TABLE 1 Characteristics of STEC O103 serogroup strains

| Strain | Source (prefecture in Japan) | Yr | Sporadic/outbreak[a] | Clinical status or symptom(s)[b] | H serotyping[c] | *fliC* genotype[d] | *stx* gene | $stx_1$ integration site | Intimin type | LEE integration site | *ehx* gene |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 072676 | Miyazaki | 2007 | Outbreak (5) | Di, Fe, BD | H11 | [H11] | $stx_1$ | ND[e] | Beta1 | *pheU*[f] | + |
| 081163 | Yamaguchi | 2008 | Sporadic | Di, AP, Fe, BD | H11 | [H11] | $stx_1$ | ND | Beta1 | *pheU* | + |
| 081319 | Kanagawa | 2008 | No data | Di, BD | H11 | [H11] | $stx_1$ | ND | Beta1 | *pheU* | + |
| 082111 | Miyagi | 2008 | Sporadic | Di, AP | H11 | [H11] | $stx_1$ | *torS/T*[g] | Beta1 | *pheU* | + |
| 100207 | Miyazaki | 2010 | No data | Di, AP, Fe | H11 | [H11] | $stx_1$ | *torS/T* | Beta1 | *pheU* | – |
| 100952 | Fukuoka | 2010 | Outbreak (2) | Di, AP, BD | H11 | [H11] | $stx_1$ | ND | Beta1 | *pheU* | + |
| 102394 | Gifu | 2010 | Outbreak (2) | Di | H11 | [H11] | $stx_1$ | *torS/T* | Beta1 | *pheU* | + |
| 101624 | Saitama | 2010 | Sporadic | Di, AP | HUT | [H11] | $stx_1$ | *torS/T* | Beta1 | *pheU* | - |
| 110780 | Miyagi | 2011 | Sporadic | Di, AP, Fe | HUT | [H11] | $stx_1$ | *torS/T* | Beta1 | *pheU* | + |
| 071049 | Osaka | 2007 | Sporadic | Di, AP | H- | [H11] | $stx_1$ | ND | Beta1 | *pheU* | – |
| 080056 | Nagasaki | 2008 | Outbreak (3) | AP | H- | [H11] | $stx_1$ | *sbcB* | Beta1 | *pheU* | – |
| 090688 | Yamaguchi | 2009 | Sporadic | Di, Fe | H25 | [H25] | $stx_1$ | ND | Theta | ND | + |
| 070373 | Miyagi | 2007 | Sporadic | Di, AP, BD | HUT | [H25] | $stx_1$ | ND | Theta | ND | + |
| 080984 | Yamagata | 2008 | Outbreak (2) | Di, BD | HUT | [H25] | $stx_1$ | ND | Theta | ND | + |
| 082332 | Mie | 2008 | Sporadic | Di, AP | HUT | [H25] | $stx_1$ | ND | Theta | ND | + |
| 080455 | Nara | 2008 | Sporadic | Di, AP, Fe, BD | HUT | [H25] | $stx_1$ | ND | Theta | ND | + |
| 082589 | Yamagata | 2008 | Sporadic | Di, AP, Vo, BD | H2 | [H2] | $stx_1$ | *torS/T* | Epsilon | ND | + |
| 092412 | Nagano | 2009 | Sporadic | AP, BD | H2 | [H2] | $stx_1$ | *torS/T* | Epsilon | ND | + |
| 071556 | Fukuoka | 2007 | Outbreak (2) | Di, AP, BD | H2 | [H2] | $stx_1$ | *torS/T* | Epsilon | ND | + |
| 111471 | Kagoshima | 2011 | Sporadic | BD | H2 | [H2] | $stx_1$ | *torS/T* | Epsilon | ND | + |
| 111336 | Miyagi | 2011 | Sporadic | Di, AP | H2 | [H2] | $stx_1$ | *torS/T* | Epsilon | ND | + |
| 111155 | Kagoshima | 2011 | Outbreak (4) | BD | HUT | [H2] | $stx_1$ | *torS/T* | Epsilon | ND | + |

[a] The numbers in parentheses indicate the numbers of confirmed patients (including asymptomatic carrier) in each outbreak.
[b] Di, diarrhea; AP, abdominal pain; Fe, fever; Vo, vomiting; BD, bloody diarrhea.
[c] HUT, untypeable; H–, nonmotile.
[d] Types listed in square brackets were determined by sequence comparison of the *fliC* gene.
[e] ND, not determined.
[f] tRNA gene.
[g] *torS/T*, *torS-torT* intergenic region.

tively. In addition, the sequences of five whole-genome-sequenced STEC strains were used: O157:H7 Sakai (accession number BA000007) (10), O26:H11 11368 (AP010953), O103:H2 12009 (AP010958) and O111:H– 11128 (AP010960) (24), and O104:H4 TY-2482 (AFVR01000000) (33).

**PCR analysis of virulence markers.** The following 13 pathotype-associated genes were detected by PCR: $stx_1$ and $stx_2$ (4), *ehxA* (encoding enterohemolysin) (27) and *eae* (26), associated with enterohemorrhagic *E. coli* (EHEC) and/or enteropathogenic *E. coli* (EPEC); *bfpA* (encoding bundle-forming pilus) (9), associated with typical EPEC; *elt* (encoding heat-labile enterotoxin) and *est* (heat-stable enterotoxin) (39), associated with enterotoxigenic *E. coli*; *astA* (encoding heat-stable enterotoxin EAST1) (44) and *aggR* (encoding transcriptional activator of aggregative adherence fimbriae I expression) (6), associated with enteroaggregative *E. coli* (EAEC); *ipaH* (encoding invasive plasmid antigen H) (36), associated with enteroinvasive *E. coli*; *cdtV* (encoding cytolethal distending toxin [CDT] V, a member of the CDT family, associated with tissue damage [3]) (5); *subAB* (encoding subtilase cytotoxin) (22); and *saa* (encoding STEC autoagglutinating adhesin) (28). All PCRs were performed according to the protocols described previously.

**Sequencing of *fliC*, *eae*, and seven housekeeping genes.** The H type was genetically determined by sequence comparison of the *fliC* gene. The entire coding region of *fliC* was amplified and sequenced using the primers F-FLIC-out (5′-TTAAATCCAGACCTGACCCGA-3′) and R-FLIC-out (5′-CCACAGCGAGTGTTTATCCAT-3′), and an additional primer F-FLIC1 (8) was used for internal sequencing of *fliC*. The entire coding region of *eae* was amplified and sequenced using two primer pairs: cesT-F9 and eae-R3 for N-terminal protein, and eae-F1 and escD-R1 for C-terminal protein (11). The internal regions of the seven housekeeping genes (*adk*, *fumC*, *gyrB*, *icd*, *mdh*, *purA*, and *recA*) were PCR amplified and sequenced using the primers and protocol specified on the *E. coli* multilocus sequence typing (MLST) website (http://mlst.ucc.ie/mlst/dbs/ecoli).

**MLSA.** The concatenated sequences (3,423 bp) of seven housekeeping genes (*adk*, *fumC*, *gyrB*, *icd*, *mdh*, *purA*, and *recA*) from O103 strains were used for multilocus sequence analysis (MLSA). In addition, the sequences of five whole-genome-sequenced STEC strains (O157:H7, O26:H11, O103:H2, O111:H–, and O104:H4) and three well-characterized STEC serotype strains (O121:H19, O165:H–, and O145:H–) were included in the analysis. *E. coli* reference strains, the ECOR collection, were also used for MLSA. Multiple alignments of sequences were constructed by using the CLUSTAL W program (41) in the MEGA4 software (40), and then neighbor-joining trees were generated by using the Tamura-Nei model. A bootstrap test with 1,000 replicates was used to estimate the confidence of the branching patterns of the tree. Sequences of the ECOR collection for MLSA and the sequence type (ST) of the STEC O103 strains were obtained from the *E. coli* MLST database (http://mlst.ucc.ie/mlst/dbs/ecoli).

**Determination of Stx1 phage and LEE integration sites.** Thus far, seven genomic loci (*torS-torT* intergenic region, *wrbA*, *yehV*, *prfC*, *sbcB*, *argW*-tRNA, and *ssrA*-tmRNA) have been identified as integration sites of $stx_1$-containing bacteriophages (Stx1 phages) (23). To determine integration sites for Stx1 phages on the chromosome, a universal PCR primer (Pstx1A-F, 5′-AAACCGCCCTTCCTCTGGAT-3′) targeted to the $stx_{1A}$ gene on the prophage and seven primers (Pstx1_tosRS-R, 5′-TTCAGGCTTTGTGCGGTGAG-3′; Pstx1_wrbA-R, 5′-CTCTCTGTTAACGGCGCTGGAT-3′; Pstx1_yehV-R, 5′-TGCCAGCGTGACAGAAGTTG-3′; Pstx1_prfC-R, 5′-ATCGGCATCATCACCAACGG-3′; Pstx1_sbcB-R, 5′-GCGGAACATCAATCAACGCCA-3′; Pstx1_argW-R, 5′-TCAACTTCTGGTTGGTCTCGC-3′; and Pstx1_ssrA-R, 5′-TCCTACCCGTACCCGCAAGTT-3′) targeted to the outside of each prophage region were designed on the basis of the genome sequences of the STEC strains O157:H7



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TABLE 2 Primers used for multiplex PCR

| Target | Primer | Sequence (5′–3′) | Observed amplicon size (bp) |
|---|---|---|---|
| Universal forward primer for *fliC* | fliC_univ_F | ATGGCACAAGTCATTAATAC | |
| *fliC* (H11) | fliC_H11_R | TATTCTTAGCCGCTGCTGC | 755 |
| *fliC* (H2) | fliC_H2_R | TATCCTGATCAGAAGCCAGCA | 417 |
| *fliC* (H25) | fliC_H25_R | TGCGGGATAGATGTGATAGCA | 559 |
| *wzy* (O103) | O103_wzy_F | CTCTTGCTGCTATGAGCTTTG | 297 |
| | O103_wzy_R | GCGGGGTCTTGTCATTTAAT | |

Sakai, O26:H11 11368, O103:H2 12009, and O111:H− 11128. In addition, integration sites (*pheV*, *pheU*, and *selC* tRNA gene loci) of LEE elements were screened by primers described elsewhere (25). Long-range PCR screenings were performed by using TaKaRa LA *Taq* polymerase (TaKaRa Bio, Inc., Ohtsu, Japan).

**Sequence analysis of the O103-antigen biosynthesis gene cluster and its flanking region.** The O103-antigen biosynthesis gene cluster and its flanking regions were amplified using a PCR primer pair, O55re-1F and O55re-1R (12). Each PCR product was sequenced by the shotgun method. Sequences were aligned using Sequencher software (v4.9; Gene Code Corp., Michigan), and sequence comparisons were performed by using *in silico* molecular cloning software (In Silico Biology, Yokohama, Japan).

