Biochemical features and virulence gene profiles of non-O157/O26 enterohemorrhagic Escherichia coli strains from humans in Yamaguchi Prefecture, Japan

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Biochemical features and virulence gene profiles of non-O157/O26 enterohemorrhagic
Escherichia coli strains from humans in Yamaguchi Prefecture, Japan

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SUMMARY

The biochemical features and virulence gene profiles of 37 enterohemorrhagic *Escherichia coli* (EHEC) strains belonging to serogroups other than O157 and O26 (non-O157/O26 EHEC) were investigated. All strains were isolated from humans between 2002 and 2013 in Yamaguchi Prefecture. Serogroup O111 strains were most common, followed by O103, O121, and O145. Most strains (84%) were negative for sorbose fermentation, whereas only one and two were negative for sorbitol and rhamnose fermentation, respectively. Two strains lacked β-D-glucuronidase activity. *stx* subtyping revealed six genotypes: *stx1a* (n=20), *stx1a + stx2a* (n=8), *stx2a* (n=4), *stx2b* (n=3), *stx2a + stx2c* (n=1), and *stx2a + stx2d* (n=1). Polymerase chain reaction screening of other toxin and adherence genes showed that *astA*, *subA*, and *cdtB* were present in five, two, and two strains, respectively. The intimin gene, *eae*, was present in 30 strains (81%). Of the seven *eae*-negative strains, *saa* and *eibG* were found in three and two strains, respectively; no adherence factors were detected in the remaining two strains. The antimicrobial susceptibility profiles of the strains to 12 drugs were examined and 11 strains (30%) showed resistant to one or more drugs. Our results revealed that non-O157/O26 EHEC strains show various biochemical phenotypes and carry several toxins and adherence factor genes.

INTRODUCTION

Enterohemorrhagic *Escherichia coli* (EHEC) is a human pathogen that causes bloody diarrhea, hemorrhagic colitis, and hemolytic uremic syndrome (HUS) (1). A broad range of EHEC serotypes have been associated with these illnesses and, in Japan, two major serogroups (O157 and O26) account for 57% and 23% of all human
isolates in 2012, respectively (2). However, infections caused by EHEC strains belonging to serogroups other than O157 and O26 (non-O157/O26 EHEC) are being reported with increasing frequency (3).

EHEC O157, O26, and O111 strains generally do not ferment sorbitol, rhamnose, and sorbose, respectively (4-6), biochemical features that are often used to identify the three serogroups from clinical or environmental specimens. Serogroup O157 also cannot produce β-D-glucuronidase (7). However, some strains with atypical phenotypes, such as sorbitol-positive or β-D-glucuronidase-positive O157, have recently emerged (8). Although much is known about these more common serogroups, information of the biochemical features of other EHEC serogroups is lacking.

Shiga toxin (Stx) is a major virulence factor of EHEC and is divided into two groups, Stx1 and Stx2 (9). The corresponding genes, stx1 and stx2, are further subdivided into three (stx1a, stx1c, and stx1d) and seven (stx2a–g) subtypes, respectively (10). Recent studies revealed that a set of EHEC strains produce other toxins, such as enteroaggregative E. coli heat-stable toxin (EAST1) (11), subtilase cytotoxin (SubAB) (12), and cytolethal distending toxin (CDT) (13), along with Stx. The majority of EHEC strains belonging to serogroups O157, O26, and O111 produce intimin. Intimin is encoded by eae, which is located in the locus of enterocyte effacement pathogenicity island, and is associated with intimate adhesion of the bacteria to cultured epithelial cells (14). However, some strains lack eae (15). Although almost all eae-negative strains have been isolated from asymptomatic carriers, some of these strains have been implicated in outbreaks of HUS (1). These eae-negative strains possess alternative adherence factors such as autoagglutinating adhesin (Saa) (16) and immunoglobulin-binding protein G (EibG) (15). In addition, some eae-negative EHEC
strains demonstrate aggregative adhesion to HEp-2 cells, in a manner similar to that of enteroaggregative *E. coli* (EAggEC) (17).

In the present study, biochemical features and distribution of virulence toxins and adhesion factor genes were investigated in non-O157/O26 EHEC strains isolated in Yamaguchi Prefecture.

