Comparison of Three Molecular Subtyping Methods among O157 and Non-O157 Shiga Toxin-Producing Escherichia coli Isolates from Japanese Cattle

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SUMMARY: To determine the infection source, route, and extent of an outbreak, it is important to subtype Shiga toxin-producing Escherichia coli (STEC) isolates belonging to the same serotype for clustering into clonally related groups. In this study, we compared 3 molecular subtyping methods—multilocus sequence typing (MLST), pulsed-field gel electrophoresis (PFGE), and multiple-locus variable-number of tandem repeat analysis (MLVA)—using O157 and non-O157 STEC isolates from Japanese beef cattle. A total of 73 STEC isolates belonging to 9 O-serogroups were analyzed. By means of 3 molecular subtyping methods, the strains were subdivided into 9 MLST sequence types (STs), 23 PFGE types, and 26 MLVA types. The STEC classification by O-serogrouping and MLST was almost identical. Furthermore, PFGE and MLVA could systematically classify STEC isolates of the same serotypes and STs. MLVA and PFGE were found to be highly efficient subtyping methods after O-serogrouping for the classification of not only O157 but also non-O157 STEC isolates in an outbreak investigation.

INTRODUCTION

Cattle are an important reservoir of Shiga toxin-producing Escherichia coli (STEC) infection, and their feces containing STEC act as a source of contamination for a variety of foods and environments (1,2). As a result, STEC infection is caused by ingestion of raw beef, offal, milk, or dairy products or through direct contact with calves or cattle. In addition, vegetables and fruits contaminated with cattle feces containing these microorganisms are also a source of infection (1,2). In Japan, the number of STEC-infected patients (including asymptomatic human carriers) has been reported to be ~3,000–5,000 per year (3). Although STEC of serogroups O157 are the major causative agents of human STEC infection in most parts of the world including Japan (1,3), non-O157 STEC-associated infections have also been increasingly reported (1), with some of these cases being foodborne (4), such as the large outbreak of Stx2-producing enteraggregative Escherichia coli serotype O104:H4 that occurred in Germany in 2011 (5,6). That is, non-O157 STEC strains also cause significant human illness (7–9). Cattle are known to be the major reservoir for STEC: not only O157 but also non-O157 STEC (1). Although over 380 different STEC O:H serotypes have been implicated in human infections, and many of these serotypes as well as others have been recovered from animals (2), only a limited number of these serotypes appear to be associated with the majority of human diseases. It is important to subtype bovine STEC isolates to determine the infection source, route, and extent of an outbreak.

Molecular subtyping methods are useful for identifying the relatedness of STEC isolates during outbreaks and surveillance investigations. Pulsed-field gel electrophoresis (PFGE) and multiple-locus variable-number tandem repeat analysis (MLVA) have been used in outbreak investigations for classifying the same STEC serotype groups (10). PFGE is considered the standard method for typing of STEC isolates and was selected by PulseNet (http://www.pulsenetinternational.org/) as the primary genotyping methodology in the mid-1990s owing to its robustness and reliability. In Japan, human STEC isolates have been monitored by PFGE since 1996 (11). Nonetheless, in recent years, MLVA has been reported to show higher efficiency in subtyping of the same serotypes (especially O157) (11). Consequently, MLVA has been employed for typing of strains O157, O26, and O111 STEC (12), and PFGE has been used for the classification of non-O157, O26, and O111 strains. Although PFGE has strong discriminatory power, it is limited by certain intra- and interlaboratory reproducibility issues (13). In contrast, multilocus sequence typing (MLST) is easy to apply to interlaboratory and worldwide comparisons of E. coli isolates, and currently, there are 7,116 sequence types (STs; available at <http://pubmlst.org/databases.shtml>, accessed September 20, 2017). Although the prevalence and virulence profiles of bovine non-O157 STEC isolates have been reported (14–19), little is known about the application and comparison of several typing methods involving bovine isolates, and there are many publications reporting application these subtyping methods to non-O157 STEC...
strains (20–22). There are no reports comparing bovine STEC isolates by major subtyping methods (that is PFGE, MLVA, and MLST).

