Original Article

Evaluation of Automated Ribotyping System for Characterization and Identification of Verocytotoxin-Producing *Escherichia coli* Isolated in Japan

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SUMMARY: The usefulness of an automated ribotyping system (RiboPrinter) was evaluated for characterizing and identifying clinical isolates of 37 verocytotoxin-producing *Escherichia coli* (VTEC) strains and 16 non-VTEC strains. All strains were successfully ribotyped with satisfactory reproducibility and stability and characterized into 10 different ribogroups. All VTEC O157 strains were characterized into a specific ribogroup and correctly typed into the specific DuPont ID for VTEC O157:H7, while all of the non-VTEC O157 strains were clearly distinguished from VTEC O157. VTEC O26 and O111 strains, the most prevalent VTEC serotypes after O157, were also well characterized into specific ribogroups and identified. These results suggest that the RiboPrinter may have an advantage over other typing systems in that it can rapidly and easily discriminate VTEC from non-VTEC strains of the most prevalent VTEC serotypes in Japan, even though it provides a lesser degree of discrimination than pulsed-field gel electrophoresis (PFGE). With a hierarchical or sequential typing combining the RiboPrinter and PFGE, rapid and accurate typing can be achieved during an outbreak of VTEC, which may be useful in clinical and public health settings.

INTRODUCTION

Verocytotoxin (VT)-producing *Escherichia coli* (VTEC) causes diarrhea, hemorrhagic colitis, and hemolytic-uremic syndrome (HUS) (1). The most common VTEC serotype is O157:H7. However, recent reports have pointed out the emergence of VTEC with non-O157 serotypes that also cause HUS. More than 50 non-O157 serotypes have been identified as pathogens causing bloody diarrhea or HUS in humans. The most common non-O157:H7 serotypes associated with human disease include O26:H11, O103:H2, O111:HNM, and O113:H21 (2). *E. coli* O157:H7 is easily differentiated from other *E. coli* strains by its inability to rapidly ferment sorbitol and a high resistance to potassium tellurite, while non-O157:H7 VTEC are phenotypically similar to commensal nonpathogenic *E. coli* and are not detected by sorbitol MacConkey agar (3). Current methods for the detection of VTEC depend primarily on the presence of VT and strain isolation, followed by serotyping. However, slide agglutination testing with enteropathogenic or saprophytic non-diarrheagenic *E. coli* serogroup-specific antisera will result in cross-reactions with the respective VTEC serogroups. Because early diagnosis of VTEC can reduce the extent of outbreaks, there is an urgent need for rapid, sensitive, and simple procedures to detect O157:H7 and non-O157:H7

VTEC in clinical and public health settings.

Recently, an automated ribotyping system, the commercially available RiboPrinter (Qualicon, Wilmington, Del., USA), has made it possible for up to 32 strains to be typed within an 8-h period (4). This system allows virtually all species of bacteria to be characterized according to their specific ribotype and to be identified according to existing reference patterns. In this study, we evaluated the RiboPrinter as a typing method for characterizing and identifying 37 VTEC strains with various O serogroups, and compared the results to those for non-VTEC strains, including six VT-negative O157 strains.

MATERIALS AND METHODS

Strains: Thirty-seven VTEC strains, representing eight different O groups and 10 O:H serotypes, were used in this study (Table 1). The strains were originally isolated in different geographic areas in Japan between 1992 and 1997. Six VT non-producing O157 strains and 10 strains of non-diarrheagenic *E. coli* (NDEC) were used for comparison. Strains were stored at −80°C in skim milk until used in this study.

VT profiles: The VT profiles for the diarrheagenic *E. coli* strains were determined by using a reversed passive latex agglutination kit (Denka Seiken, Tokyo) according to the manufacturer's instructions (5). VT genotypes were identified by PCR as previously described (6).