**MATERIALS AND METHODS**

**Strains**

A total of 539 EHEC isolates were sent to our laboratory from hospitals and health care centers in Yamaguchi Prefecture, Japan. All samples were isolated between 2002 and 2013 from humans by the respective health care facilities. For confirmation of motility of the isolates, cultured colonies were inoculated into sulfide-indole motility (SIM) medium (Eiken Chemical, Tokyo, Japan) and incubation for 48 h at 25°C. The serotype was determined using commercial *E. coli* O and H antisera (Denka Seiken, Tokyo, Japan). The isolates being untypeable on the basis of O or H antigen testing were identified at the National Institute of Infectious Diseases in Tokyo, Japan.

**Biochemical features**

Carbohydrate fermentation using lactose, sorbitol, rhamnose, and sorbose was tested in semisolid media tube based on phenol red broth base (Becton, Dickinson and Company, Sparks, MD, USA) containing 0.3% (w/v) Bacto agar (Becton, Dickinson and Company) and 1.0% (w/v) of each carbohydrate. Inoculated tubes were incubated for 72 h at 37°C. Production of indole, lysine decarboxylase (LDC), and β-D-glucuronidase was confirmed in SIM medium, lysine indole motility medium (Kyokuto Pharmaceutical Industries, Tokyo, Japan), and cellobiose lactose indole β-D-glucuronidase medium.
(Kyokuto Pharmaceutical Industries), respectively, following incubation for 20 ± 2 h at 37°C (18).

**Stx production and stx subtyping**

Stx1 and Stx2 production was assessed using a reversed passive latex agglutination test (VTEC-RPLA; Denka Seiken, Tokyo, Japan) according to the manufacturer’s instructions. Detection and subtyping of stx genes was performed by polymerase chain reaction (PCR) (10) using genomic DNA prepared using a QIAamp DNA blood mini kit (Qiagen Sciences, MD, USA) according to the manufacturer’s instructions.

**Detection of virulence toxins and adherence factor genes**

The toxin-encoding genes astA, cdtB, and subA and adherence factor genes eae, aggR, saa, and eibG were detected by PCR (11, 12, 15, 19-22). Additional toxin genes lt, sth, and stp, coding for heat-labile enterotoxin (LT) and heat-stable enterotoxins of the human (STh) or porcine (STp) variety, respectively, were also screened by PCR using commercial primer sets (Takara Bio, Shiga, Japan) according to the manufacturer’s instructions.

**Susceptibility to antimicrobial agents**

The susceptibility of all strains to various antimicrobial agents was tested by employing the Kirby-Bauer disk diffusion method (23) on Mueller-Hinton agar (Oxoid, Hampshire, UK) plates containing the following antimicrobial agents (Nippon Becton Dickinson, Fukushima, Japan): ampicillin (AMP), cephalothin (CEF), cefotaxime (CTX), streptomycin (STR), kanamycin (KAN), gentamicin (GEN), tetracycline (TET), chloramphenicol (CHL), nalidixic acid (NAL), ciprofloxacin (CIP), fosfomycin (FOM), and trimethoprim-sulfamethoxazole (SXT).
RESULTS

Non-O157/O26 EHEC strains

Of the 539 isolates collected, 423 (78.5%) were identified as serogroup O157, and 72 (13.4%) were O26. Of the remaining 44 non-O157/O26 isolates, a total of 37 epidemiologically-unrelated strains, consisted of 33 strains were from sporadic cases and one representative strain from four each outbreaks, were subjected to further analysis (Table 1). Serogroup O111 was dominant (n=15), followed by O103 (n=6), O121 (n=4), and O145 (n=3). One strain, 09Y56, was O antigen untypable (OUT), and four strains, 07Y16, 07Y30, 06Y27 and 06Y32, were H antigen untypable (HUT). Eighteen strains were non-motile (NM). Twenty-nine (65%) of the strains were isolated from patients, while the remaining isolates were from asymptomatic carriers. Strains were isolated from patients displaying the following clinical symptoms: watery diarrhea (n=23), abdominal pain (n=20), fever (n=10), bloody diarrhea (n=9), vomiting (n=4), and intussusception (n=1). HUS was not observed in any of the patients. Of the strains isolated from asymptomatic carriers, O91:H14 and O146:NM accounted for two strains each, while one strain was identified from each of the serotypes O111:NM, O112ab:H25, O169:H9, and OUT:NM.

Biochemical features

All strains were positive for lactose fermentation and indole production. Most strains (84%), including all O111 isolates, were negative for sorbose fermentation, whereas only one (OUT:NM) and two (O111:NM and O103:H11) strains were sorbitol- and rhamnose-negative, respectively. Seventeen strains, including all serogroup O111 isolates, were negative for LDC production, and two strains (O103:H2 and O103:H25)
lacked β-D-glucuronidase activity (Table 1).