In the present study, we evaluated the 3 molecular subtyping methods—PFGE, MLST, and MLVA—on O157 and non-O157 STEC isolates from Japanese beef cattle.

MATERIALS AND METHODS

Bacterial isolates: A total of 96 rectal samples (one sample from one animal) of Japanese beef cattle raised on 13 farms were acquired from the Osaka Municipal Slaughter Center from August to November 2011. The samples were directly spread on CHROMagar STEC plates (CHROMagar Microbiology, Paris, France), and 3–10 mauve colonies of each sample were selected. The identification of E. coli species and stx-positive strains was confirmed by biochemical tests (utilization of sucrose, lactose, and glucose, production of gas, H₂S and indole on TSI and SIM media [Nissui, Tokyo, Japan]; and secretion of lysine decarboxylase on lysine decarboxylase media [self-made]) and a verotoxin gene PCR screening set (Takara Bio Inc., Kusatsu, Japan). Shiga toxin production was also confirmed by a reverse passive latex agglutination (RPLA) assay using VTEC-RPLA (Denka-Seiken, Tokyo, Japan). Shiga toxin production was also confirmed by a reverse passive latex agglutination (RPLA) assay using VTEC-RPLA (Denka-Seiken, Tokyo, Japan). Serotypes and virulence genes among bovine STEC isolates: Among 96 rectal samples from cattle raised on 13 farms, 73 STEC isolates were obtained from 27 (28.1%) samples. Among them, 63 were classified into 9 O-serogroups (O5:HNM, O109:HNM, O113:H4, O136:H16, O156:HNM, O157:H7, O157:H19, CVN004, CVN007, and CVN011) and the other 10 could not be assigned to any known O-serogroups (OUT) (Table 1, Fig. 2). The 3 major O-serogroups were O157 (n = 19 from 5 farms), O113 (n = 11 from 3 farms), and O157 (n = 10 from 2 farms). All the strains were positive for either stxl or stx2, except for the O157:H7 strain and 5 O113:H4 strains that had both stxl and stx2. A total of 30 strains belonging to O5, O156, O157, O182, and OUT were eae-positive, 48 strains belonging to O5, O109, O156, O157, O182, and OUT were hlyA-positive, and 3 strains belonging to O109 and OUT were both hlyA- and subA-positive, respectively. On the other hand, all the strains tested negative for saa, eibG, aggR, and CVD432. Based on the source sample, serotype, and virulence gene pattern, the STEC strains were grouped into 30 types (Table 1).

RESULTS

Serotypes and virulence genes among bovine STEC isolates: Among 96 rectal samples from cattle raised on 13 farms, 73 STEC isolates were obtained from 27 (28.1%) samples. Among them, 63 were classified into 9 O-serogroups (O5:HNM, O109:HNM, O113:H4, O136:H16, O156:HNM, O157:H7/HNM, O157:H7/HNM, O168:H51, O174:H21/HNM, and O182:H19/HUT), and the other 10 could not be assigned to any known O-serogroups (OUT) (Table 1, Fig. 2). The 3 major O-serogroups were O157 (n = 19 from 5 farms), O113 (n = 11 from 3 farms), and O157 (n = 10 from 2 farms). All the strains were positive for either stxl or stx2, except for the O157:H7 strain and 5 O113:H4 strains that had both stxl and stx2. A total of 30 strains belonging to O5, O156, O157, O182, and OUT were eae-positive, 48 strains belonging to O5, O109, O156, O157, O182, and OUT were hlyA-positive, and 3 strains belonging to O109 and OUT were both hlyA- and subA-positive, respectively. On the other hand, all the strains tested negative for saa, eibG, aggR, and CVD432. Based on the source sample, serotype, and virulence gene pattern, the STEC strains were grouped into 30 types (Table 1).

