Evaluation of a PCR-Restriction Fragment Length Polymorphism (PCR-RFLP) Assay for Molecular Epidemiological Study of Shiga Toxin-Producing Escherichia coli

Norihiko SUGIMOTO1,3, Kensuke SHIMA1,**, Atsushi HINENOYA1), Masahiro ASAKURA1,3), Akio MATSUHISA1,3), Haruo WATANABE2) and Shinji YAMASAKI1)*

1)Graduate School of Life and Environmental Sciences, Osaka Prefecture University, 1–58 Rinku Ourai-kita, Izumisano, Osaka 598–8531, 2)Department of Bacteriology, National Institute of Infectious Diseases, 1–23–1 Toyama, Shinjuku, Tokyo 162–8640 and 3)Research and Development Center, Fuso Pharmaceutical Industries, Ltd., 2–3–30 Morinomiya, Jouto-ku, Osaka 536–8523, Japan

(Received 11 January 2011/Accepted 28 January 2011/Published online in J-STAGE 10 February 2011)

ABSTRACT. In this study, we have evaluated our recently developed polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) assay for the molecular subtyping of Shiga toxin-producing Escherichia coli (STEC). A total of 200 STEC strains including O157 (n=100), O26 (n=50), O111 (n=10), and non-O26/O111/O157 (n=40) serogroups isolated during 2005–2006 in Japan, which were identified to be clonally different by pulse-field gel electrophoresis (PFGE) were further analyzed by the PCR-RFLP assay in comparison to PFGE. Ninety-five of O157, 48 of O26, five of O111 and 19 of non-O26/O111/O157 STEC strains yielded one to three amplification patterns for STEC strains [2, 15, 23, 35]. For instance, PFGE requires expensive and elaborate equipment, and skilled labor. PFGE is labor-intensive and fairly time-consuming. PFGE profiles of some strains cannot be analyzed because of smeared profiles, which may be associated with degradation of genomic DNA due to free radical produced during electrophoresis [8, 18, 37]. PFGE pattern of a single isolate of STEC could be altered by repeated subcultures in vitro [13, 36]. Passage through bovine or human gastrointestinal tract could also cause variation in PFGE patterns for STEC strains [2, 15]. Thus, there is a chance of misinterpretation of clonality of the same strain. In addition, it is not possible to handle large number of samples at a time [16].

Sato et al. identified 6 characteristic regions (I to VI) in Shiga toxin phage genome [33]. Among them, region V, which is located in the upstream region of the Stx2 operons, was identified to be the most distinct portion in the entire phage genome and may be a good target for molecular sub-

**PRESENT ADDRESS: SHIMA, K., Institute of Medical Microbiology and Hygiene, University of Lübeck, Ratzeburger Allee 160, 23538 Lübeck, Germany.

*CORRESPONDENCE TO: YAMASAKI, S., Graduate School of Life and Environmental Sciences, Osaka Prefecture University, 1–58 Rinku Ourai-kita, Izumisano, Osaka 598–8531, Japan. e-mail: shinji@vet.osakafu-u.ac.jp

**PRESENT ADDRESS: SHIMA, K., Institute of Medical Microbiology and Hygiene, University of Lübeck, Ratzeburger Allee 160, 23538 Lübeck, Germany.
typing of STEC strains. For this reason, we have developed a rapid and simple DNA fingerprinting method, PCR-restriction fragment length polymorphism (PCR-RFLP) assay based on the nucleotide sequence diversity within the region V of Stx-phage, for molecular epidemiological study of STEC strains [34–37]. In the present study, we have evaluated the PCR-RFLP assay targeting the region V of Stx-phages by using a variety of defined STEC strains including O157 and non-O157, which were identified to be clonally different by PFGE.