**Multiplex PCR assay.** The primers used for multiplex PCR and the lengths of the amplicons are listed in Table 2. A universal primer designed on the basis of the N-terminal sequences of *fliC* and specific primers designed on the basis of the highly diversified sequences (middle part) of each *fliC* gene were used. In addition, primers targeting the *wzy* (O103) gene were also used for control amplification. Multiplex PCR was performed with a 15-μl reaction mixture containing 10 ng of genomic DNA, 1× Kapa *Taq* buffer, each deoxynucleoside triphosphate at 0.3 mM, 2.5 mM $MgCl_2$, 0.25 μM fliC_univ_F primer, 0.25 μM fliC_H2_R primer, 0.25 μM fliC_H25_R primer, 0.38 μM fliC_H11_R primer, 0.5 μM O103_wzy_F primer, 0.5 μM O103_wzy_R primer, and 0.4 U of Kapa *Taq* DNA polymerase (Kapa Biosystems, Woburn, MA). The thermocycling condition was 25 cycles of 94°C for 20 s, 57°C for 20 s, and 72°C for 30 s. The PCR products (2 μl) were electrophoresed in 1.5% in agarose gels in 0.5× TBE (25 mM Tris borate, 0.5 mM EDTA) and photographed under UV light after the gel was stained with ethidium bromide.

**Nucleotide sequence accession number.** The sequences of the two O103-antigen gene clusters from O103:H25 and O103:H11, and of the *adk*, *fumC*, *gyrB*, *icd*, *mdh*, *purA*, and *recA* genes were deposited in GenBank/EMBL/DDBJ database under accession numbers AB704860, AB704861, and AB704965 to AB705139, respectively.

## RESULTS

**Characterization of O103:non-H2 strains.** Seventeen STEC O103:non-H2 strains isolated from patients in Japan were investigated (Table 1). Six strains were isolated from disease outbreaks, nine were from sporadic cases, and two were from cases for which no information was available. Seven strains were classified as H11 type and one as H25 type by using agglutination assays. Seven additional strains were classified as HUT, because their H types could not be determined due to no or low agglutination, or because aggregation was observed for multiple anti-H antisera. The remaining two strains showed no motility.

***fliC* analysis.** The sequence analysis of *fliC* from all O103 strains examined showed that the amino acid sequences (487 amino acids [aa]) of two HUT (101624 and 110780) and two H− (071049 and 080056) strains were identical to those of H11-expressing O103 strains. The sequences (443 aa) of four HUT strains (070373, 080984, 082332, and 080455) were identical to that of H25-expressing O103 strain 090688, except for one amino acid difference in 080984. In addition, the sequence (494 aa) of OUT strain 111155 was identical to that of H2-expressing O103 strains and that of the fully sequenced O103:H2 strain. These results indicated that all of the control and experimental O103 strains were one of the following three H types: H2/[*fliC*-H2], H11/[*fliC*-H11], or H25/[*fliC*-H25] (Table 1). By comparison, the sequence identities of FliC between H2 and H11, between H11 and H25 (090688), and between H25 and H2 were 55.4, 50.4, and 49.4%, respectively.

**PCR screening of virulence-related genes.** PCR-based screening for *E. coli* virulence-related genes showed that all O103 strains possessed $stx_1$ and *eae* and that 18 of the strains examined carried *ehx* (Table 1). The remaining 10 genes ($stx_2$, *bfpA*, *elt*, *est*, *astA*, *aggR*, *ipaH*, *cdtV*, *subAB*, and *saa*) included in the screen were absent from all strains examined.

***eae* typing.** The results of sequence analysis of *eae* from all O103 strains are shown in Table 1. The amino acid sequences of the H2/[H2] and H11/[H11] strains (948 and 939 aa, respectively) were identical to those of O103:H2 strain 12009 and O26:H11 strain 11368, respectively, indicating that H2/[H2] and H11/[H11] strains possess the *eae* genes encoding epsilon and beta1 subclass intimins (*eae*-epsilon and *eae*-beta1, respectively). In addition, the sequences (935 aa) of H25/[H25] strains were identical to that of O111:H− strain 11128, indicating that H25/[H25] strains possess *eae*-theta.

**Integration site of Stx1 phages and LEE elements.** Long-range PCR screening targeting seven alternative integration sites of Stx1 phages was performed. All six H2/[H2] and five H11/[H11] strains were found to contain the Stx1 phage in the *torS*-*torT* intergenic region, and one H11/[H11] strain contained it in the *sbcB* locus (Table 1). The integration site in the other strains was not determined by these methods (Table 1). PCR screening analysis for three alternative integration sites of LEE showed that all H11/[H11] strains possess LEE elements in the *pheU* locus. The integration site of LEE in H2/[H2] and H25/[H25] was not determined (Table 1).

**Phylogenetic relationship of O103 strains.** We analyzed the phylogenetic relationships among O103 strains and well-known strains from the STEC serotype collection. As shown in Fig. 1, the O103:H11/[H11] and O103:H25/[H25] strains formed two distinct groups, different from that of O103:H2/[H2] strains. The O103:H11/[H11] strains formed two groups with one nucleotide difference and were closely related to STEC O26:H11, while the O103:H25/[H25] strains were associated with Shiga toxin-producing EAEC O104:H4. The O103:H2/[H2] strains belonged to ST17 and the O103:H25/[H25] strains to ST343 (Fig. 1). One group of O103:H11/[H11] strains belonged to ST21, which was associated with O26:H11, and the other belonged to ST723 (Fig.

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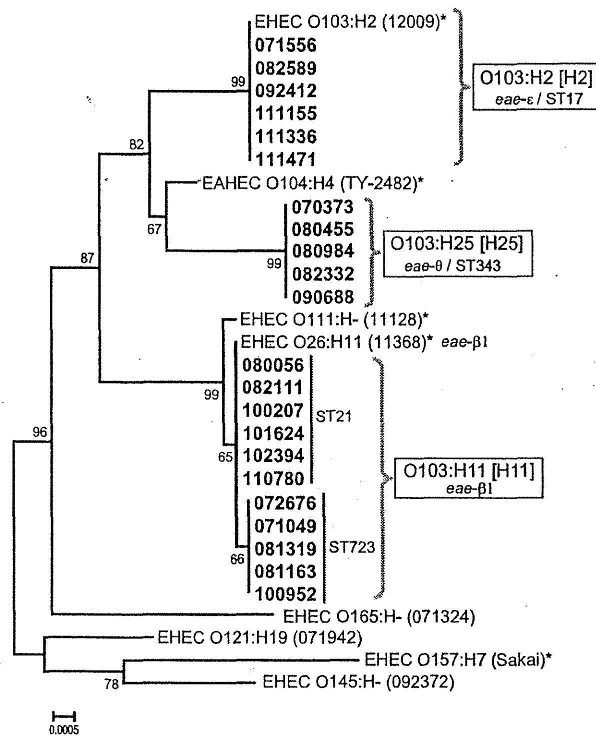


FIG 1 Phylogenetic relationships of O103-serogroup strains among eight well-characterized STEC serotypes strains. The phylogenetic tree was constructed on the basis of the concatenated sequences of seven housekeeping genes by using the neighbor-joining algorithm. Bootstrap analysis was performed with 1,000 replicates.

1). Compared to the sequences of the ECOR collection strains, three O103 groups belonged to the B1 phylogroup (data not shown). Pulsed-field gel electrophoresis pattern analysis revealed diverse populations of STEC O103:H11 and O103:H25 strains. For the O103:H11/[H11] classification, however, two strains (071049 and 101624) and three strains (082111, 100207, and 110780) differed by fewer than four bands within each of these two groups, indicating that they were genetically closely related (data not shown).

**Sequences of the O103-antigen biosynthesis gene cluster.** To gain more information about the genetic similarity of the O103-antigen encoding region among the three lineage groups, the sequences of the O103-antigen gene cluster of a representative strain from each lineage (072676 for O103:H11/[H11] and 080984 for O103:H25/[H25]) were determined and compared to that of STEC O103:H2 12009. The gene organization of the O103-antigen gene cluster was identical among the three strains, and their sequences were highly conserved except for three genes (*ugd*, *rmlB*, and *galF*) in the O103:[H25] strains (Fig. 2). In addition, the sequences of the O-antigen gene cluster and its flanking regions of O103:H11 were compared to those of O26:H11, which is closely related to O103:H11. As shown in Fig. 2, in addition to the flanking genes, three upstream genes (*wzz*, *ugd*, and *gnd*) and two downstream genes (*rmlB* and *rmlF*) in the O-antigen gene cluster were conserved between the O103 and O26 strains (94.1 to 99.7% identity).

**Multiplex PCR.** We developed a multiplex PCR system for classifying the pathogenic O103 strains that were confirmed to possess the *stx* and/or *eae* gene(s). Because *fliC* alleles encoding each of the H2, H11, and H25 antigens were lineage-specific among the STEC O103 strains (Fig. 1), this multiplex PCR method targeting *fliC* provided a rapid way to classify STEC O103 strains into three clonal groups. On the basis of the sequences of the O103-antigen gene clusters obtained in the present study, primers targeting the *wzy* (O103) gene were also designed for control amplification. The validity of the multiplex PCR system was confirmed using 22 STEC O103 control strains and three different H-antigen serotype control strains (O128:H2, O130:H11, and O156:H25). All PCR products matched the predicted sizes of the *fliC* (H2) (417 bp), *fliC* (H11) (755 bp), *fliC* (H25) (559 bp), and *wzy* (O103) (297 bp) genes, and the expected band patterns (Fig. 3).

## DISCUSSION

Although STEC O103:H2, O26:H11 and O111:H− strains belong to the *E. coli* B1 phylogroup and are closely related, especially O26:H11 and O111:H-, genomic analyses support the hypothesis that independent acquisition of Stx phages, LEE elements and many other virulence-related genes has driven the emergence of each STEC (24).