**Characterization of virulence factors**

As shown in Table 1, the eight O111 strains produced both Stx1 and Stx2, while other strains produced either Stx1 or Stx2. Six stx genotypes were identified: stx1a (n=20), in Stx1-producing strains, stx1a + stx2a (n=8), in Stx1 and Stx2-producing strains, and stx2a (n=4), stx2a + stx2c (n=1), stx2a + stx2d (n=1), and stx2b (n=3), in Stx2-producing strains.

Of the toxin genes, astA was detected in O121:H19 (n=3), O146:NM (n=1), and O169:H9 (n=1), subA was detected in two O91:H14 strains, and cdtB was found in one strain each of O111:NM and O112ab:H25. The adherence factor gene eae was detected in 30 strains (81.1%). Of the seven eae-negative strains, saa was detected in two O91:H14 and one O112ab:H25 strains, and cibG was found in two O146:NM strains. No adherence factor genes were detected in two strains, O169:H9 and OUT:NM.

**Antimicrobial agent susceptibility**

Of the 37 tested strains, 11 (29.7%) showed resistance to one or more antimicrobials (Table 2). The seven O111 strains were resistant to AMP, STR, and TET, and two of these strains, 06Y27 and 12Y03, exhibited additional resistance to KAN and NAL, and CEF and KAN, respectively. The three O121:H19 strains were resistant to STR and TET, and an OUT:NM strain showed resistance to TET.

**Discussion**

We investigated various biochemical phenotypes used in identification of non-O157/O26 EHEC strains. Sorbose can be used an indicator for the isolation of EHEC O111; however, most other strains were also negative for sorbose fermentation.
A few strains with sorbitol- or rhamnose-negative phenotypes were also found. Interestingly, strain 10Y02 (serotype O111:NM) was negative for both rhamnose and sorbose fermentation. Two of the 37 non-O157/O26 strains were not able to produce β-glucuronidase, a characteristic shared by serogroup O157 strains. We have previously encountered β-D-glucuronidase-negative EHEC O26:H11 infections (18). Therefore, these findings show that it is important to consider atypical phenotypic characteristics when identifying EHEC isolates.

We identified a group of non-O157/O26 strains that produced Stx along with either EAST1, SubAB, or CDT. Although EAST1 was first discovered in an EAggEC strain, the toxin is also present in various other types of E. coli (11). EHEC O157 often harbors ast4, coding for EAST1 (24), and in this study, serogroups O121, O146, and O169 also carried this toxin gene. SubAB is a newly-identified toxin that was first identified in an eae-negative, saa-positive EHEC O113:H21 strain that was responsible for a small outbreak of HUS in South Australia (25). A recent study revealed that the various serogroups of eae-negative EHEC, including O91, produce SubAB (12). In the current study, the two O91:H14 strains carried the toxin gene. CDT has been detected in eae-negative EHEC strains that were isolated from humans and domestic animals (13, 26). In this study, two cdt-carrying strains were obtained, and of those, one was eae-negative (O112ab:H25) and the other was eae-positive (O111:NM). The cdt gene in E. coli is usually located either on the chromosome or on a large plasmid (13). The location of cdt in the strains used in the current study requires further study.

The intimin, encoded by eae, is a virulence factor for EHEC. In the present study, the eae was detected in 30 strains (81.1%), and all strains from patients (n=29) were eae-positive. The observation indicates the presence of eae may strongly associate
with disease in humans. \textit{eae}-negative EHEC is occasionally associated with severe diseases that are indistinguishable from those caused by \textit{eae}-positive strains (1). In this study, seven \textit{eae}-negative strains were found, but all were obtained from asymptomatic carriers. \textit{saa} and \textit{eibG} are commonly found in multiple serotypes of \textit{eae}-negative EHEC, and were originally detected in serogroup O113 and O91 strains, respectively (15, 16). In the present study, \textit{saa} was found in serogroup O91 and O112ab isolates, while \textit{eibG} was amplified from O146:NM strains. While no adherence factor gene was found in isolates belonging to serotypes O169:H9 or OUT:NM, an alternative adherence factor might exist in these strains.

Antimicrobial susceptibility testing showed that 29.7\% of the strains showed resistance to one or more antibiotics. Seto et al. (27) demonstrated that 40.5\% of non-O157/O26/O111 strains were resistant to at least one antibiotic, a resistance ratio that is much higher than that of our study. No resistance was observed towards the drugs used for treatment of EHEC infection, such as fosfomycin and fluoroquinolones. Recently extended-spectrum cephalosporin-resistant EHEC O26:H11 emerged in Japan (28). This finding, along with results of the current study, indicates that continuous monitoring of antimicrobial susceptibility in EHEC strains is needed.