MATERIALS AND METHODS

Bacterial strains and growth media: A total of 200 STEC strains of various serogroups, including O157 (n=100), O26 (n=50), O111 (n=10) and non-O26/O111/O157 (n=40) were randomly selected from 1,795 strains which were provided from the prefectural and municipal health centers and public health institutes (PHIs) in Japan during 2005 to 2006. Serology was determined and all strains were identified to be different clone by PFGE at the National Institute of Infectious Diseases under the National Epidemiological Surveillance of Infectious Diseases undertaken in compliance with the Law Concerning the Prevention of Infectious Diseases and Medical Care for Patients of Infections [25, 26]. STEC strains used in this study were cultured either on L-agar or in L-broth.

Chemicals and enzymes: Chemicals were purchased either from Nacalai Tesque (Kyoto, Japan), Wako Pure Chemical Industries (Tokyo, Japan), or Sigma Chemical Co. (St. Louis, MO, U.S.A.). Restriction enzymes, Takara LA Taq, and LA PCR kit version 2 were purchased from Takara Bio (Shiga, Japan). Bacto tryptone and yeast extract were purchased from Becton, Dickinson and Company (Franklin Lakes, NJ, U.S.A.). Pulsed-field certified, low-melting point preparative-grade, Seakem GTG and Seakem HGT (for high gelling temperature) agaroses, were either from Bio-Rad Laboratories, Inc. (Hercules, CA, U.S.A.) or Takara Bio. Molecular weight makers were purchased from Takara Bio.

Detection of stx1 and stx2 genes by multiplex PCR: Presence of stx1 and stx2 genes was examined by multiplex PCR using EVT-1 and EVT-2 primers, and EVS-1 and EVC-2 primers, respectively, as described in Table 1 [30]. Briefly, 50 µl of overnight culture of STEC strains was added to 450 µl of TE buffer and the mixture was boiled for 10 min and snap cooled on ice. After centrifugation at 10,000 g for 10 min, supernatant was collected and stored at −30°C for further use as boiled template. PCR was carried out in 20 µl of reaction mixture for each tube containing 1 µl of DNA template, 1 × PCR buffer (10 mM Tris-HCl [pH 8.3], 50 mM KCl, and 1.5 mM MgCl2), 0.2 mM of dNTP, 0.625 U of rTaq polymerase (Takara Bio Inc.) by using GeneAmp PCR system 2400 (Perkin-Elmer, Wellesley, MA, U.S.A.). PCR primer and conditions are described in Table 1. The PCR products were subjected to 2.0% agarose gel electrophoresis in TAE (40 mM Tris-acetate [pH 8.0], 1 mM EDTA) buffer followed by staining in ethidium bromide (2 µg/ml) and destaining in distilled water for 5–10 min each. Images were captured by Gel-Doc 2000 (Bio-Rad Laboratories).

PCR-RFLP: PCR was performed in a GeneAmp PCR System 2400 (Perkin-Elmer) using primer sets targeted to the region V or upstream region of stx genes in Stx-phage as shown in Fig. 1 and Table 1. The PCR products were analyzed by 0.4% agarose gel electrophoresis using HGT agarose and/or by field inversion gel electrophoresis using 1.0% pulsed-field certified agarose gel in 0.5 × TBE (45 mM Tris-borate [pH 8.0], 1.0 mM EDTA) buffer for 16 hr followed by staining with ethidium bromide solution (2 µg/ml) and destaining in distilled water for 5–10 min each. The run condition was generated by the autoalgorithm mode of CHEF Mapper pulsed-field gel electrophoresis (PFGE) system with a size range of 6 to 15 kb. The PCR products were further restric-
tion digested for 2 hr either by 10 U of BglII or 8 U of EcoRV. The digested products were then analyzed by 1.5% agarose gel electrophoresis and/or by field inversion gel electrophoresis using 1.0% pulsed-field certified agarose gel in 0.5 × TBE buffer for 18 hr. A 1-kb DNA ladder and a 2.5-kb DNA ladder (Takara Bio. Inc.) were used as molecular mass standards. The run condition was generated by the autoalgorithm mode of CHEF Mapper PFGE system with a size range of 1 to 10 kb for the BglII digest or 1 to 6 kb for the EcoRV digest. A model 1000 Mini-Chiller (Bio-Rad Laboratories) was used to maintain the temperature of the buffer at 14°C. The gels were stained and destained by similar way as mentioned above. The photographs of the electrophoretic patterns of the digested DNA (PCR-RFLP) were captured and recorded digitally using gel documentation system (Gel-Doc 2000, Bio-Rad Laboratories).

