Jpn. J. Infect. Dis., 55, 19-22, 2002

Short Communication

High Genomic Diversity of Enterohemorrhagic *Escherichia coli* Isolates in Japan and Its Applicability for the Detection of Diffuse Outbreak

Jun Terajima, Hidemasa Izumiya, Sunao Iyoda, Kazumichi Tamura and Haruo Watanabe*

Department of Bacteriology, National Institute of Infectious Diseases, Toyama 1-23-1, Shinjuku-ku, Tokyo 162-8640, Japan

(Received December 4, 2001. Accepted February 19, 2002)

SUMMARY: Genotyping of 1,102 enterohemorrhagic *Escherichia coli* isolates by the use of pulsed-field gel electrophoresis (PFGE) carried out from January to November 2000 has revealed the high genomic diversity of these isolates in Japan. By combining the results of genotyping of the isolates with the information from other epidemiological investigations of the cases, we identified a diffuse outbreak in Japan in the year 2000 that seemed to be sporadic but was actually linked. Isolates with only the Shiga toxin 2 gene derived from patient specimens and the contaminated food involved in this diffuse outbreak showed an indistinguishable PFGE profile and the same phage type. Based on the diversity of genotypes among the isolates of enterohemorrhagic *E. coli* O157:H7/- in Japan, we suggest the presence of a few other possible diffuse outbreaks due to the organisms, showing indistinguishable genotypes.

The outbreak of enterohemorrhagic *Escherichia coli* (EHEC) O157:H7 infection in 1996 that affected more than 15,000 persons and caused the death of at least five children has led to the establishment of several measures for the control and prevention of EHEC infection in Japan (1). EHEC infection was listed as a specially designated communicable disease and notification of both symptomatic and asymptomatic cases has become compulsory since August of the same year. The disease was classified as a category III infectious disease under the Law Concerning the Prevention of Infectious Diseases and Medical Care of Patients with Infections enacted in April of 1999 in Japan (2).

Laboratory-based surveillance, especially the molecular subtyping of EHEC isolates, is an established procedure that has received increased support after multiple large-scale outbreaks occurred in 1996 in Japan. Among several molecular subtyping techniques, we applied pulsed-field gel electrophoresis (PFGE) to obtain further discrimination of isolates required for the investigation of the outbreak; other methods included evaluation of amplified-fragment length polymorphism (AFLP) analysis (3) and random amplification of polymorphic DNA methods.

PFGE analysis has proven to be useful in discriminating isolates of the same serotype such as O157:H7. This method has led to an understanding of the prevalence throughout Japan of the divergent clones of O157:H7 (4). Furthermore, because bacteriophage typing is easy to perform and has already been internationally standardized, we included phage typing as a subtyping method, in addition to PFGE and *stx* typing to obtain maximum strain discrimination; this approach has enhanced the reliability of the analysis (5,6).

By combining the results of the subtyping of these isolates with information about other epidemiological investigations of the cases, it became possible to detect diffuse outbreaks that only appeared sporadic but were in fact linked to one another. In 1998, analyses using PFGE and phage typing combined with other epidemiological data eventually revealed a diffuse outbreak involving 49 symptomatic patients and 13 asymptomatic carriers dispersed in seven prefectures and municipalities in Japan (7). It is urgent and important to have a functioning surveillance network for the detection of foodborne diseases based on reliable and established methods (8-10), because contaminations of mass-produced or widely distributed food products are apparently emerging as a serious threat to public health (11).

We report here that there exists genomic diversity among the isolates of EHEC in Japan and that the results of subtyping of EHEC isolates, combined with other information from epidemiological investigations, have enabled us to link widely dispersed outbreaks and sporadic cases to one another.

Since 1996, we have been collecting clinical isolates of EHEC from each municipal public health institute in Japan. PFGE analysis has revealed 1,483 isolates of EHEC O157 in 1998, 1,605 in 1999, and 1,102 in 2000 as of November 17. Fifty-seven O157:H- isolates were included among 1,102 O157:H7/-. Biochemical identification of specimens that were sent to our institute was reconfirmed by standard tests such as growth on MacConkey sorbitol agar with cefixime-potassium tellurite medium (CT-SMAC) including serotyping for somatic and flagellar antigen identification. In terms of Shiga toxin production, 57% of the isolates were positive for both *stx*1 and *stx*2, 41% were positive only for *stx*2, and 2% were positive only for *stx*1. All of the specimens were subjected to PFGE analysis according to a previously described method (4). PFGE profiles were recorded as digitalized data for further analysis by a computer software, GelCompar II from Applied Maths (Kortrijk, Belgium). As for the isolates of EHEC O157 from the indicated period in 2000, we have analyzed a total of 1,102 of EHEC O157:H7/- isolates that were derived from 756 incidents in Japan during that period. The PFGE patterns showed high diversity of these isolates,

