  8  |  9  | 13  |  8   |  9   |      |      |      |
|          | Total   |          | 145| 29  | 18  | 12  | 26  | 25  | 24   | 23   | 18   | 15   | 13   |

(a) Intact or 204-bp-deletion norV gene. b) “−” indicates non-clade 8 isolates.

Table 2. *stx* genotypes of intact or deletion norV-type isolates

| norV type(a) | *stx1a* | *stx2a* | *stx2c* | *stx1a*/*stx2a* | *stx1a*/*stx2c* | *stx2a*/*stx2c* | *stx2a*/*stx2c* |
|--------------|---------|---------|---------|-----------------|-----------------|-----------------|-----------------|
| Intact       | 0       | 21      | 32      | 0               | 14              | 1               | 21              |
| Deletion     | 3       | 6       | 0       | 105             | 0               | 0               | 0               |
| Total        | 3       | 27      | 32      | 105             | 14              | 1               | 21              |

(a) Intact or 204-bp-deletion norV gene.
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