In the present study, 17 STEC O103-serogroup strains were classified into three distinct clonal groups coincident with variations in their *fliC* and *eae* genes (Fig, 1). A key finding was that strains belonging to the O103:H11/[H11] group were closely related to STEC O26:H11, suggesting that the STEC O103:H11 and STEC O26:H11 clones evolved from a common ancestor with one or more exchange(s) of the region encoding O-antigen biosynthesis. It is known that EHEC O157:H7 emerged from an O55:H7-like EPEC ancestor by specific events including acquisition of the O157-antigen biosynthesis gene cluster by horizontal gene trans-

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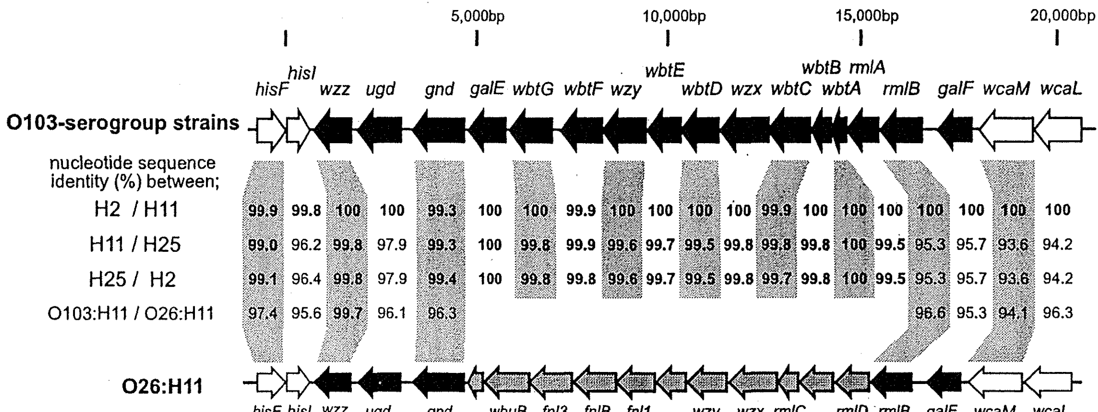

FIG 2 Comparison of O103-antigen biosynthesis gene clusters and their flanking regions. The genetic organization of the O103-antigen gene cluster and its flanking regions from O103 serotype strains is shown at top and that from STEC O26:H11 11368 (AP010953) is shown at the bottom. Genes associated with O-antigen biosynthesis are indicated by black arrows, and flanking genes are indicated by white arrows. O26-specific genes are indicated by gray arrows. Nucleotide sequence identities (%) between O103:H2 and O103:H11, between O103:H11 and O103:H25, and between O103:H25 and O103:H2 are shown in the middle. In addition, sequence identities between O103:H11 and O26:H11 are also shown.

fer (43), and a previous genome-wide sequence comparison showed that a large region of up to 130 kb including the O-antigen gene cluster was replaced by the result of recombination events (17). From the present sequence comparison of the O-antigen gene cluster and its flanking region between STEC O103:H11 and STEC O26:H11, a level of sequence conservation comparable to that of housekeeping genes (representing the backbone of the chromosome and nearly 100% conserved on the basis of the sequences of genes for MLSA) was not observed in the neighboring genes except for *wzz* (99.7%), suggesting that replacement of the region containing the O-antigen gene cluster occurred across a larger region.

Beutin et al. (1) demonstrated considerable diversity among STEC/EPEC O103 strains, which was investigated by MLST and *eae* typing. O103:H2 strains were predominantly positive for *eae*-epsilon, whereas an O103:H11 strain, whose MLST profile was different from those of the O103:H2 strains, was positive for *eae*-beta1. Ogura et al. (23) demonstrated that LEE elements are generally found at specific loci within the clonal groups and, among all six STEC O26:H11/H− strains tested, LEE elements with *eae*-beta1 were located at the *pheU*-tRNA locus. The O103:H11/[H11] strains tested also carried LEE elements with *eae*-beta1 at the *pheU* locus (Table 1), suggesting that, after acquiring a LEE element with *eae*-beta1 in the *pheU* locus, a LEE-positive common ancestor divided into the two clonal groups of STEC O26:H11 and O103:H11. On the other hand, the presence and location of Stx phages are known to be unsteady even within a clonal group. Stx1 phages in O157:H7 strains have been found in at least three different loci: *sbcB*, *yehV*, and *argW* (23). It is known that STEC O26:H11 strains carried the Stx1 phage at the *wrbA* locus (23); in contrast, five of the O103:H11/[H11] strains studied here carried the Stx1 phage in the *torS-torT* intergenic region, which was previously found to be an integration site in STEC O103:H2 (23), and one O103:H11/[H11] strain carried the Stx1 phage at the *sbcB* locus, which was found to be an integration site in O157:H7 (23). The remaining 10 strains characterized here had unknown integration sites. These results suggested that the Stx1 phage has integrated into different sites of the genome even among closely related strains, and it is not clear when the lineages associated with STEC O26:H11 and O103:H11 acquired the Stx1 phage(s).

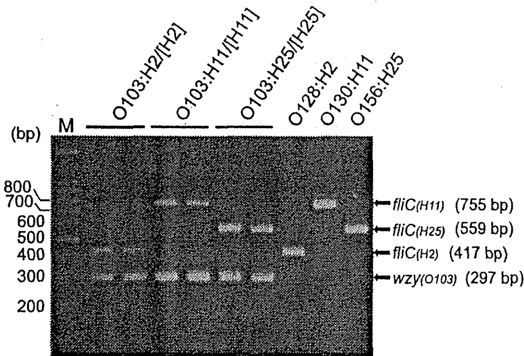


FIG 3 Multiplex PCR products of STEC O103 strains representing three groups. The strains used were 082589 and 111155 for O103:H2/[H2], 072676 and 071049 for O103:H11/[H11], 090688 and 082332 for O103:H25/[H25] and three non-O103 strains expressing either H2, H11, or H25 antigen. M, 100-bp DNA ladder markers.

A few cases of infection associated with STEC O103:H25 have been reported (30, 31, 42), and most isolates were found to be Stx1-producing strains. In 2006, however, an outbreak caused by Stx2-producing O103:H25 strains in Norway was reported (35). Among the 17 cases, 10 were children who developed HUS. The sequences of seven housekeeping genes for MLSA from Stx2-producing O103:H25 NVH-734 (GenBank accession no. AGSG01000000) (15) were identical to those of the Stx1-producing O103:H25 strains that we investigated, indicating that they belonged to the same clonal group (data not shown).

Although serotypes O103:H11 and O103:H25 are rare causes of EHEC disease, these serotypes used here were obtained from patients with diarrhea and hemorrhagic colitis. Because these O103 strains were the only bacteria known to cause these conditions, it is likely that the isolated strains caused these conditions. Thus, these serotype strains could be a threat to human health, and caution should be exercised around them. The clinical isolates



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characterized here were geographically and temporally dispersed, suggesting that these pathogens are widespread throughout Japan. Precise O/H serotyping of STEC strains isolated from human and food sources is required for validation. In many cases, the O-serogroup classification of STEC strains provides enough information to presume its clonal relatedness to well-known O-serogroup strains. Our STEC O103 clinical isolates, however, belonged to three distinct clonal groups. Despite the fact that these strains had diverse genetic backgrounds, they all carried the EHEC marker genes $stx_1$, *eae*, and/or *ehx*. Although the H type can be a useful phenotypic marker for classifying strains, we could not determine the H type of some O103 isolates, because of unclear agglutination or lack of bacterial motility. As many researchers have shown before (8, 18, 29), sequence variation in the *fliC* gene could be a proxy for these agglutination tests. In the present study, on the basis of sequence variation in *fliC* genes, we developed a multiplex PCR method for such classification of STEC O103 strains. The PCR-based methodologies described in the present study may be utilized to aid clinical and epidemiological studies of the STEC O103 serogroup strains.

In conclusion, we demonstrated that STEC O103 from patients formed three distinct groups, and the group comprising O103:H11 strains was closely related to STEC O26:H11. These findings suggest that the STEC O103:H11 and O26:H11 clones evolved from a common ancestor and provide further insights into the high variability of STEC strains with emerging new serotypes.

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Members of the EHEC Study Group include the following: Kazumi Horikawa, Yoshiki Etoh, and Shachiko Ichihara, Fukuoka Institute of Health and Environmental Sciences; Junji Seto, Yamagata Prefectural Institute of Public Health; Shuji Yoshino, Miyazaki Prefectural Institute for Public Health and Environment; Yutaka Shiraki, Gifu Prefectural Research Institute for Health and Environmental Sciences; Kiyoshi Tominaga, Yamaguchi Prefectural Institute of Public Health and Environment; Takashi Hatakeyama, Miyagi Prefectural Institute of Public Health and Environmental; Madoka Hamada, Kagoshima Prefectural Institute for Environmental Research and Public Health; Hiromi Nakamura, Osaka City Institute of Public Health and Environmental Sciences; Sumie Kohori, Saitama City Institute of Health Science and Research; Yuji Migita, Nagasaki Prefectural Institute for Environmental Research and Public Health; Hitomi Kasahara, Nagano Environmental Conservation Research Institute; Misao Hashida, Nara Prefectural Institute for Hygiene and Environment; Yuki Nagai, Mie Prefecture Health and Environment Research Institute; members of the Sagamihara City Laboratory of Public Health; and Kazuko Seto, Osaka Prefectural Institute of Public Health.

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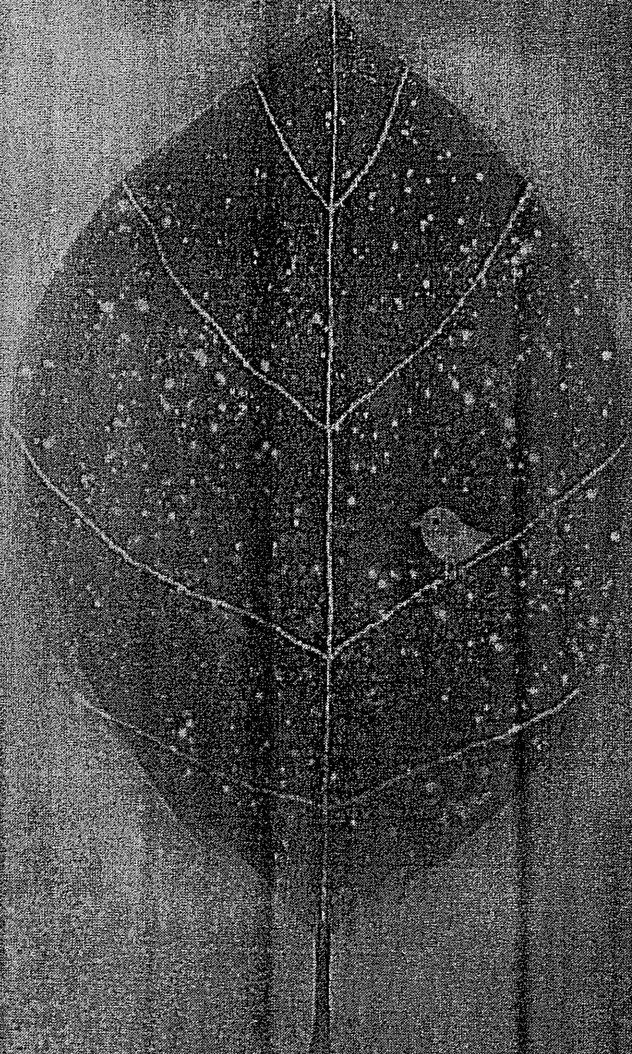
日本医師会生涯教育シリーズ