Ribotyping: The RiboPrinter was used for ribotyping as previously described (4). In brief, all strains were plated on brain heart infusion agar (Nihon Becton Dickinson, Tokyo).

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Table 1. Verocytotoxin-producing *E. coli* (VTEC) strains used in this study and their characterization and identification

Strain[1]	Serotype[2]	Origin	VT type[3]	Ribogroup	DUP ID[4]
NL2416	O1:HNM	Aichi	VT2	A	3005EC
NL2437	O6:H34	Shiga	VT2	B	3006EC
NL2419	O26:H11	Aichi	VT1	C	3011EC
NL2420	O26:H11	Aichi	VT1	C	3011EC
NL2443	O26:H11	Osaka	VT1	C	3011EC
NL2444	O26:H11	Osaka	VT1	C	3011EC
NL2451	O26:H11	Tokyo	VT1	C	3011EC
NL2452	O26:H11	Tokyo	VT1	C	3011EC
NL2463	O26:H11	Hokkaido	VT1	C	3011EC
NL2320	O26:HNM	Aichi	VT1	C	3011EC
NL2321	O26:HNM	Aichi	VT1	C	3011EC
NL2423	O26:HNM	Aichi	VT1	C	3011EC
NL2424	O26:HNM	Aichi	VT1	C	3011EC
NL2432	O26:HNM	Aichi	VT1	C	3011EC
NL2438	O26:HNM	Toyama	VT1	C	3011EC
NL2457	O26:HNM	Shizuoka	VT1	C	3011EC
NL2459	O103:H2	Aomori	VT1	B	3006EC
NL2323	O111:HNM	Aichi	VT1,VT2	D	3016EC
NL2324	O111:HNM	Aichi	VT1,VT2	D	3006EC
NL2425	O111:HNM	Aichi	VT1	D	3006EC
NL2426	O111:HNM	Aichi	VT1,VT2	D	3006EC
NL2442	O111:HNM	Osaka	VT1	D	3006EC
NL2453	O111:HNM	Tokyo	VT1	D	3006EC
NL2454	O111:HNM	Tokyo	VT1	D	3006EC
NL2455	O111:HNM	Shizuoka	VT1,VT2	D	3006EC
NL2456	O111:HNM	Shizuoka	VT1	D	3006EC
NL2462	O111:HNM	Hokkaido	VT1,VT2	E	NI
NL2445	O118:H2	Ishikawa	VT1	B	3006EC
NL2446	O118:H2	Ishikawa	VT1	B	3006EC
NL2327	O157:H7	unknown	VT1,VT2	F	3064EC O157:H7
NL2328	O157:H7	unknown	VT1,VT2	F	3064EC O157:H7
NL2329	O157:H7	unknown	VT1,VT2	F	3064EC O157:H7
NL2330	O157:H7	unknown	VT1,VT2	F	3064EC O157:H7
NL2464	O157:HNM	Aichi	VT2	F	3064EC O157:H7
NL2465	O157:H7	Aichi	VT2	F	3064EC O157:H7
NL2466	O157:H7	Aichi	VT2	F	3064EC O157:H7
NL2406	O157:H42	Aichi	Neg	G	3006EC
NL2411	O157:H42	Aichi	Neg	G	3006EC
NL2397	O157:H45	Aichi	Neg	H	NI
NL2396	O157:HUT	Aichi	Neg	H	NI
NL2398	O157:HUT	Aichi	Neg	I	3039EC
NL2400	O157:HUT	Aichi	Neg	I	3039EC
NL2441	O165:H25	Toyama	VT2	J	3051EC
NDEC(7)		Aichi	Neg	A	3005EC
NDEC(3)		Aichi	Neg	B	3006EC

[1]: NDEC, non-diarrhoeagenic *Escherichia coli*.
[2]: NM, nonmotile; UT, untypeable.
[3]: VT, Verocytotoxin; Neg, negative.
[4]: DUP ID, DuPont Identification Pattern; EC, *Escherichia coli*; NI, no identification.