Data analysis: The discriminatory power of the PCR-RFLP typing for different O serogroup was evaluated using the Simpson’s index of diversity as described previously [12].

RESULTS

Detection of stx1 and stx2 genes by multiplex PCR: A total of 200 STEC strains including 100 of O157, 50 of O26, 10 of O111 and 40 of other serogroups were analyzed for the presence of stx1 and stx2 genes by the multiplex PCR [30]. In O157 serogroup, 5, 46 and 49 strains were found to be positive for stx1, stx2 and both stx1 and stx2 genes whereas in O26 serogroup 48 and 2 strains were positive for stx1 and both stx1 and stx2 genes, respectively. In case of O111, 6 were positive for stx1 and 4 for both stx1 and stx2 genes, while in case of non-O26/O111/O157, 20, 14 and 6 were positive for stx1, stx2 and both stx1 and stx2 genes, respectively (Table 2).

PCR-RFLP for Shiga toxin-producing E. coli: Boiled templates of 200 STEC strains including 100 of O157, 50 of O26, 10 of O111 and 40 of non-O26/O111/O157 were amplified by primer set RV-F and RV-R and one to three amplicons ranging from 6.0 to 15.5 kb in size were yielded from 95 of O157, 48 of O26, 5 of O111 and 19 of non-O26/O111/O157 strains (Figs. 2A and 3A, Table 2). Subsequently PCR products obtained from 167 STEC strains were digested with either EcoRV or BglII and the RFLP patterns were compared to each other among each category of serogroups. Figs. 2B and 3B show the representative RFLP patterns of EcoRV digest whose discrimination ability is higher than those of BglII digest. In the case of O157, EcoRV digest yielded 4 to 11 fragments ranging from 450 bp to 5.7 kb in size (Fig. 2B) and BglII digest yielded 2 to 9 fragments ranging from 500 bp to 9.6 kb in size (data not shown). Although RFLP patterns of O157–30 (lane 31) and O157–31 (lane 32) by EcoRV were identical, those were differentiated by BglII digest (data not shown). In the case of O26, EcoRV digest yielded 4 to 14 fragments ranging from 150 bp to 4.4 kb in size (Fig. 3B, Lanes 2–9) while in O111, 5 to 6 fragments ranging from 900 bp to 8.5 kb were obtained by EcoRV digest (Fig. 3B, Lanes 10–13). In the case of non-O26/O111/O157, 2 to 12 fragments ranging from 150 bp to 5.7 kb were obtained by EcoRV digest (Fig. 3B, Lanes 14–30). Based on the RFLP profiles, 95 O157, 48 O26, 5 O111 and 19 non-O26/O111/O157 strains were classified into 41, 8, 4 and 17 groups, respectively (Tables 2 and 3).

DISCUSSIONS

Molecular typing methods have played important roles to trace the route of infection and identify the strain associated with outbreak, in particular diffuse outbreak [40]. Among them, PFGE is currently the most widely used molecular subtyping method for detecting outbreaks of E. coli O157:H7 because of its high resolution and reproducibility. However, there are several disadvantages and limitations in PFGE as described above. Therefore, the use of PCR-based method such as PCR-RFLP may be an alternative because of several benefits. For example, the PCR-RFLP (1) does not require special equipment, although FIGE was used in this study, a simple mini gel electrophoresis unit such as MUPID (Advance Co., Ltd., Tokyo) can be applicable, (2) can analyze large number of strains at the same time, (3) can complete rapidly within a day, (4) can analyze the RFLP without isolation of the strain [34], (5) can avoid smearing due to free radical produced during electrophoresis [37], and (6) is not affected by repeated subculture of a single isolate in vitro and passage through bovine or human gastrointestinal tract [35, 36]. In addition, (7) it is not necessary to send STEC strains but genomic DNA is enough in order to compare the RFLP patterns at two different laboratories, which is safer and easier to ship. For this reason, we have developed a simple, rapid and easy molecular subtyping method such as PCR-RFLP assay on the basis of genetic diversity of region V in Stx-phage and have shown its various utilities...
and usefulness [34–37]. Shima et al. [37] initially evaluated the PCR-RFLP assay by using 204 STEC strains from our laboratory collections. The data showed that 202 STEC strains out of 204 yielding PCR amplicons were classified into 24 groups. PFGE patterns of representative STEC O157 strains also divided into 24 groups and, hence, were