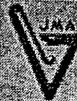
日本医師会●編

# 小児・思春期診療 最新マニュアル

【監修】
五十嵐　隆

【編集】
児玉　浩子
早乙女智子
平岩　幹男
松平　隆光



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ス性髄膜炎では急性に，結核性・真菌性髄膜炎では亜急性〜慢性に出現する．けいれんをしばしば伴う．ただし乳児では，髄膜刺激症候を認めにくいことが多い．新生児では症状がさらに非特異的で，何となく元気がない（not doing well）程度の例も多い．

**臨床検査，画像検査**

まず，血液検査を行う．細菌性髄膜炎では通常，白血球増多やCRP強陽性などの炎症所見がみられる．その他の髄膜炎でも軽度〜中等度の炎症反応がある．細菌性髄膜炎の疑いがあれば血液培養を施行する．

確定診断のため髄液検査を行う．病原体同定には培養が最も確実だが，数日〜数週を要する．迅速診断には細菌（グラム染色）・真菌（PAS・墨汁染色）の鏡検，病原体の抗原（免疫学的検出法）・核酸（PCR法）の検出を行う．

頭部CT・MRIにより脳浮腫，脳梗塞・出血，硬膜下膿瘍・水腫などの合併病態を調べる．

## 治療のポイント

**原因療法**

細菌性髄膜炎には，起因菌が感受性を有し，髄液移行が良く，殺菌的な抗菌薬を選んで静脈内投与する．多くの菌に対して第三世代セフェム（セフトリアキソンなど）が第一選択であるが，リステリアに対してはペニシリン（アンピシリン），ペニシリン耐性肺炎球菌に対してはカルバペネム系薬かバンコマイシンが選択される．投与開始の24〜36時間後に髄液を再検して，細菌が陰性化したか否かにより初期効果を判定する．抗菌薬は少なくとも10日間，菌種に応じ2〜3週間以上投与する．

結核性・真菌性髄膜炎に対しては病原に応じた化学療法を行う．

**支持療法**

細菌性・結核性髄膜炎の重症例では，呼吸・循環を含めた全身管理が重要である．けいれんに対しては抗けいれん薬を，脳浮腫に対しては浸透圧利尿薬を投与する．またインフルエンザ菌，肺炎球菌，結核菌による髄膜炎では，副腎皮質ステロイドが難聴や神経学的後遺症の軽減目的に併用される．

ウイルス性髄膜炎は，安静・輸液などの対症療法のみで軽快することが多い．

**手術療法**

硬膜下膿瘍・水腫，水頭症などの合併症に対し，脳室ドレナージや脳室腹腔シャント術を行う．

**予後**

細菌性髄膜炎の死亡率は低年齢ほど高い．水頭症，知的障害，運動麻痺，てんかん，難聴などの後遺症がしばしば残る．結核性髄膜炎も神経学的後遺症を残す例が多い．ウイルス性髄膜炎の予後は，大多数で良好である．

**予防**

Hib，肺炎球菌，ムンプス髄膜炎は，ワクチンで予防可能である．

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（水口　雅）

# 急性脳炎・急性脳症

## 疾患の概要

**急性脳炎とは**

脳実質の急性炎症である．原因の多くは感染（ウイルス，マイコプラズマなど），一部がワクチン接種である．ウイルス性急性脳炎は概念的に，ウイルスの直接侵襲による一次性脳炎（単純ヘルペス脳炎など）と自己免疫による二次性脳炎（急性散在性脳脊髄炎など）に大別される．ワクチン接種後脳炎は二次性脳炎である．

**急性脳症とは**

感染症を契機に，脳炎以外の機序で広範囲の脳浮腫が生じる病態である．意識障害や頭蓋内圧亢進症候を呈し，しばしばけいれんを伴う．

## 診断のポイント

### ●臨床症状と経過

感染症に続発する脳炎・脳症では，発熱，発疹，感冒様症状など感染症の症候と共に，脳機能障害の症状が出現する．一次性脳炎や急性脳症は感染症の急性期（有熱期）に，二次性脳炎は解熱後の亜急性期（発疹が出現したり，抗体が上昇する時期）に発症することが多い．

けいれん・意識障害がしばしばみられる．頭蓋内圧亢進症候は全脳炎や急性脳症の際に強く出やすい．一方，神経学的局所症状は局所性・散在性脳炎の際に目立ちやすい．

脳炎か脳症か，脳炎・脳症の病原・病型を診断し，治療に結びつける．ほとんどの場合，複数の検査の所見を組み合わせて診断する．

### ●臨床検査

鼻咽頭スワブ，便・血液などの検体を用いて，脳炎・脳症の契機となった感染症の病因診断を行う．特に一次性脳炎の診断においては，髄液検査が重要である．ウイルスの分離・培養，ウイルス抗原の検出（酵素免疫測定法など），ウイルスゲノム検出（PCR 法など），髄液の抗体価測定などを行う．

血液検査では感染症の所見に加え，重症の脳炎・脳症では全身の臓器障害や DIC の所見もみられる．髄液検査では脳炎の多くで細胞数が増加する．蛋白もしばしば上昇する．

### ●画像検査，生理検査

頭部 CT・MRI により，びまん性脳浮腫や局所性病変を描出する．側頭葉・前頭葉下部病変は単純ヘルペス脳炎に，視床，基底核・黒質病変は日本脳炎に，多発性・散在性病変は急性散在性脳脊髄炎に，両側視床の対称性病変は急性壊死性脳症に，大脳皮質下白質の遅発性拡散低下はけいれん重積型急性脳症にそれぞれ特徴的である．

脳波検査も有用である．基礎波の異常として高振幅徐波が全脳炎や急性脳症ではびまん性に，局所性脳炎では局所性に出現する例が多い．突発性異常もしばしばみられる．

## 治療のポイント

### ●支持療法

けいれんや意識障害に対する対症療法を行う．重症例では呼吸・循環の安定化のため，気道・血管を確保し，輸液や人工呼吸を開始する．けいれんを止め，その再発を予防し，頭蓋内圧降下療法（高浸透圧利尿薬など）を行う．全身・頭部を冷却し，感染症の治療を行う．体液バランス異常（血糖・血清電解質の異常，代謝性アシドーシス）を補正し，血液学的異常（凝固異常や DIC）があれば輸血その他で対処する．

### ●原因療法，特異的治療

一次性ウイルス脳炎のうち，単純ヘルペス脳炎には抗ウイルス薬（アシクロビル，ビダラビン）を投与する．自己免疫性の脳炎に対しては副腎皮質ステロイドや免疫グロブリンを用いた免疫抑制療法を行う．

急性脳症のうち，急性壊死性脳症など高サイトカイン血症を伴う病型には副腎皮質ステロイドを投与する．重症例に対して脳低体温療法や血液浄化などが試みられることもある．

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（水口　雅）

# 脳性麻痺

## 脳性麻痺とは

厚生労働省の定義は，「受胎から生後 28 日以内に生じた脳の非進行性病変に基づく運動および姿勢の異常」であり，進行性疾患や生後 28 日以降の疾患や外傷によるものは含めない．一方，出産時の原因であっても腕神経叢損傷のような末梢神経障害は脳性麻痺には入れない．しかし，実際には，後天性や末梢性の運動障害を

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# Molecular Characterization Reveals Three Distinct Clonal Groups among Clinical Shiga Toxin-Producing Escherichia coli Strains of Serogroup O103

**Atsushi Iguchi, Sunao Iyoda and Makoto Ohnishi**
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# Molecular Characterization Reveals Three Distinct Clonal Groups among Clinical Shiga Toxin-Producing *Escherichia coli* Strains of Serogroup O103

**Atsushi Iguchi,[a] Sunao Iyoda,[b] and Makoto Ohnishi[b] on behalf of the EHEC Study Group**
Interdisciplinary Research Organization, University of Miyazaki, Miyazaki, Japan,[a] and Department of Bacteriology I, National Institute of Infectious Diseases, Tokyo, Japan[b]

**Shiga toxin-producing *Escherichia coli* (STEC) is one of the most important groups of food-borne pathogens, and STEC strains belonging to the serotype O103:H2 can cause diarrhea, hemorrhagic colitis, and hemolytic-uremic syndrome in humans. STEC O103:non-H2 strains are also sometimes isolated from human patients, but their genetic characteristics and role in significant human enteric disease are not yet understood. Here, we investigated 17 STEC O103:non-H2 strains, including O103:H11, O103:H25, O103:HUT (UT [untypeable]), and O103:H− (nonmotile) isolated in Japan, and their characteristics were compared to those of STEC O103:H2 and other serotype STEC strains. Sequence analyses of *fliC* and *eae* genes revealed that strains possessed any of the following combinations: *fliC*-H2/*eae*-epsilon, *fliC*-H11/*eae*-beta1, and *fliC*-H25/*eae*-theta, where *fliC*-H2, -H11, and -H25 indicate *fliC* genes encoding H2, H11, and H25 flagella antigens, respectively, and *eae*-epsilon, -beta1, and -theta indicate *eae* genes encoding epsilon, beta1, and theta subclass intimins, respectively. Phylogenetic analysis based on the sequences of seven housekeeping genes demonstrated that the O103:H11/[*fliC*-H11] and O103:H25/[*fliC*-H25] strains formed two distinct groups, different from that of the O103:H2/[*fliC*-H2] strains. Interestingly, a group consisting of O103:H11 strains was closely related to STEC O26:H11, which is recognized as a most important non-O157 serotype, suggesting that the STEC O103:H11 and STEC O26:H11 clones evolved from a common ancestor. The multiplex PCR system for the rapid typing of STEC O103 strains described in the present study may aid clinical and epidemiological studies of the STEC O103:H2, O103:H11, and O103:H25 groups. In addition, our data provide further insights into the high variability of STEC stains with emerging new serotypes.**

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Shiga toxin-producing *Escherichia coli* (STEC) is one of the most important groups of food-borne pathogens worldwide because it can cause gastroenteritis that may be complicated by hemorrhagic colitis or hemolytic-uremic syndrome (HUS) (21). STEC O157:H7 is the main serotype responsible for outbreaks and sporadic cases of hemorrhagic colitis and HUS, but non-O157 serogroups (such as O26, O103, O111, and O145) can also be associated with severe illness in humans (16, 32).

Serotype O103:H2 is one of the most frequently isolated non-O157 STEC. It was first identified as a causative agent of HUS in 1992 (19), and since then both outbreaks and sporadic cases of diarrhea and HUS caused by STEC O103:H2 have been reported worldwide (2, 7, 14, 20, 34). STEC O103 strains expressing H antigens other than H2 are sometimes isolated from human patients. Sporadic cases of human infections with O103:H11 in Japan (37) and Canada (38) have been described previously, and it was recently shown that O103:H25 was responsible for outbreaks of HUS in Norway (35). Thus, STEC O103:non-H2 serotype strains have also become a threat to public health.