Classification of bovine STEC strains by 3 molecular subtyping methods: Figure 1 shows the phylogenetic tree of 30 representative STEC strains analyzed by MLST (these 30 strains are shown in Table 1). The classification of these 30 strains was closely associated with their O-serogroups (Fig. 1). By means of MLST, 60 STEC isolates were classified into 9 STs; ST10, ST11, ST300, ST329, ST333, ST39, ST441, ST677, and ST770 (Table 1, Figs. 1 and 2). The remaining 13 could not be assigned to any known STs but were grouped into 4 identical sequences associated with their serotypes O5:HNM, O168:H51, and OUT:HNM (Table 1). A total of 72 isolates showed 35 PFGE patterns and were clearly classified into 23 types based on Tenover's criteria. Nevertheless, in one of 9 STEC OUT:HNM
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strains, the PFGE pattern could not be obtained because of DNA degradation (strain ID 13 in Table 1). As shown in Fig. 3 and Table 1, isolates from the same animal represented the same PFGE types with variation of a few bands. Among STEC O174 strains, 6 PFGE types were confirmed (Fig. 3A), and among them, the PFGE patterns of 7 O174 isolates from 2 animals (D-26 and D-27) raised on farm D belonged to the same PFGE type 23. Among STEC O113 strains, 3 PFGE types were confirmed (Fig. 3B). The PFGE patterns of 3 STEC O113 isolates from 2 heads of cattle (M-17 and N-18) from 2 different farms M and N belonged to the same type 15. Two patterns were confirmed among 10 STEC O157 isolates. On the same farm (farm N), 9 STEC O157 isolates from 3 heads of cattle (N-18, N-19, and N-21) had indistinguishable patterns.

MLVA classified 73 isolates into 26 MLV A types (Fig. 2). Two to 6 MLV A types were confirmed among the same O-serogroups (Fig. 2). It must be noted that MLV A showed almost the same discriminatory power as PFGE did, except for 5 O156, 4 O157, and 7 OUT isolates.

In other words, although 5 STEC O156 isolates were determined to be PFGE type 6, they were subdivided into 3 groups (m5, m6, and m7) by MLVA. Similarly, 4 STEC O157 isolates were found to be PFGE type 17 but were subdivided into 2 groups (m10 and m11) by MLVA. In contrast, 4 isolates classified into m2 by MLVA were grouped into PFGE types 16 and 3. In the case of STEC O174 and O113 isolates, MLVA classification depended on the sample sources, except for m18 of O174 strains, which were isolated from 2 heads of cattle (D-26 and D-27) on farm D (Fig. 2).

**DISCUSSION**

Subtyping of STEC isolates after O-serogrouping is important for epidemiological studies as well as surveillance of STEC during outbreaks. In this study, we attempted to use 3 molecular subtyping methods for classifying O157 and non-O157 strains, including O174 and O113. In one study, Pei et al. (11) reported that MLV A has greater discriminatory power than PFGE did, except for 5 O156, 4 O157, and 7 OUT isolates.