Table 2. Subtyping of STEC strains by PCR-RFLP

<table>
<thead>
<tr>
<th>Serogroup</th>
<th>Serotype</th>
<th>PCR-RFLP No. of PCR amplicons (kb)</th>
<th>Str</th>
<th>PCR-RFLP No. of PCR amplicons (kb)</th>
<th>Str</th>
</tr>
</thead>
<tbody>
<tr>
<td>O157</td>
<td>O157:H7</td>
<td>8.2/10.5</td>
<td></td>
<td>O26</td>
<td></td>
</tr>
<tr>
<td>O157</td>
<td>O157:H7</td>
<td>11.6</td>
<td></td>
<td>O26</td>
<td></td>
</tr>
<tr>
<td>O157</td>
<td>O157:H7</td>
<td>13.1</td>
<td></td>
<td>O26</td>
<td></td>
</tr>
<tr>
<td>O157</td>
<td>O157:H7</td>
<td>13.1</td>
<td></td>
<td>O26</td>
<td></td>
</tr>
<tr>
<td>O157</td>
<td>O157:H7</td>
<td>11.6</td>
<td></td>
<td>O26</td>
<td></td>
</tr>
<tr>
<td>O157</td>
<td>O157:H7</td>
<td>11.6</td>
<td></td>
<td>O26</td>
<td></td>
</tr>
<tr>
<td>O157</td>
<td>O157:H7</td>
<td>8.2/13.1</td>
<td></td>
<td>O111</td>
<td></td>
</tr>
<tr>
<td>O157</td>
<td>O157:H7</td>
<td>11.6/12.7</td>
<td></td>
<td>O26</td>
<td></td>
</tr>
<tr>
<td>O157</td>
<td>O157:H7</td>
<td>11.7/14.3</td>
<td></td>
<td>O26</td>
<td></td>
</tr>
<tr>
<td>O157</td>
<td>O157:H7</td>
<td>11.6</td>
<td></td>
<td>O26</td>
<td></td>
</tr>
<tr>
<td>O157</td>
<td>O157:H7</td>
<td>11.6</td>
<td></td>
<td>O26</td>
<td></td>
</tr>
<tr>
<td>O157</td>
<td>O157:H7</td>
<td>11.6</td>
<td></td>
<td>O26</td>
<td></td>
</tr>
<tr>
<td>O157</td>
<td>O157:H7</td>
<td>8.2/13.1</td>
<td></td>
<td>O111</td>
<td></td>
</tr>
<tr>
<td>O157</td>
<td>O157:H7</td>
<td>11.6/12.7</td>
<td></td>
<td>O26</td>
<td></td>
</tr>
<tr>
<td>O157</td>
<td>O157:H7</td>
<td>11.7/14.3</td>
<td></td>
<td>O26</td>
<td></td>
</tr>
<tr>
<td>O157</td>
<td>O157:H7</td>
<td>11.6</td>
<td></td>
<td>O26</td>
<td></td>
</tr>
<tr>
<td>O157</td>
<td>O157:H7</td>
<td>11.6</td>
<td></td>
<td>O26</td>
<td></td>
</tr>
<tr>
<td>O157</td>
<td>O157:H7</td>
<td>11.6</td>
<td></td>
<td>O26</td>
<td></td>
</tr>
<tr>
<td>O157</td>
<td>O157:H7</td>
<td>8.2/13.1</td>
<td></td>
<td>O111</td>
<td></td>
</tr>
<tr>
<td>O157</td>
<td>O157:H7</td>
<td>11.6/12.7</td>
<td></td>
<td>O26</td>
<td></td>
</tr>
<tr>
<td>O157</td>
<td>O157:H7</td>
<td>11.7/14.3</td>
<td></td>
<td>O26</td>
<td></td>
</tr>
<tr>
<td>O157</td>
<td>O157:H7</td>
<td>11.6</td>
<td></td>
<td>O26</td>
<td></td>
</tr>
<tr>
<td>O157</td>
<td>O157:H7</td>
<td>11.6</td>
<td></td>
<td>O26</td>
<td></td>
</tr>
<tr>
<td>O157</td>
<td>O157:H7</td>
<td>11.6</td>
<td></td>
<td>O26</td>
<td></td>
</tr>
<tr>
<td>O157</td>
<td>O157:H7</td>
<td>8.2/13.1</td>
<td></td>
<td>O111</td>
<td></td>
</tr>
<tr>
<td>O157</td>
<td>O157:H7</td>
<td>11.6/12.7</td>
<td></td>
<td>O26</td>
<td></td>
</tr>
<tr>
<td>O157</td>
<td>O157:H7</td>
<td>11.7/14.3</td>
<td></td>
<td>O26</td>
<td></td>
</tr>
<tr>
<td>O157</td>
<td>O157:H7</td>
<td>11.6</td>
<td></td>
<td>O26</td>
<td></td>
</tr>
<tr>
<td>O157</td>
<td>O157:H7</td>
<td>11.6</td>
<td></td>
<td>O26</td>
<td></td>
</tr>
<tr>
<td>O157</td>
<td>O157:H7</td>
<td>11.6</td>
<td></td>
<td>O26</td>
<td></td>
</tr>
</tbody>
</table>

