**FULL PAPER** *Public Health*

# Distribution of IS*629* and *stx* genotypes among enterohemorrhagic *Escherichia coli* O157 isolates in Yamaguchi Prefecture, Japan, 2004–2013

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ABSTRACT. Patterns of insertion sequence (IS)*629*, *norV* genotype, and Shiga toxin (Stx) genotype distribution were investigated amongst 203 enterohemorrhagic *Escherichia coli* O157 isolates collected in Yamaguchi Prefecture, Japan, between 2004 and 2013. A total of 114 IS*629* patterns were identified; these were divided into eight IS groups (A–H). Ninety isolates carried an intact *norV* gene, whereas 113 isolates carried a *norV* with a 204-bp deletion. Other than one isolate from IS group G, all isolates with an intact *norV* belonged to groups A–F, whereas isolates with a mutant *norV* belonged to IS groups G and H. Seven *stx* genotypes were identified, and of those, *stx1a*/*stx2a* was predominant (n=105), followed by *stx2c* (n=32) and *stx2a* (n=27). The *stx1a*/*stx2a* genotype was associated with the mutant *norV* isolates, whereas isolates with an intact *norV* had the *stx2c* genotype. Therefore, certain combinations of IS type and *stx* genotype appear to be more frequent among O157 clades which may be useful for detection of predominant subtypes in the interest of public health.

KEY WORDS: enterohemorrhagic *Escherichia coli*, insertion sequence *629*, *norV*, O157, *stx* genotype

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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. IS*629*, a member of the IS*3* 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 IS*629* [2, 10]. O157 isolates show diverse patterns of IS*629* insertion, and therefore, IS*629* variability can be used for fingerprinting O157 isolates. Ooka *et al*. [12] developed a multiplex PCR tool for screening the distribution of IS*629* 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 IS*629* 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 IS*629* among O157 isolates from Yamaguchi Prefecture, Japan, has not been investigated. In the present study, we investigated the distribution of IS*629* 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-motile and were therefore classified as O157:NM.

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*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 IS*629* 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 IS*629* 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 IS*629* 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 IS*629* 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 IS*629* 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 IS*629* 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