After overnight growth, colonies were picked from individual agar plates, suspended in lysis buffer, transferred to a sample carrier, and loaded into the RiboPrinter. DNA was digested with *Eco*RI, after which the restriction fragments were separated by electrophoresis, transferred to nylon membranes, and hybridized with a chemiluminescent-labeled DNA probe containing the *E. coli rrn*B rRNA operon. The chemiluminescent pattern was then electronically imaged, processed, and compared to other patterns in the RiboPrinter database. Through the customized software of this system, similarity coefficients between the strains were calculated on the basis of band position, weight, and intensity. Strains were judged to have the same pattern if the similarity coefficient between their patterns was ≥0.93, and assigned to a specific ribogroup. If the pattern was quite close (i.e., 0.85 or higher similarity) to an existing reference pattern (DuPont Identification Pattern [DuPont ID]) in the RiboPrinter database, which included 65 *E. coli* patterns, the RiboPrinter automatically assigned an identification at a specific genus, species, and strain level. Quality control (QC) and reproducibility were ensured by replicate testing of four QC strains: *E. coli* ATCC 51739, *Staphylococcus aureus* ATCC 51740, *Salmonella*

choleraesuis ATCC 51741, and *Listeria innocua* ATCC 51742.

The banding patterns were compared by using Dendron software version 2.3 (Solltech, Iowa City, Iowa, USA). The dendrogram was generated by the unweighted pair group method using the arithmetic averages (UPGMA) method.

RESULTS

Reproducibility and stability: Reproducibility of the characterization was assessed by repeat testing of 20 different strains. The mean similarity of ribotype patterns between runs was 0.93 (range, 0.86 to 0.98). Stability of the characterization was evaluated after the 5th and 15th serial subculture of four different strains. The mean similarity of ribotype patterns between subcultures was 0.94 (range, 0.91 to 0.96).

Reproducibility and stability of the automated identification showed a reproducibility of 0.85 (17 of 20) and a stability of 1.00 (12 of 12).

Characterization: All 53 *E. coli* strains used in this study were successfully typed by the RiboPrinter. The RiboPrinter characterized these strains into 10 different ribogroups, which were tentatively designated A to J (Table 1). Each of the patterns contained 8 to 11 fragments, ranging in size from 0.9 to 44 kbp (Fig. 1). The dendrogram presented in Fig. 1 shows two main clusters. One cluster includes all strains with the most prevalent VTEC serotypes in Japan, O26, O111, and O157, although this cluster also includes seven NDEC strains.

All VTEC O26 strains (seven H11and seven HNM strains) belonged to ribogroup C, and all but one VTEC O111 strain to ribogroup D. Only one strain (O111:HNM, NL2462) belonged to ribogroup E. These three ribogroups (C, D, and E) formed a second cluster, showing genetic relatedness with a similarity coefficient of 0.9 or higher (Fig. 1).

All VTEC O157 (six H7 and one HNM strains) belonged to ribogroup F. However, the six VT non-producing O157 strains were typed into three ribogroups (G, H, and I) that were distinct from the ribogroups of the VTEC O157 strains. In fact, the dendrogram clearly shows that ribogroups comprising VT non-producing O157 strains (G and H) were genetically distinct from the ribogroup of VTEC O157 strains (F).

The other VTEC strains belonged to five different O groups

represented by three ribogroups; VTEC O1:HNM belonged to ribogroup A, VTEC O165:H25 belonged to ribogroup J, and VTEC O6:H34, O103:H2, and two strains of O118:H2 belonged to ribogroup B. The 10 NDEC strains belonged to two different ribogroups (A and B), which were grouped into distinct clusters (Fig. 1). Ribogroups A and B included both VTEC and NDEC strains, while ribogroups C, D, and F each included only VTEC strains O26, O111, and O157, respectively.