Our previous studies (12, 13) demonstrated that *E. coli* strains with the same O serogroup but different H types sometimes belong to different evolutionary lineages. Furthermore, most STEC strains possess various combinations of virulence genes and exhibit allelic variations of some genes, such as the *stx* gene on lambda-like prophages and *eae* (encoding the adhesin intimin) on the locus of enterocyte effacement (LEE) element, which may affect the pathogenicity of strains. Because O103:H2 is a major serotype of STEC, the prevalence and genotypic characteristics of these strains have been investigated in detail; however, little is known about the characteristics of STEC O103:non-H2 strains.

The aim of the present study was to compare STEC O103:non-H2 strains isolated from Japanese patients infected with STEC O103:H2 and other serotype STEC strains to identify their genetic characteristics and to explore their phylogenetic relationships to determine whether pathogenic non-H2 strains share similar molecular characteristics with other, better-characterized O103 strains.

## MATERIALS AND METHODS

**Bacterial strains.** The relevant characteristics of the 22 STEC O103 strains, including five O103:H2 strains used in the present study, are listed in Table 1. The strains were isolated from patients with gastrointestinal disease (including diarrhea and hemorrhagic colitis) from 2007 to 2011 in various prefectures of Japan. O serogroups of each strain were determined by agglutination tests with the anti-O103 serum (Denka Seiken Co., Ltd., Tokyo, Japan) according to the manufacturer's instructions. H types were determined using a set of anti-H sera purchased from Statens Serum Institut (Statens Serum Institut, Copenhagen, Denmark). Three STEC strains, O145:H− (092372), O121:H19 (071942), and O165:H− (071324), obtained from Osaka Prefectural Institute of Public Health and three different kinds of *E. coli* serotype strains, O128:H2 (100923), O130:H11 (102608), and O156:H25 (110085), obtained from Fukuoka Institute of Health and Environmental Sciences were used as controls for the phylogenetic analysis and the multiplex PCR assay described below, respec-

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Address correspondence to Atsushi Iguchi, iguchi@med.miyazaki-u.ac.jp.
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TABLE 1 Characteristics of STEC O103 serogroup strains

| Strain | Source (prefecture in Japan) | Yr | Sporadic/outbreak[a] | Clinical status or symptom(s)[b] | H serotyping[c] | *fliC* genotype[d] | *stx* gene | $stx_1$ integration site | Intimin type | LEE integration site | *ehx* gene |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 072676 | Miyazaki | 2007 | Outbreak (5) | Di, Fe, BD | H11 | [H11] | $stx_1$ | ND[e] | Beta1 | *pheU*[f] | + |
| 081163 | Yamaguchi | 2008 | Sporadic | Di, AP, Fe, BD | H11 | [H11] | $stx_1$ | ND | Beta1 | *pheU* | + |
| 081319 | Kanagawa | 2008 | No data | Di, BD | H11 | [H11] | $stx_1$ | ND | Beta1 | *pheU* | + |
| 082111 | Miyagi | 2008 | Sporadic | Di, AP | H11 | [H11] | $stx_1$ | *torS/T*[g] | Beta1 | *pheU* | + |
| 100207 | Miyazaki | 2010 | No data | Di, AP, Fe | H11 | [H11] | $stx_1$ | *torS/T* | Beta1 | *pheU* | – |
| 100952 | Fukuoka | 2010 | Outbreak (2) | Di, AP, BD | H11 | [H11] | $stx_1$ | ND | Beta1 | *pheU* | + |
| 102394 | Gifu | 2010 | Outbreak (2) | Di | H11 | [H11] | $stx_1$ | *torS/T* | Beta1 | *pheU* | + |
| 101624 | Saitama | 2010 | Sporadic | Di, AP | HUT | [H11] | $stx_1$ | *torS/T* | Beta1 | *pheU* | - |
| 110780 | Miyagi | 2011 | Sporadic | Di, AP, Fe | HUT | [H11] | $stx_1$ | *torS/T* | Beta1 | *pheU* | + |
| 071049 | Osaka | 2007 | Sporadic | Di, AP | H- | [H11] | $stx_1$ | ND | Beta1 | *pheU* | – |
| 080056 | Nagasaki | 2008 | Outbreak (3) | AP | H- | [H11] | $stx_1$ | *sbcB* | Beta1 | *pheU* | – |
| 090688 | Yamaguchi | 2009 | Sporadic | Di, Fe | H25 | [H25] | $stx_1$ | ND | Theta | ND | + |
| 070373 | Miyagi | 2007 | Sporadic | Di, AP, BD | HUT | [H25] | $stx_1$ | ND | Theta | ND | + |
| 080984 | Yamagata | 2008 | Outbreak (2) | Di, BD | HUT | [H25] | $stx_1$ | ND | Theta | ND | + |
| 082332 | Mie | 2008 | Sporadic | Di, AP | HUT | [H25] | $stx_1$ | ND | Theta | ND | + |
| 080455 | Nara | 2008 | Sporadic | Di, AP, Fe, BD | HUT | [H25] | $stx_1$ | ND | Theta | ND | + |
| 082589 | Yamagata | 2008 | Sporadic | Di, AP, Vo, BD | H2 | [H2] | $stx_1$ | *torS/T* | Epsilon | ND | + |
| 092412 | Nagano | 2009 | Sporadic | AP, BD | H2 | [H2] | $stx_1$ | *torS/T* | Epsilon | ND | + |
| 071556 | Fukuoka | 2007 | Outbreak (2) | Di, AP, BD | H2 | [H2] | $stx_1$ | *torS/T* | Epsilon | ND | + |
| 111471 | Kagoshima | 2011 | Sporadic | BD | H2 | [H2] | $stx_1$ | *torS/T* | Epsilon | ND | + |
| 111336 | Miyagi | 2011 | Sporadic | Di, AP | H2 | [H2] | $stx_1$ | *torS/T* | Epsilon | ND | + |
| 111155 | Kagoshima | 2011 | Outbreak (4) | BD | HUT | [H2] | $stx_1$ | *torS/T* | Epsilon | ND | + |

[a] The numbers in parentheses indicate the numbers of confirmed patients (including asymptomatic carrier) in each outbreak.
[b] Di, diarrhea; AP, abdominal pain; Fe, fever; Vo, vomiting; BD, bloody diarrhea.
[c] HUT, untypeable; H−, nonmotile.
[d] Types listed in square brackets were determined by sequence comparison of the *fliC* gene.
[e] ND, not determined.
[f] tRNA gene.
[g] *torS/T*, *torS-torT* intergenic region.

tively. In addition, the sequences of five whole-genome-sequenced STEC strains were used: O157:H7 Sakai (accession number BA000007) (10), O26:H11 11368 (AP010953), O103:H2 12009 (AP010958) and O111:H− 11128 (AP010960) (24), and O104:H4 TY-2482 (AFVR01000000) (33).

**PCR analysis of virulence markers.** The following 13 pathotype-associated genes were detected by PCR: $stx_1$ and $stx_2$ (4), *ehxA* (encoding enterohemolysin) (27) and *eae* (26), associated with enterohemorrhagic *E. coli* (EHEC) and/or enteropathogenic *E. coli* (EPEC); *bfpA* (encoding bundle-forming pilus) (9), associated with typical EPEC; *elt* (encoding heat-labile enterotoxin) and *est* (heat-stable enterotoxin) (39), associated with enterotoxigenic *E. coli*; *astA* (encoding heat-stable enterotoxin EAST1) (44) and *aggR* (encoding transcriptional activator of aggregative adherence fimbriae I expression) (6), associated with enteroaggregative *E. coli* (EAEC); *ipaH* (encoding invasive plasmid antigen H) (36), associated with enteroinvasive *E. coli*; *cdtV* (encoding cytolethal distending toxin [CDT] V, a member of the CDT family, associated with tissue damage [3]) (5); *subAB* (encoding subtilase cytotoxin) (22); and *saa* (encoding STEC autoagglutinating adhesin) (28). All PCRs were performed according to the protocols described previously.

**Sequencing of *fliC*, *eae*, and seven housekeeping genes.** The H type was genetically determined by sequence comparison of the *fliC* gene. The entire coding region of *fliC* was amplified and sequenced using the primers F-FLIC-out (5′-TTAAATCCAGACCTGACCCGA-3′) and R-FLIC-out (5′-CCACAGCGAGTGTTTATCCAT-3′), and an additional primer F-FLIC1 (8) was used for internal sequencing of *fliC*. The entire coding region of *eae* was amplified and sequenced using two primer pairs: cesT-F9 and eae-R3 for N-terminal protein, and eae-F1 and escD-R1 for C-terminal protein (11). The internal regions of the seven housekeeping genes (*adk*, *fumC*, *gyrB*, *icd*, *mdh*, *purA*, and *recA*) were PCR amplified and sequenced using the primers and protocol specified on the *E. coli* multilocus sequence typing (MLST) website (http://mlst.ucc.ie/mlst/dbs/ecoli).

**MLSA.** The concatenated sequences (3,423 bp) of seven housekeeping genes (*adk*, *fumC*, *gyrB*, *icd*, *mdh*, *purA*, and *recA*) from O103 strains were used for multilocus sequence analysis (MLSA). In addition, the sequences of five whole-genome-sequenced STEC strains (O157:H7, O26:H11, O103:H2, O111:H−, and O104:H4) and three well-characterized STEC serotype strains (O121:H19, O165:H−, and O145:H−) were included in the analysis. *E. coli* reference strains, the ECOR collection, were also used for MLSA. Multiple alignments of sequences were constructed by using the CLUSTAL W program (41) in the MEGA4 software (40), and then neighbor-joining trees were generated by using the Tamura-Nei model. A bootstrap test with 1,000 replicates was used to estimate the confidence of the branching patterns of the tree. Sequences of the ECOR collection for MLSA and the sequence type (ST) of the STEC O103 strains were obtained from the *E. coli* MLST database (http://mlst.ucc.ie/mlst/dbs/ecoli).