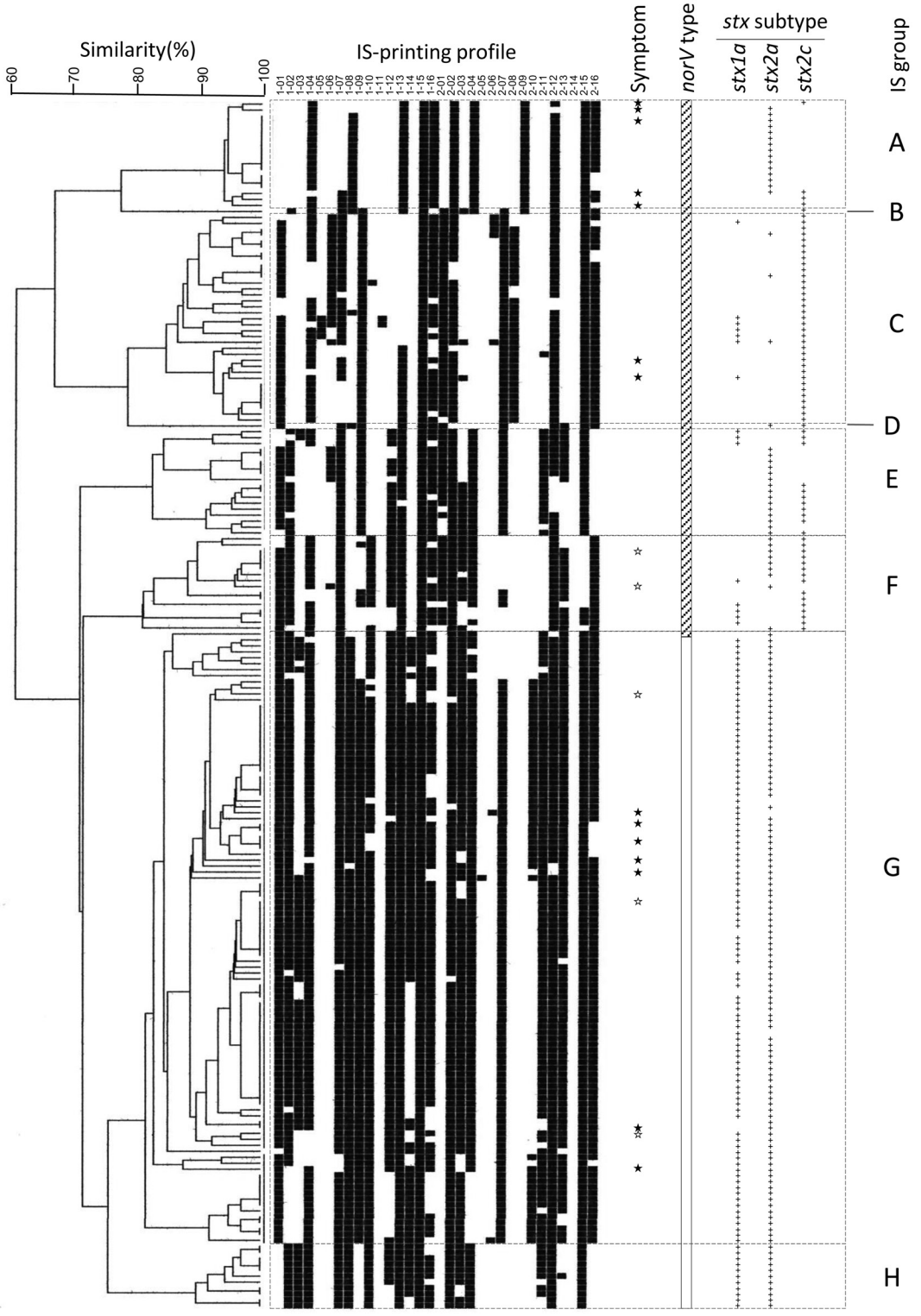


Fig. 1. Cluster analysis of 203 enterohemorrhagic *Escherichia coli* O157 isolates based on IS-printing profiles. Isolates with >80% similarity were considered to belong to the same group. The hatched bar indicates the intact *norV*, and the open bar indicates the 204-bp-deletion *norV*. A "☆" indicates a HUS patient, and "★" indicates an asymptomatic carrier.

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

| IS group | *norV*[a)] | Clade[b)] | n | No. of isolates in each year | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | 2004 | 2005 | 2006 | 2007 | 2008 | 2009 | 2010 | 2011 | 2012 | 2013 |
| A | Intact | - | 18 | 5 | | | 4 | 2 | 2 | 4 | | 1 | |
| B | Intact | - | 1 | | | | 1 | | | | | | |
| C | Intact | - | 35 | 4 | 1 | 1 | 5 | 7 | 5 | 3 | 3 | 3 | 3 |
| D | Intact | - | 1 | 1 | | | | | | | | | |
| E | Intact | - | 3 | | | | 1 | | | 2 | | | |
| | | 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 | | | | | |
| | Deletion | - | 102 | 14 | 15 | 8 | 10 | 9 | 13 | 8 | 8 | 9 | |
| H | Deletion | - | 11 | | | 1 | | 3 | 2 | | 2 | 2 | 1 |
| 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)] | No. of isolates in each *stx* genotype | | | | | | |
|---|---|---|---|---|---|---|---|
| | *stx1a* | *stx2a* | *stx2c* | *stx1a/ stx2a* | *stx1a/ stx2c* | *stx1a/ 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.

found that *stx* type alone cannot account for the variation in hospitalization and HUS rates by clade.

In the present study, the diversity of IS*629* 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 IS*629* distribution and *stx* genotype. Because analysis of patterns of IS*629* 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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