In conclusion, non-O157/O26 strains show various biochemical phenotypes and carry several toxin and adherence factor genes. The phenotypic characteristic of being negative for sorbose fermentation might be useful for the identification of non-O157/O26 EHEC strains, although this requires confirmation through further screening of a large number of non-O157/O26 strains. In addition, the potential role of EAST1, SubAB, and CDT in the pathogenicity of EHEC should be investigated.
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Conflict of interest

None to declare.

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| Strain no. | Serotype | Year of isolation | Clinical features | Production subtype | Other virulence toxin gene | Adherence factor gene | Lactose | Sorbitol | Rhamnose | Sorbose | β-D-glucuronidase | Fermentation of 2) | \( β \)-D-glucuronidase |
|-----------|----------|------------------|-------------------|-------------------|---------------------------|----------------------|---------|---------|----------|---------|----------------|---------------------|-------------------|
| 02Y01     | O111:NM  | 2002             | DB, F, V          | 1, 2              | 1a, 2a                    | eae                  | ++      | +       | -        | -       | +             | ++                  | ++                |
| 06Y04     | O111:NM  | 2006             | AP, DW            | 1, 2              | 1a, 2a                    | eae                  | +       | +       | +        |        | -             | ++                  | ++                |
| 07Y16     | O111:HUT | 2007             | AP, DB, DW, F     | 1, 2              | 1a, 2a                    | eae                  | +       | +       | +        |        | -             | ++                  | ++                |
| 07Y30     | O111:HUT | 2007             | DB                | 1, 2              | 1a, 2a                    | eae                  | +       | +       | +        |        | -             | ++                  | ++                |
| 09Y36     | O111:NM  | 2009             | AP, DB, DW        | 1, 2              | 1a, 2a                    | eae                  | +       | +       | +        |        | -             | ++                  | ++                |
| 10Y02     | O111:NM  | 2010             | –                 | 1, 2              | 1a, 2a                    | eae                  | +       | +       | +        |        | -             | ++                  | ++                |
| 11Y14     | O111:NM  | 2011             | AP, DW            | 1, 2              | 1a, 2a                    | eae                  | +       | +       | +        |        | -             | ++                  | ++                |
| 11Y18     | O111:NM  | 2011             | AP, DB            | 1, 2              | 1a, 2a                    | eae                  | +       | +       | +        |        | -             | ++                  | ++                |
| 02Y11     | O111:NM  | 2002             | DW                | 1                 | 1a                        | eae                  | +       | +       | +        |        | -             | ++                  | ++                |
| 04Y29     | O111:NM  | 2004             | AP, DB, DW        | 1                 | 1a                        | eae                  | +       | +       | +        |        | -             | ++                  | ++                |
| 06Y35     | O111:NM  | 2006             | DW, F             | 1                 | 1a                        | cdtB                  | +       | +       | +        |        | -             | ++                  | ++                |
| 06Y27     | O111:HUT | 2006             | AP, DW, F         | 1                 | 1a                        | eae                  | +       | +       | +        |        | -             | ++                  | ++                |
| 09Y17     | O111:NM  | 2009             | AP, DW            | 1                 | 1a                        | eae                  | +       | +       | (+)      |        | -             | ++                  | ++                |
| 12Y03     | O111:NM  | 2012             | AP, DW, F, V      | 1                 | 1a                        | eae                  | +       | +       | -        |        | -             | ++                  | ++                |
| 12Y24     | O111:NM  | 2012             | DW                | 1                 | 1a                        | eae                  | +       | +       | +        |        | -             | ++                  | ++                |
| 10Y24     | O103:H2  | 2010             | AP, DW, F         | 1                 | 1a                        | eae                  | +       | +       | +        |        | -             | ++                  | ++                |
| 10Y42     | O103:H2  | 2010             | AP, DW, F         | 1                 | 1a                        | eae                  | +       | +       | +        |        | -             | ++                  | ++                |
| 08Y07     | O103:H2  | 2008             | AP, DW            | 1                 | 1a                        | eae                  | +       | +       | -        |        | +             | ++                  | ++                |
| 12Y05     | O103:H2  | 2012             | DW                | 1                 | 1a                        | eae                  | +       | +       | +        |        | -             | ++                  | ++                |
| 09Y09     | O103:H11 | 2008             | AP, DB, DW, F     | 1                 | 1a                        | eae                  | +       | +       | -        |        | +             | ++                  | ++                |
| 09Y07     | O103:H25 | 2009             | DW, F             | 1                 | 1a                        | eae                  | +       | +       | (+)      |        | -             | ++                  | ++                |
| 07Y06     | O121:H19 | 2007             | AP, I, V          | 2                 | 2a                        | astA                  | +       | +       | +        |        | +             | ++                  | ++                |
| 07Y09     | O121:H19 | 2007             | DW                | 2                 | 2a                        | astA                  | +       | +       | +        |        | +             | ++                  | ++                |
| 07Y11     | O121:H19 | 2007             | AP, DW, V         | 2                 | 2a                        | astA                  | +       | +       | +        |        | +             | ++                  | ++                |
| 11Y11     | O121:H19 | 2011             | AP, DW            | 2                 | 2a                        | astA                  | +       | +       | +        |        | +             | ++                  | ++                |
| 07Y42     | O145:NM  | 2007             | AP, DW            | 1                 | 1a                        | eae                  | +       | +       | +        |        | -             | ++                  | ++                |
| 07Y46     | O145:NM  | 2007             | DW                | 1                 | 1a                        | eae                  | +       | +       | +        |        | -             | ++                  | ++                |
| 11Y12     | O145:NM  | 2011             | AP, DB            | 1                 | 1a                        | eae                  | +       | +       | (+)      |        | -             | ++                  | ++                |
| 06Y34     | O115:H10 | 2006             | AP, F             | 1                 | 1a                        | eae                  | +       | +       | -        |        | +             | ++                  | ++                |
| 06Y32     | O165:HUT | 2006             | AP, DB, DW        | 2                 | 2a, 2c                    | eae                  | +       | +       | (+)      |        | -             | ++                  | ++                |
| 09Y35     | O91:H14  | 2009             | –                 | 1                 | 1a                        | subA                  | (+)     | +       | +        |        | -             | ++                  | ++                |
| 10Y33     | O91:H14  | 2010             | –                 | 1                 | 1a                        | subA                  | +       | +       | +        |        | -             | ++                  | ++                |
| 10Y39     | O112ab:H25| 2010            | –                 | 2                 | 2a, 2d                    | cdtB                  | +       | +       | +        |        | -             | ++                  | ++                |
| 11Y22     | O146:NM  | 2011             | –                 | 2                 | 2b                        | astA                  | eibG     | +       | (+)      |        | -             | ++                  | ++                |
| 12Y12     | O146:NM  | 2012             | –                 | 2                 | 2b                        | astA                  | eibG     | +       | (+)      |        | -             | ++                  | ++                |
| 10Y40     | O169:H9  | 2010             | –                 | 1                 | 1a                        | astA                  | None     | +       | +        | (+)     | +             | ++                  | ++                |
| 09Y56     | OUT:NM   | 2009             | –                 | 2                 | 2b                        | None                  | -        | -       | +        | (+)     | -             | ++                  | ++                |