Identification: The feasibility of automated identification by the RiboPrinter was evaluated for 53 *E. coli* strains, 50 of which were correctly identified as *E. coli* (Table 1). Among the three strains that were not identified, one was O111:HNM (NL2462), which was the only strain in ribogroup E. The other two strains were non-VTEC O157 strains (NL2397 and NL2396).

All 14 VTEC O26 strains were identified as 3011 *E. coli* (DuPont ID 3011EC), and all seven VTEC O157 strains as 3064 *E. coli* ser. O157:H7. However, four VT non-producing O157 strains were identified as two different reference ribotypes (3006EC and 3039EC), and two were unidentified. VTEC O1 (1 strain) and NDEC (7/10 strains) were identified as 3005EC. Similarly, VTEC O111 (8/10 strains), O6 (1 strain), O103 (1 strain), O118 (2 strains), and NDEC (3/10 strains) were identified as 3006EC. VTEC O111:HNM (NL2323) was identified as 3016EC, and O111:HNM (NL2462), as mentioned earlier, was unidentified.

Only reference ribotype 3006EC was included in three ribogroups (B, D, and G), which were not characterized as one cluster (Fig. 1). On the other hand, the only strains in ribogroup D were identified as two reference ribotypes (3006EC and 3016EC).

DISCUSSION

In outbreaks of VTEC, rapid identification of the source of infection may prevent the further spread of disease and reduce the number of victims. VTEC O157:H7 can be isolated and identified easily with sorbitol MacConkey agar. However, identification of the source of infection is difficult in many cases. Identification of the route of infection by non-O157 VTEC is much more difficult because no rapid and
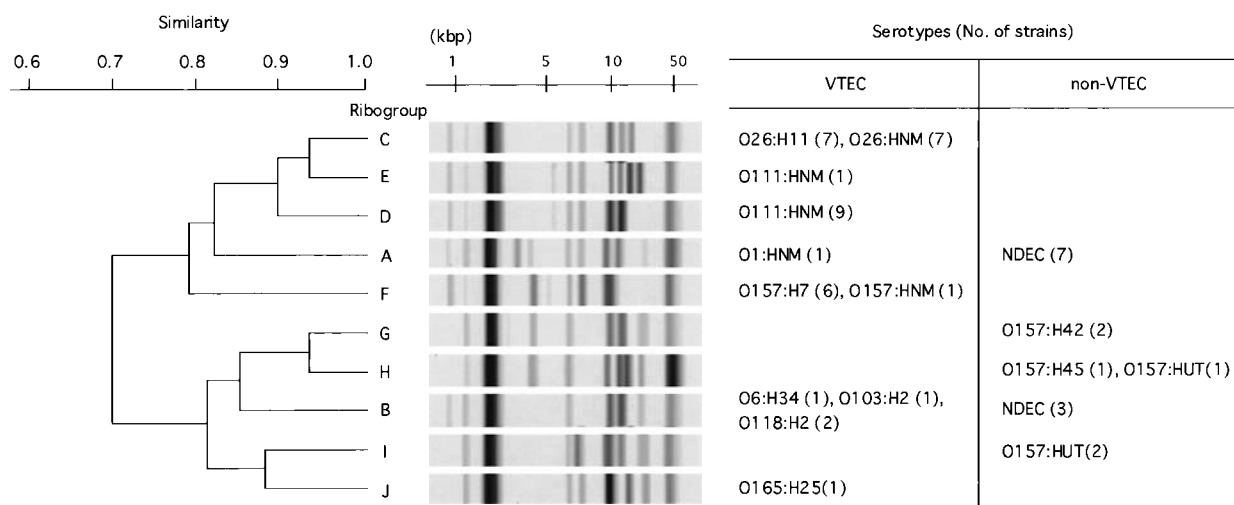

Fig. 1. Dendrogram based on UPGMA cluster analysis of ribotypes of the10 ribogroups generated from the *Escherichia coli* strains used in this study. Tentative designation of the ribogroups used in this study are shown at the right of the dendrogram, and serotypes with the number of strains in parentheses.

reliable method for identification is available. The RiboPrinter can directly and immediately characterize organisms isolated from clinical specimens or food on the selection agar, and subsequently assigns these strains to a specific ribotype. Several reports comparing food or environmental- and human-derived strains using this system have already been published (7-11).