**Table 1. Characteristics of bovine STEC used in this study**

| Sample ID | Sampling date | No. of isolates | Serotype | Possession of virulence genes | ST | PFGE type | MLV A type | Strain ID |
|-----------|---------------|-----------------|----------|-------------------------------|----|-----------|------------|-----------|
| O-23      | Sep. 27       | 5               | O5:HNM   | + - - - - - - + -             | ND | 20        | m1         | 64        |
| J-14      | Sep. 12       | 1               | O109:HNM | + - - - - - - + +             | 339| 12        | m23        | 31        |
| N-12      | Sep. 5        | 1               | O109:HNM | + - - - - - - + +             | 339| 10        | m22        | 27        |
| J-13      | Sep. 20       | 3               | O113:H4  | + - - - - - - - -             | 10 | 11-a,b    | m25        | 28        |
| M-17      | Sep. 20       | 1               | O113:H4  | + - - - - - - - -             | 10 | 15-a      | m24        | 41        |
| N-18      | Sep. 27       | 2               | O113:H4  | + - - - - - - - -             | 10 | 15-a,b    | m24        | 49        |
| N-20      | Sep. 27       | 5               | O113:H4  | + - - - - - - - -             | 10 | 18        | m26        | 57        |
| N-11      | Sep. 5        | 1               | O136:H16 | + - - - - - - - -             | 329| 9         | m3         | 26        |
| E-7       | Aug. 16       | 4               | O156:HNM | + - + - - - - - +             | 300| 6-a       | m5,m6      | 16        |
| F-8       | Aug. 22       | 1               | O156:HNM | + - - - - - - - -             | 300| 6-b       | m7         | 21        |
| B-4       | Aug. 8        | 1               | O157:HNM | + - - - - - - - + -           | 11 | 4         | 12         | 8         |
| N-18      | Sep. 27       | 3               | O157:H7  | - + - - - - - - -             | 11 | 17-a      | m10        | 46        |
| N-19      | Sep. 27       | 5               | O157:H7  | - + - - - - - - -             | 11 | 17-a,b,c  | m10        | 51        |
| N-21      | Sep. 27       | 1               | O157:H7  | - + - - - - - - -             | 11 | 17-a      | m11        | 62        |
| G-9       | Aug. 29       | 1               | O168:H51 | + - - - - - - - -             | ND | 7         | m19        | 22        |
| A-1       | Aug. 8        | 3               | O174:H21 | - + - - - - - - -             | 677| 1-a,b     | m14        | 1         |
| D-25      | Oct. 3        | 4               | O174:H21 | - + - - - - - - -             | 677| 22-a,b     | m13        | 71        |
| D-26      | Oct. 3        | 4               | O174:H21 | - + - - - - - - -             | 677| 23-a,b,c   | m18        | 75        |
| D-27      | Oct. 3        | 3               | O174:H21 | - + - - - - - - -             | 677| 23-a       | m18        | 79        |
| G-24      | Oct. 3        | 2               | O174:HNM | - + - - - - - - -             | 677| 21         | m17        | 69        |
| H-10      | Aug. 29       | 2               | O174:H21 | - + - - - - - - -             | 677| 8         | m15        | 24        |
| N-22      | Sep. 27       | 1               | O174:HNM | - + - - - - - - -             | 677| 19         | m16        | 63        |
| L-15      | Sep. 12       | 3               | O182:HUT | + - + + - - - + -             | 300| 13         | m9         | 32        |
| L-15      | Sep. 12       | 1               | O182:HUT | + - + + - - - + -             | 300| 13         | m9         | 34        |
| L-16      | Sep. 12       | 5               | O182:H19 | + - + + - - - + -             | 300| 14         | m8         | 36        |
| A-3       | Aug. 8        | 3               | OUT:HNM  | - + - - - - - - -             | ND | 3-a,b     | m2         | 5         |
| C-5       | Aug. 8        | 1               | OUT:HNM  | - + - - - - - - -             | 441| ND         | m21        | 13        |
| D-6       | Aug. 16       | 1               | OUT:HNM  | - + - - - - - - -             | 333| 5         | m4         | 14        |
| M-17      | Sep. 20       | 4               | OUT:HNM  | - + - - - - - - -             | ND | 16-a,b,c   | m2         | 42        |
| A-2       | Aug. 8        | 1               | OUT:HUT  | - + - - - - - - -             | 770| 2         | m20        | 4         |

(1) UT, untypeable; NM, non-motile.
(2) ND: not determined.
(3) representative strain used in phylogenetic analysis (Fig. 3).
does with respect to STEC O157 isolates. In the present study, MLV A was found to be a useful method for subtyping non-O157 STEC isolates such as O113 and O174. It has been reported that STEC O174 and O113 strains are associated with severe illness in humans (34,35) and have been isolated from patients with hemolytic uremic syndrome (36). Furthermore, STEC serotype O113:H4, associated with individual cases of diarrheal illness in Ireland, has been demonstrated to increase in prevalence since 2007 (37). These strains have the \textit{stx2d} gene, have been grouped into ST10 by MLST, and are generally \textit{eae}-negative (37), and the STEC O113:H4 detected in the present study showed features similar to the characteristics of these isolates. In addition, Geue et al. (38) reported that bovine STEC O156 isolates are characterized as ST300 by MLST, which could probably be a potential reservoir of human STEC O156 infection. Therefore, more attention must be given to these O-serogroup STEC strains in clinical, food, and environmental studies.