**Determination of Stx1 phage and LEE integration sites.** Thus far, seven genomic loci (*torS-torT* intergenic region, *wrbA*, *yehV*, *prfC*, *sbcB*, *argW*-tRNA, and *ssrA*-tmRNA) have been identified as integration sites of $stx_1$-containing bacteriophages (Stx1 phages) (23). To determine integration sites for Stx1 phages on the chromosome, a universal PCR primer (Pstx1A-F, 5′-AAACCGCCCTTCCTCTGGAT-3′) targeted to the $stx_{1A}$ gene on the prophage and seven primers (Pstx1_tosRS-R, 5′-TTCAGGCTTTGTGCGGTGAG-3′; Pstx1_wrbA-R, 5′-CTCTCTGTTAACGGCGCTGGAT-3′; Pstx1_yehV-R, 5′-TGCCAGCGTGACAGAAGTTG-3′; Pstx1_prfC-R, 5′-ATCGGCATCATCACCAACGG-3′; Pstx1_sbcB-R, 5′-GCGGAACATCAATCAACGCCA-3′; Pstx1_argW-R, 5′-TCAACTTCTGGTTGGTCTCGC-3′; and Pstx1_ssrA-R, 5′-TCCTACCCGTACCCGCAAGTT-3′) targeted to the outside of each prophage region were designed on the basis of the genome sequences of the STEC strains O157:H7



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TABLE 2 Primers used for multiplex PCR

| Target | Primer | Sequence (5′–3′) | Observed amplicon size (bp) |
|---|---|---|---|
| Universal forward primer for *fliC* | fliC_univ_F | ATGGCACAAGTCATTAATAC | |
| *fliC* (H11) | fliC_H11_R | TATTCTTAGCCGCTGCTGC | 755 |
| *fliC* (H2) | fliC_H2_R | TATCCTGATCAGAAGCCAGCA | 417 |
| *fliC* (H25) | fliC_H25_R | TGCGGGATAGATGTGATAGCA | 559 |
| *wzy* (O103) | O103_wzy_F | CTCTTGCTGCTATGAGCTTTG | 297 |
| | O103_wzy_R | GCGGGGTCTTGTCATTTAAT | |

Sakai, O26:H11 11368, O103:H2 12009, and O111:H− 11128. In addition, integration sites (*pheV*, *pheU*, and *selC* tRNA gene loci) of LEE elements were screened by primers described elsewhere (25). Long-range PCR screenings were performed by using TaKaRa LA *Taq* polymerase (TaKaRa Bio, Inc., Ohtsu, Japan).

**Sequence analysis of the O103-antigen biosynthesis gene cluster and its flanking region.** The O103-antigen biosynthesis gene cluster and its flanking regions were amplified using a PCR primer pair, O55re-1F and O55re-1R (12). Each PCR product was sequenced by the shotgun method. Sequences were aligned using Sequencher software (v4.9; Gene Code Corp., Michigan), and sequence comparisons were performed by using *in silico* molecular cloning software (In Silico Biology, Yokohama, Japan).

**Multiplex PCR assay.** The primers used for multiplex PCR and the lengths of the amplicons are listed in Table 2. A universal primer designed on the basis of the N-terminal sequences of *fliC* and specific primers designed on the basis of the highly diversified sequences (middle part) of each *fliC* gene were used. In addition, primers targeting the *wzy* (O103) gene were also used for control amplification. Multiplex PCR was performed with a 15-μl reaction mixture containing 10 ng of genomic DNA, 1× Kapa *Taq* buffer, each deoxynucleoside triphosphate at 0.3 mM, 2.5 mM $MgCl_2$, 0.25 μM fliC_univ_F primer, 0.25 μM fliC_H2_R primer, 0.25 μM fliC_H25_R primer, 0.38 μM fliC_H11_R primer, 0.5 μM O103_wzy_F primer, 0.5 μM O103_wzy_R primer, and 0.4 U of Kapa *Taq* DNA polymerase (Kapa Biosystems, Woburn, MA). The thermocycling condition was 25 cycles of 94°C for 20 s, 57°C for 20 s, and 72°C for 30 s. The PCR products (2 μl) were electrophoresed in 1.5% in agarose gels in 0.5× TBE (25 mM Tris borate, 0.5 mM EDTA) and photographed under UV light after the gel was stained with ethidium bromide.

**Nucleotide sequence accession number.** The sequences of the two O103-antigen gene clusters from O103:H25 and O103:H11, and of the *adk*, *fumC*, *gyrB*, *icd*, *mdh*, *purA*, and *recA* genes were deposited in GenBank/EMBL/DDBJ database under accession numbers AB704860, AB704861, and AB704965 to AB705139, respectively.

## RESULTS

**Characterization of O103:non-H2 strains.** Seventeen STEC O103:non-H2 strains isolated from patients in Japan were investigated (Table 1). Six strains were isolated from disease outbreaks, nine were from sporadic cases, and two were from cases for which no information was available. Seven strains were classified as H11 type and one as H25 type by using agglutination assays. Seven additional strains were classified as HUT, because their H types could not be determined due to no or low agglutination, or because aggregation was observed for multiple anti-H antisera. The remaining two strains showed no motility.

***fliC* analysis.** The sequence analysis of *fliC* from all O103 strains examined showed that the amino acid sequences (487 amino acids [aa]) of two HUT (101624 and 110780) and two H− (071049 and 080056) strains were identical to those of H11-expressing O103 strains. The sequences (443 aa) of four HUT strains (070373, 080984, 082332, and 080455) were identical to that of H25-expressing O103 strain 090688, except for one amino acid difference in 080984. In addition, the sequence (494 aa) of OUT strain 111155 was identical to that of H2-expressing O103 strains and that of the fully sequenced O103:H2 strain. These results indicated that all of the control and experimental O103 strains were one of the following three H types: H2/[*fliC*-H2], H11/[*fliC*-H11], or H25/[*fliC*-H25] (Table 1). By comparison, the sequence identities of FliC between H2 and H11, between H11 and H25 (090688), and between H25 and H2 were 55.4, 50.4, and 49.4%, respectively.

**PCR screening of virulence-related genes.** PCR-based screening for *E. coli* virulence-related genes showed that all O103 strains possessed $stx_1$ and *eae* and that 18 of the strains examined carried *ehx* (Table 1). The remaining 10 genes ($stx_2$, *bfpA*, *elt*, *est*, *astA*, *aggR*, *ipaH*, *cdtV*, *subAB*, and *saa*) included in the screen were absent from all strains examined.

***eae* typing.** The results of sequence analysis of *eae* from all O103 strains are shown in Table 1. The amino acid sequences of the H2/[H2] and H11/[H11] strains (948 and 939 aa, respectively) were identical to those of O103:H2 strain 12009 and O26:H11 strain 11368, respectively, indicating that H2/[H2] and H11/[H11] strains possess the *eae* genes encoding epsilon and beta1 subclass intimins (*eae*-epsilon and *eae*-beta1, respectively). In addition, the sequences (935 aa) of H25/[H25] strains were identical to that of O111:H− strain 11128, indicating that H25/[H25] strains possess *eae*-theta.

**Integration site of Stx1 phages and LEE elements.** Long-range PCR screening targeting seven alternative integration sites of Stx1 phages was performed. All six H2/[H2] and five H11/[H11] strains were found to contain the Stx1 phage in the *torS*-*torT* intergenic region, and one H11/[H11] strain contained it in the *sbcB* locus (Table 1). The integration site in the other strains was not determined by these methods (Table 1). PCR screening analysis for three alternative integration sites of LEE showed that all H11/[H11] strains possess LEE elements in the *pheU* locus. The integration site of LEE in H2/[H2] and H25/[H25] was not determined (Table 1).

**Phylogenetic relationship of O103 strains.** We analyzed the phylogenetic relationships among O103 strains and well-known strains from the STEC serotype collection. As shown in Fig. 1, the O103:H11/[H11] and O103:H25/[H25] strains formed two distinct groups, different from that of O103:H2/[H2] strains. The O103:H11/[H11] strains formed two groups with one nucleotide difference and were closely related to STEC O26:H11, while the O103:H25/[H25] strains were associated with Shiga toxin-producing EAEC O104:H4. The O103:H2/[H2] strains belonged to ST17 and the O103:H25/[H25] strains to ST343 (Fig. 1). One group of O103:H11/[H11] strains belonged to ST21, which was associated with O26:H11, and the other belonged to ST723 (Fig.

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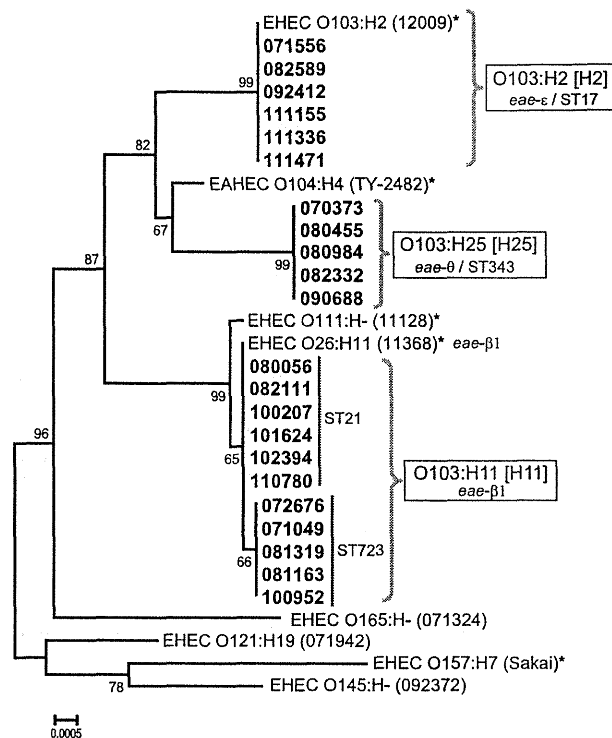


FIG 1 Phylogenetic relationships of O103-serogroup strains among eight well-characterized STEC serotypes strains. The phylogenetic tree was constructed on the basis of the concatenated sequences of seven housekeeping genes by using the neighbor-joining algorithm. Bootstrap analysis was performed with 1,000 replicates.

1). Compared to the sequences of the ECOR collection strains, three O103 groups belonged to the B1 phylogroup (data not shown). Pulsed-field gel electrophoresis pattern analysis revealed diverse populations of STEC O103:H11 and O103:H25 strains. For the O103:H11/[H11] classification, however, two strains (071049 and 101624) and three strains (082111, 100207, and 110780) differed by fewer than four bands within each of these two groups, indicating that they were genetically closely related (data not shown).

**Sequences of the O103-antigen biosynthesis gene cluster.** To gain more information about the genetic similarity of the O103-antigen encoding region among the three lineage groups, the sequences of the O103-antigen gene cluster of a representative strain from each lineage (072676 for O103:H11/[H11] and 080984 for O103:H25/[H25]) were determined and compared to that of STEC O103:H2 12009. The gene organization of the O103-antigen gene cluster was identical among the three strains, and their sequences were highly conserved except for three genes (*ugd*, *rmlB*, and *galF*) in the O103:[H25] strains (Fig. 2). In addition, the sequences of the O-antigen gene cluster and its flanking regions of O103:H11 were compared to those of O26:H11, which is closely related to O103:H11. As shown in Fig. 2, in addition to the flanking genes, three upstream genes (*wzz*, *ugd*, and *gnd*) and two downstream genes (*rmlB* and *rmlF*) in the O-antigen gene cluster were conserved between the O103 and O26 strains (94.1 to 99.7% identity).