NA: not applicable, UT: untypable.

a) One strain of 26–8 and one strain of 103–2 shows the same PCR-RFLP typing pattern.
Fig. 3. (A) Field inversion gel electrophoresis of LA-PCR products obtained from 29 representative STEC non-O157 strains differentiated by the PCR-RFLP assay. Lanes: 1 and 31, 2.5-kb DNA ladder; 2, 26–1; 3, 26–2; 4, 26–3; 5, 26–4; 6, 26–5; 7, 26–6; 8, 26–7; 9, 26–8; 10, 111–1; 11, 111–2; 12, 111–3; 13, 111–4; 14, 38; 15, 63; 16, 80; 17, 84–1; 18, 84–2; 19, 103–1; 20, 103–2; 21, 121; 22, 128; 23, 145; 24, 153; 25, 165; 26, 169; 27, UT-1; 28, UT-2; 29, UT-3; 30, UT-4. (B) Field inversion gel electrophoresis of EcoRV digest of LA-PCR products obtained from 29 representative STEC non-O157 strains differentiated by the PCR-RFLP assay. Lanes: 1 and 31, 1-kb DNA ladder; 2, 26–1; 3, 26–2; 4, 26–3; 5, 26–4; 6, 26–5; 7, 26–6; 8, 26–7; 9, 26–8; 10, 111–1; 11, 111–2; 12, 111–3; 13, 111–4; 14, 38; 15, 63; 16, 80; 17, 84–1; 18, 84–2; 19, 103–1; 20, 103–2; 21, 121; 22, 128; 23, 145; 24, 153; 25, 165; 26, 169; 27, UT-1; 28, UT-2; 29, UT-3; 30, UT-4.
to discriminate among different serogroups isolated at different time frame and from distal geographic area must be used in comparison to PFGE.