In the present study, MLV A and PFGE were found to be useful subtyping methods, when compared with MLST, not only for O157 but also for O113 and O174 strains. In another study, Jenke et al. (39) compared MLV A with MLST based on their ability to differentiate hemolytic-uremic-syndrome-associated STEC strains. They found that MLV A was suitable for short-range epidemiological studies owing to its ability to differentiate closely related strains. Accordingly, in the present study, because the STEC strains were collected within a short period of time (~4 months), MLV A was found to be better than MLST.

The STEC isolates with the same PFGE or MLV A patterns were predominantly obtained from the same farm as well as from the same animal. In a cow-calf pasture-based production system, STEC strains may be transmitted between animals, but not necessarily from a dam to its calf (14). Horizontal STEC transmission may occur on a cattle farm. In this study, the PFGE pattern (15-a or 15-b) and MLV A type obtained from 3 STEC O113 (ST10) isolates from different farms were indistinguishable (M-17 and N-18; Fig. 3B). It must be noted that farms M and N are geographically separated in Japan (~800 km). Besides, these cattle are traced via an individual identification number, not through their place of birth and breeding.

**Fig. 1.** Phylogenetic tree of 30 bovine STEC strains by MLST. Strain ID, O serogroup, sequence type (ST) are indicated. Black bar indicated the same sequence strains. The tree was constructed on the basis of the concatenated sequences of 7 housekeeping genes by the neighbor-joining algorithm by MEGA 5.0 software. Bootstrap analysis was performed with 1,000 replications. ND, Not determined; UT, untypable.

**Fig. 2.** Dendrogram of the MLVA results. MLVA type, PFGE type, ST, Serotype, and Sample ID are indicated. Black bar indicated the same MLVA types. The dendrogram was constructed by BioNumerics software using categorical data and the Ward algorism based on allele numbers defined the MLVA allele string. ND, Not determined; UT, untypable; NM, non-motile.
In our study, the prevalence of STEC isolates among healthy beef cattle was 28.1% (27 of 96). Various studies have shown 27.5% and 34% STEC prevalence among healthy cattle in the USA (19) and France (18), respectively. Similarly, in Japan, STEC isolates have been recovered from 79 (22.1%) of 358 healthy cattle (40) in 1998, and from 39 (14.2%) of 274 beef cattle in 2012–2013 (16). In these study, 62.8% and 64.2% of the enrichment cultures of fecal samples were found to be stx-positive by PCR, respectively (16,40). In the present study, although we tried to obtain STEC isolates from bovine fecal samples by a direct plating method, the detection of STEC isolates was not significantly different from that of enrichment procedure. Blanco et al. (41) reported that most of the STEC strains of bovine origin appear to belong to 20 O-serogroups: O₄, O₈, O₂₂, O₂₅, O₃₂, O₄₅, O₈₂, O₈₄, O₁₀₃, O₁₁₁, O₁₁₃, O₁₁₆, O₁₂₁, O₁₃₆, O₁₄₆, O₁₅₃, O₁₅₇, O₁₇₁, O₁₇₂, and O₁₇₄ (OX3). In the present study, 40 of 73 STEC isolates belonged to 3 major serogroups: O₁₇₄, O₁₁₃, and O₁₁₅. Particularly, Mekata et al. (16) reported O₁₁₃ and O₁₁₅ as the major serogroups, which may be the recent major serogroups among beef cattle in Japan.

In conclusion, we characterized 73 STEC isolates from Japanese beef cattle and compared 3 subtyping methods. As far as we have determined, these STEC isolates have no adhesion factor gene (aggR, CVD432, saa, and eibG), except for the eae gene. Because all the O₁₅₇ strains possess genes eae and hlyA, it can be concluded that O₁₅₇ is still an important serogroup found in Japanese cattle and causes human STEC infection. Both MLVA and PFGE were found to be useful subtyping methods for classifying not only O₁₅₇ but also O₁₁₃ and O₁₇₄ STEC from cattle.

Conflict of interest None to declare.
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