**Multiplex PCR.** We developed a multiplex PCR system for classifying the pathogenic O103 strains that were confirmed to possess the *stx* and/or *eae* gene(s). Because *fliC* alleles encoding each of the H2, H11, and H25 antigens were lineage-specific among the STEC O103 strains (Fig. 1), this multiplex PCR method targeting *fliC* provided a rapid way to classify STEC O103 strains into three clonal groups. On the basis of the sequences of the O103-antigen gene clusters obtained in the present study, primers targeting the *wzy* (O103) gene were also designed for control amplification. The validity of the multiplex PCR system was confirmed using 22 STEC O103 control strains and three different H-antigen serotype control strains (O128:H2, O130:H11, and O156:H25). All PCR products matched the predicted sizes of the *fliC* (H2) (417 bp), *fliC* (H11) (755 bp), *fliC* (H25) (559 bp), and *wzy* (O103) (297 bp) genes, and the expected band patterns (Fig. 3).

## DISCUSSION

Although STEC O103:H2, O26:H11 and O111:H− strains belong to the *E. coli* B1 phylogroup and are closely related, especially O26:H11 and O111:H-, genomic analyses support the hypothesis that independent acquisition of Stx phages, LEE elements and many other virulence-related genes has driven the emergence of each STEC (24).

In the present study, 17 STEC O103-serogroup strains were classified into three distinct clonal groups coincident with variations in their *fliC* and *eae* genes (Fig, 1). A key finding was that strains belonging to the O103:H11/[H11] group were closely related to STEC O26:H11, suggesting that the STEC O103:H11 and STEC O26:H11 clones evolved from a common ancestor with one or more exchange(s) of the region encoding O-antigen biosynthesis. It is known that EHEC O157:H7 emerged from an O55:H7-like EPEC ancestor by specific events including acquisition of the O157-antigen biosynthesis gene cluster by horizontal gene trans-





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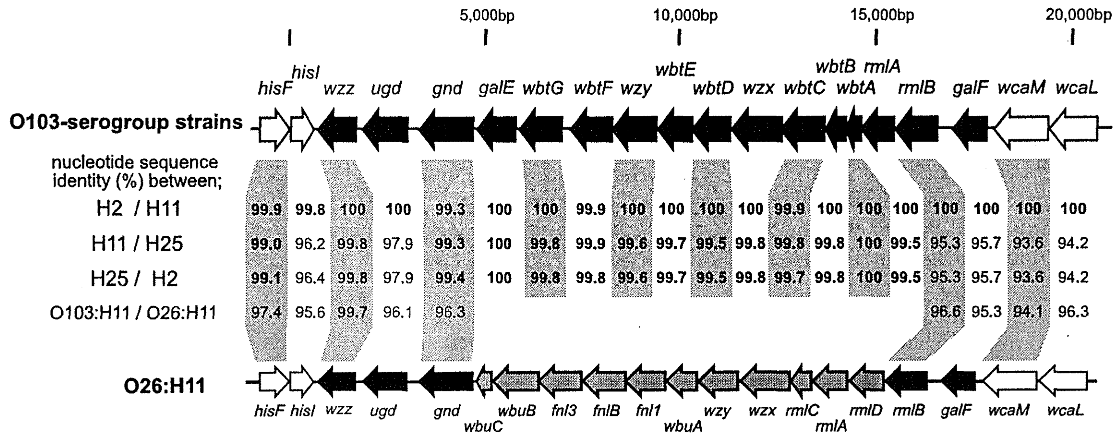


FIG 2 Comparison of O103-antigen biosynthesis gene clusters and their flanking regions. The genetic organization of the O103-antigen gene cluster and its flanking regions from O103 serotype strains is shown at top and that from STEC O26:H11 11368 (AP010953) is shown at the bottom. Genes associated with O-antigen biosynthesis are indicated by black arrows, and flanking genes are indicated by white arrows. O26-specific genes are indicated by gray arrows. Nucleotide sequence identities (%) between O103:H2 and O103:H11, between O103:H11 and O103:H25, and between O103:H25 and O103:H2 are shown in the middle. In addition, sequence identities between O103:H11 and O26:H11 are also shown.

fer (43), and a previous genome-wide sequence comparison showed that a large region of up to 130 kb including the O-antigen gene cluster was replaced by the result of recombination events (17). From the present sequence comparison of the O-antigen gene cluster and its flanking region between STEC O103:H11 and STEC O26:H11, a level of sequence conservation comparable to that of housekeeping genes (representing the backbone of the chromosome and nearly 100% conserved on the basis of the sequences of genes for MLSA) was not observed in the neighboring genes except for *wzz* (99.7%), suggesting that replacement of the region containing the O-antigen gene cluster occurred across a larger region.

Beutin et al. (1) demonstrated considerable diversity among STEC/EPEC O103 strains, which was investigated by MLST and *eae* typing. O103:H2 strains were predominantly positive for *eae*-epsilon, whereas an O103:H11 strain, whose MLST profile was different from those of the O103:H2 strains, was positive for *eae*-beta1. Ogura et al. (23) demonstrated that LEE elements are generally found at specific loci within the clonal groups and, among all six STEC O26:H11/H− strains tested, LEE elements with *eae*-beta1 were located at the *pheU*-tRNA locus. The O103:H11/[H11] strains tested also carried LEE elements with *eae*-beta1 at the *pheU* locus (Table 1), suggesting that, after acquiring a LEE element with *eae*-beta1 in the *pheU* locus, a LEE-positive common ancestor divided into the two clonal groups of STEC O26:H11 and O103:H11. On the other hand, the presence and location of Stx phages are known to be unsteady even within a clonal group. Stx1 phages in O157:H7 strains have been found in at least three different loci: *sbcB*, *yehV*, and *argW* (23). It is known that STEC O26:H11 strains carried the Stx1 phage at the *wrbA* locus (23); in contrast, five of the O103:H11/[H11] strains studied here carried the Stx1 phage in the *torS-torT* intergenic region, which was previously found to be an integration site in STEC O103:H2 (23), and one O103:H11/[H11] strain carried the Stx1 phage at the *sbcB* locus, which was found to be an integration site in O157:H7 (23). The remaining 10 strains characterized here had unknown integration sites. These results suggested that the Stx1 phage has integrated into different sites of the genome even among closely related strains, and it is not clear when the lineages associated with STEC O26:H11 and O103:H11 acquired the Stx1 phage(s).

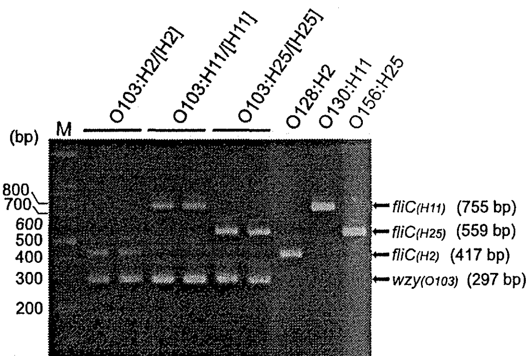


FIG 3 Multiplex PCR products of STEC O103 strains representing three groups. The strains used were 082589 and 111155 for O103:H2/[H2], 072676 and 071049 for O103:H11/[H11], 090688 and 082332 for O103:H25/[H25] and three non-O103 strains expressing either H2, H11, or H25 antigen. M, 100-bp DNA ladder markers.

A few cases of infection associated with STEC O103:H25 have been reported (30, 31, 42), and most isolates were found to be Stx1-producing strains. In 2006, however, an outbreak caused by Stx2-producing O103:H25 strains in Norway was reported (35). Among the 17 cases, 10 were children who developed HUS. The sequences of seven housekeeping genes for MLSA from Stx2-producing O103:H25 NVH-734 (GenBank accession no. AGSG01000000) (15) were identical to those of the Stx1-producing O103:H25 strains that we investigated, indicating that they belonged to the same clonal group (data not shown).

Although serotypes O103:H11 and O103:H25 are rare causes of EHEC disease, these serotypes used here were obtained from patients with diarrhea and hemorrhagic colitis. Because these O103 strains were the only bacteria known to cause these conditions, it is likely that the isolated strains caused these conditions. Thus, these serotype strains could be a threat to human health, and caution should be exercised around them. The clinical isolates

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characterized here were geographically and temporally dispersed, suggesting that these pathogens are widespread throughout Japan. Precise O/H serotyping of STEC strains isolated from human and food sources is required for validation. In many cases, the O-serogroup classification of STEC strains provides enough information to presume its clonal relatedness to well-known O-serogroup strains. Our STEC O103 clinical isolates, however, belonged to three distinct clonal groups. Despite the fact that these strains had diverse genetic backgrounds, they all carried the EHEC marker genes $stx_1$, *eae*, and/or *ehx*. Although the H type can be a useful phenotypic marker for classifying strains, we could not determine the H type of some O103 isolates, because of unclear agglutination or lack of bacterial motility. As many researchers have shown before (8, 18, 29), sequence variation in the *fliC* gene could be a proxy for these agglutination tests. In the present study, on the basis of sequence variation in *fliC* genes, we developed a multiplex PCR method for such classification of STEC O103 strains. The PCR-based methodologies described in the present study may be utilized to aid clinical and epidemiological studies of the STEC O103 serogroup strains.

In conclusion, we demonstrated that STEC O103 from patients formed three distinct groups, and the group comprising O103:H11 strains was closely related to STEC O26:H11. These findings suggest that the STEC O103:H11 and O26:H11 clones evolved from a common ancestor and provide further insights into the high variability of STEC strains with emerging new serotypes.