In this study, 37 VTEC with 8 O-serotypes and 16 non-VTEC strains were characterized with satisfactory reproducibility and stability into 10 different ribogroups. All VTEC 157 strains were characterized into specific ribogroups and identified as 3064EC (EHEC O157:H7), while all non-VTEC O157 strains were clearly discriminated from VTEC O157. The VTEC O26 and O111 strains, the most prevalent VTEC serotypes after O157, were also well characterized and identified. However, 8 of the 10 strains of VTEC O111 were identified as 3006EC, and so were VTEC with other serotypes and some non-VTEC strains that had been characterized into distinct ribogroups. This discrepancy between characterization and identification may be a result of the difference in similarity threshold between ribogrouping and identification. Nevertheless, our results suggest that the RiboPrinter may have an advantage over other typing systems in that it can rapidly discriminate VTEC from non-VTEC strains of these serotypes. However, the discriminatory power of RiboPrinter should be further examined, especially for the O26 and O111 VTEC strains, because enteropathogenic *E. coli* (EPEC) strains with these serotypes have been reported to be heterogeneous serotypes that include different clones and genetic lineages, some of which are genetically closely related to EHEC strains (12).

Strain delineation by molecular typing techniques is a very powerful tool that has contributed to our understanding and control of bacterial outbreaks. At present, many authorities consider pulsed-field gel electrophoresis (PFGE) to be the gold standard against which all other techniques should be measured (13). Indeed, PFGE is used as a reference typing tool in the national epidemiologic investigation system for food-borne infections, including VTEC (14). However, this method is time- and labor-intensive, requires considerable technical skill, and a small number of strains are difficult to type. In contrast to manual typing systems, including PFGE, the RiboPrinter system has many advantages: it is extremely easy to use, the total hands-on processing time is approximately 12 min per isolate, and up to 32 strains can be typed within 8 h. In addition, the extensive database for this system allows the laboratory to compare stored banding patterns both within a gel and between gels and to identify the ribotype clusters of related isolates. In our study, the typeability, reproducibility, and stability of the RiboPrinter were all satisfactory. The discriminatory power of this method is inferior to that of PFGE, however, because of the genomic diversity of VTEC O157:H7 strains isolated in Japan and identified by PFGE (15).

A comparative study of the RiboPrinter versus PFGE for typing clinical isolates of VTEC found that of the 47 EHEC strains, 46 were grouped into a single ribogroup with the restriction enzyme *Eco*RI (16). While this result is almost consistent with the results of our study, PFGE generated nine genotypes from this cohort. It was also reported that when *Pvu*II was used as the restriction enzyme, four patterns were generated. However, use of a substitute enzyme to increase the discrimination power cannot be recommended due to lack of an adequate database, which may result in identification failure. Moreover, even when a substitute enzyme is used, the

discriminatory power will still be less than that of PFGE. Detection of the VTEC pathogen responsible is of the highest priority in clinical and public health settings. For these reasons, we recommend a hierarchical typing approach in which isolates are initially ribotyped. When necessary, the more labor-intensive PFGE can be performed for the final determination of strain relatedness. In many cases, however, a detailed identification is not urgent.

In conclusion, the RiboPrinter appears to be very useful for rapid identification of strains of the most prevalent VTEC serotypes in Japan, O26, O111, and O157. With a hierarchical or sequential typing approach combining the RiboPrinter and PFGE, rapid and accurate typing can be achieved during an outbreak of VTEC.

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