In this study, 200 STEC strains including not only O157 but also non-O157 which were identified as clonally different were randomly selected from 1,795 strains isolated at 42 regional public health institutes in Japan. Although out of 100 of O157 and 50 of O26 STEC strains, 95 (95%) and 48 (96%) strains yielded PCR amplicons, respectively, by the LA-PCR, only 5 (50%) and 19 (48%) strains yielded PCR amplicons out of 10 O111 and 40 non-O26/O111/O157 strains, respectively (Table 3). Ninety-five O157 strains yielding PCR amplicons were further classified into 41 groups by the restriction digestion. However, PCR products obtained from 48 of O26 strains were classified into only 8 groups. Five of O111 and 19 non-O26/O111/O157 strains were classified into 4 and 17 groups, respectively. Analysis of the typed strain by Simpson’s Index indicated 0.957 of O157, 0.603 of O26, 0.900 of O111 and 0.988 of non-O26/O111/O157 (Table 3), respectively. However, positive rate of O111 and non-O26/O111/O157 strains was less than 50%.

Taken together, these data indicate that the PCR-RFLP may be useful for the molecular subtyping of O157 but not for non-O157 STEC strains (Table 3).

Apart from PFGE, sequenced-based methods, such as multilocus sequence typing (MLST), have been used as powerful subtyping tools in molecular epidemiology [42]. These methods have several advantages of being easily standardized and automated, shorter assay times and totally comparable and transferable data between laboratories [19, 20, 24]. MLST analyzes the internal fragments of housekeeping genes to ascertain genetic relatedness among isolates. Although this method was successful for the differentiation of other organisms [9, 19, 46], it was unable to discriminate among E. coli O157:H7 isolates [28]. Alternatively, multiple-locus variable-number of tandem repeats analysis (MLVA) has been developed and successfully utilized for E. coli O157:H7 [14, 17, 28, 41]. In prokaryotic genomes, a wide array of repetitive DNA elements ranging from single to multiple repeats of nucleotides is present.

MLVA is a PCR-based subtyping method that can be used to discriminate among isolates based on variable number of tandem repeats (VNTRs). In general, seven or more loci are selected and the choice of proper loci in MLVA is important for better subtyping. To obtain high discriminating result, selection of loci with high diversities is very crucial. However, more diverse loci may be more unstable and, therefore, the change in VNTRs may affect clonal turn over. Indeed a 5-day serial experiment with an E. coli O157 outbreak strain conducted by Noller et al. [27] showed a significant variation in at least one of the loci analyzed. Since VNTRs evolve so quickly, it is concerned that multiple MLVA types would emerge during an outbreak initially caused by a single clone.

PFGE alone is not sufficient and other typing methods may be necessary in epidemiological surveys [7, 10]. At this moment, MLVA would be the best but it would be better to combine the PFGE. Although MLVA can be automated and standardized, it requires DNA sequencer, which is very expensive and, hence, is not affordable by many laboratories or regional public health institutes. In any cases, each methodology has advantages and disadvantages. Therefore, the PCR-RFLP assay might be an alternative, which can be performed in an ordinal laboratory where PCR and electrophoresis are available and is not affected by clonal turn over in vitro and in vivo [35, 36].

In conclusion, the PCR-RFLP assay targeting region V is a practical method for molecular epidemiological studies especially for O157, the most important serogroup implicated in human diseases, although the discriminatory power of PCR-RFLP assay was little bit less than that of PFGE. Therefore, the PCR-RFLP assay may be useful for the initial survey before PFGE analysis or as a supplementary molecular subtyping in conjunction with PFGE while analyzing DNA fingerprints for O157:H7 STEC strains because of its simplicity, rapidity, easiness and reliability.

ACKNOWLEDGMENTS. We thank all the municipal and prefectural public health institutes for providing us EHEC isolates and valuable information of the cases. This work
was partly supported by Grant-in-Aid for Scientific Research (C) from the Japan Society for the Promotion of Science (21590487) and grant-in-aids of the Ministry of Health, Labor and Welfare of Japan (H21-Shinkou-Ippan-003). This study was performed in partial fulfillment of the requirements of a PhD thesis for N.S. from Graduate School of Life and Environmental Sciences, Osaka Prefecture University, Osaka, Japan.

REFERENCES


27. Noller, A. C., McEllistrem, M. C., Shutt, K. A. and Harrison,