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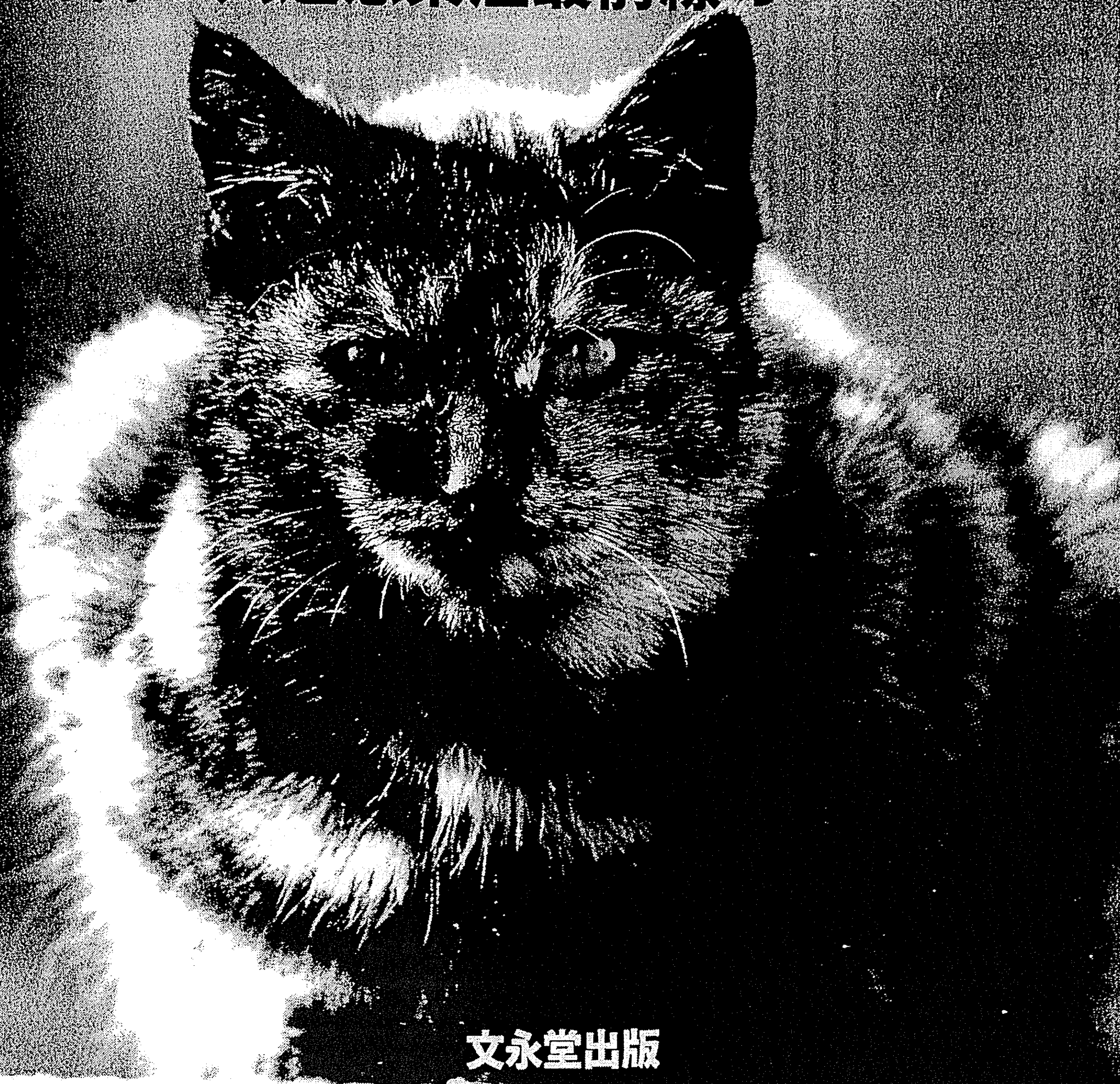
## 特集
## 人と動物の共通感染症最前線 9



文永堂出版

特集

人と動物の共通感染症最前線 9

# 腸管出血性大腸菌 O104による大規模集団食中毒事例

大西　真


## 要　約

2011年5月，ドイツ北部を中心に溶血性尿毒症症候群（HUS）が多発する事例が発生した。腸管出血性大腸菌（EHEC）O104:H4がその原因菌と同定され，さらにドイツで栽培された発芽野菜の汚染が本事例の原因であることが明らかとなった。本稿では下痢原性大腸菌およびEHECの性状を概説し，ドイツにおける血清群O104の腸管出血性大腸菌による大規模集団事例を紹介する。加えて本事例の原因株の細菌学的性状について紹介したい。


## 病原性大腸菌

大腸菌は遺伝的に多様な菌株の集団であり，病原性に関しても多彩である。大腸菌は人の腸管内の常在細菌の1つであり，多くは病原性を持たないが，特定の大腸菌は人に対する病原性を示す。また，病原性をもつ大腸菌の病原性は質的に多様である。病原性大腸菌は下痢等の原因となる腸管病原性を持つものと，膀胱炎等の原因となる腸管外病原性を持つものが存在する。下痢を引き起こす病原性大腸菌（下痢原性大腸菌）は，さらに少なくとも5つに分類される（表1）。

大腸菌の人に対する下痢原性が明らかにされはじめたのは1960年代のことである[14, 25, 29, 31, 36]。細菌性赤痢と類似の病態を示す大腸菌，あるいはコレラ様の下痢の原因となる大腸菌が存在することが知られていた[7, 29]。それぞれ腸管侵入性大腸菌 enteroinvasive *Escherichia coli*（EIEC），腸管毒素原性大腸菌 enterotoxigenic *E. coli*（ETEC）として分類されている[7, 15, 28]。さらに，腸管の微絨毛破壊を伴う特異的な病理変化を惹起する大腸菌が第3の下痢原性大腸菌として認識されることとなった（腸管病原性大腸菌 enteropathogenic *E.coli*：EPEC）[5, 37]。

EIEC，ETEC，そしてEPECを分類するには動物実験等が必要であったので，血清型による分類が下痢原性大腸菌の分類に大きな役割を果たしてきた。つまり，特定の血清型を示す大腸菌がある特定の臨床症状と関連することを利用したものであるが，血清分類に用いられる抗原そのものが病原性に関与しているわけではない。一方で，HEp-2細胞に対する付着形態から3種類に分類することが試みられた[6]。その中の1つ，HEp-2細胞上に微小コロニーを形成する付着形態はEPECあるいは後述する腸管出血性大腸菌 enterohaemorrhagic *E.coli*（EHEC）の付着形態と同一と考えられる。さらに，いわゆる"煉瓦を積み重ねた"様の付着形態を示す大腸菌と小児下痢症との関連が示され[24]，腸管出血性大腸菌に続いて5番目の下痢原性大腸菌（腸管凝集付着性大腸菌 enteroaggregative *E. coli*：EAggEC）として分類された（表1）。

## 腸管出血性大腸菌の発見

激烈な腹痛，水様性の下痢とそれに続く血性下痢を特徴とする食中毒事例が1982年米国においてに発生した。血清型O157:H7を示す大腸菌が患者検体および患者により共通に喫食された食材から分離された[27]。血清型O157:H7は既知の下痢原性大腸菌の示す血清型には合致しなかった。さらに，下痢に引き続く溶血性尿毒症症候群（HUS：溶血性貧血，血小板減少，腎機能不全を3主徴と


Makoto ONISHI：国立感染症研究所細菌第一部
〒162-8640　東京都新宿区戸山1-23-1

細菌第一部長。
1991年信州大学医学部卒業。同年～2000年細菌学助手。2001年より宮崎医科大学（現宮崎大学医学部）微生物学講師，助教授。2003～2005年ロックフェラー大学留学。2005年より国立感染症研究所細菌第一部　第五室室長。2010年より現職。病原性等における菌種内多様性に興味を持って研究に従事。

| 表1　下痢原性大腸菌の多様性 | |
|---|---|
| 下痢原性大腸菌 | 病原性の特徴 |
| 腸管侵入性大腸菌<br>enteroinvasive *E. coli* (EIEC) | 赤痢菌と同様の細胞侵入性に関与する病原因子をプラスミド上にコードしている。 |
| 腸管毒素原性大腸菌<br>ernterotoxigenic *E. coli* (ETEC) | 下痢原性に関与する毒素をコードするプラスミドを所持する。易熱性（heat-labile）毒素，耐熱性（heat-stable）毒素のいずれか，あるいは両毒素を産生する |
| 腸管病原性大腸菌<br>enteropathogenic *E. coli* (EPEC) | 典型的なEPECは初期接着に必要な束上線毛と，強固な接着に必要なLEE遺伝子群を持つ。初期接着とLEE遺伝子群の制御に必要な遺伝子はプラスミド上に存在する。<br>束上線毛を産生しない菌株は非典型的なEPECとして考えられるが，その病原性についての詳細は不明である。 |
| 腸管出血性大腸菌<br>enterohemorrahagic *E. coli* (EHEC) | 志賀毒素遺伝子はファージゲノムによってコードされている。典型的なEHECはEPEC同様，LEE依存的な細胞付着性を示す。<br>LEEを保持しないEHECの細胞付着性に関しては，不明な点が残されている。 |
| 腸管凝集付着性大腸菌<br>enteroaggregative *E. coli* (EAggEC) | 菌が相互に凝集しつつ，宿主細胞に付着する特徴的な性状を示す。この付着性状はプラスミドにコードされている。 |

する症候群）の患者便から，Vero細胞への毒性をもつ大腸菌が分離された[18]。本毒素は後に赤痢菌の産生する志賀毒素と同様のものであることが示され，加えてRileyらによって報告された大腸菌O157:H7も志賀毒素を産生することが示された。激しい腹痛と血性下痢を特徴とする病態を示す病原性大腸菌の存在と細胞毒性との関連，加えて溶血性尿毒症症候群との関連が初めて示唆されることとなった。

我が国においても1984年に志賀毒素産生性の大腸菌O145:H-による集団食中毒事例，1986年大腸菌O111:H-による集団事例が発生した。血清群O157による集団発生としては，1990年埼玉県での事例が最初である。1996年には大阪府における学童の大規模集団食中毒事件をはじめ全国的に大発生し，有症状者のみで1万人を超えた。その後，毎年3,000～4,000名程度のEHEC感染者数の報告がなされている。

## O血清群から見る腸管出血性大腸菌の多様性

日本国内で単離されるEHECは，血清群O157，O26あるいはO111を示す菌株が国内分離株の95%程度を占めている。その他の血清群としては，O103，O121，O91，O145，O165等の血清群の菌株が分離されている。EU諸国においても2009年において3600症例ほどの報告があり，血清群があきらかにされているもののうち（n＝2,565），我が国と同様O157（72%）が最も分離報告が多い。また，O26をくわえるとこの2つの血清群で80%を占めることになる。O103，O91，O145，O146，O128，O111，O103がそれぞれ1～3%の頻度で分離されている。

## 腸管出血性大腸菌の病原性因子

腸管出血性大腸菌の病原性は志賀毒素産生性によって規定される。志賀毒素は2種類存在し（Stx 1およびStx2），EHECは両者あるいはどちらか片方の遺伝子をもつ。毒素産生性に加え，腸管細胞への付着性がEHECの病原性には必須である。EHECの細胞付着性はEPECと同様の機構に依存しており，EHECはEPECが志賀毒素産生性を獲得したことでその特有の病原性を発揮するようになったと考えることが出来る。EHECの細胞付着性は，LEE（locus of enterocyte effacement）と呼ばれる染色体上に存在する遺伝子群にコードされる特異的な蛋白質輸送装置が必要とする。この強固な接着が成立する際には，菌の接着部分におけるアクチン線維の蓄積など細胞骨格系蛋白質の再構成による台座様構造の形成が起こる[12]。

しかしながら，LEE領域を保持しない非典型的なEHECが臨床検体から分離されることがある。EHEC菌株間の病原性の強弱について，さらにはLEE領域保有株と非保有株との病原性の相違について，詳細は不明な点が多く残されている。

## ドイツを中心に発生した腸管出血性大腸菌O104集団事例の概要

2011年5月初めからはじまった下痢症アウトブレイクは，3週間後の5月22日を最大発生数に達した。その後EHEC感染者数，HUS患者数ともに減少し，6月半ばには，散発的な発生を認めるだけになった[1,30]。これまでのところ最初の確定例の発症日は5月8日とされている。明ら