1) AP, abdominal pain; DB, bloody diarrhea; DW, watery diarrhea; F, fever; I, intussusception; V, vomiting; –, asymptomatic carrier
2) +, positive within 24 hrs; (+), positive after 24 to 72 hrs; -, negative at 72 hrs

Table 1 Biochemical features and virulence gene profiles of non–O157/O26 EHEC strains
| Strain | Serotype   | Antimicrobial resistance patterns ¹ |
|--------|------------|-----------------------------------|
| 06Y27  | O111:HUT   | TET, STR, AMP, KAN, NAL           |
| 12Y03  | O111:NM    | TET, STR, AMP, KAN, CEF           |
| 07Y16  | O111:HUT   | TET, STR, AMP                     |
| 11Y14  | O111:NM    | TET, STR, AMP                     |
| 11Y18  | O111:NM    | TET, STR, AMP                     |
| 04Y29  | O111:NM    | TET, STR, AMP                     |
| 12Y24  | O111:NM    | TET, STR, AMP                     |
| 07Y06  | O121:H19   | TET, STR                          |
| 07Y09  | O121:H19   | TET, STR                          |
| 07Y11  | O121:H19   | TET, STR                          |
| 09Y56  | OUT:NM     | TET                               |

¹) TET, tetracyclin; STR, streptomycin; AMP, ampicillin; KAN, kanamycin; NAL, nalidic acid; CEF, cefalothin.