*Corresponding author: Fax: +81-3-5285-1171, E-mail: haruwata@nih.go.jp

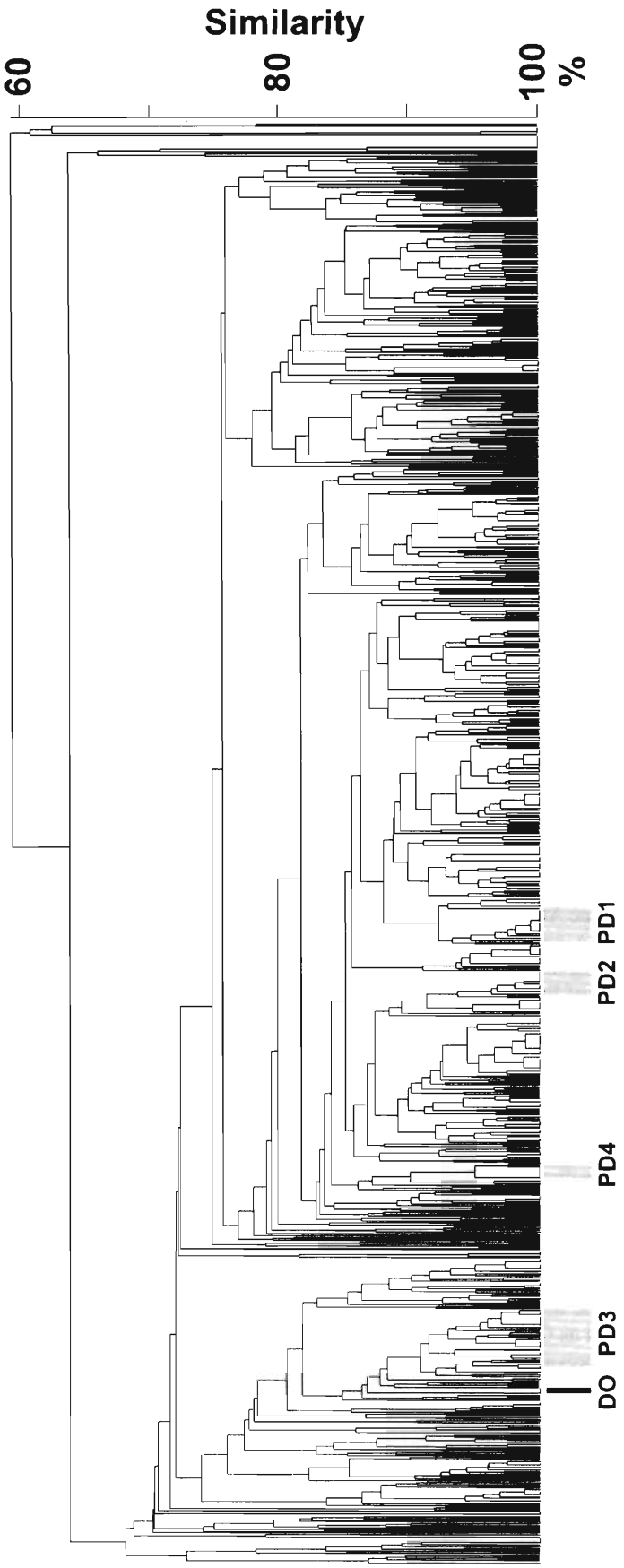

Fig. 1. The dendrogram of PFGE profiles of EHEC O157 isolates. A total of 1,102 isolates of O157 were analyzed by PFGE and their profiles were analyzed to obtain the dendrogram using the computer software GelCompar II version 2.0 (Applied Maths). The isolates belonging to each cluster are indicated as either DO, a diffuse outbreak, or as PD, one to four possible diffuse outbreaks. Dice coefficient was chosen to calculate the similarity matrix and the unweighted pair group method using arithmetic averages was selected to specify the clustering method.

as shown in Figure 1.

By analyzing 1,102 isolates of EHEC O157:H7/-, we identified several isolates with indistinguishable PFGE pattern, which may indicate epidemiological linkage. In fact, one event that included three sporadic cases was dispersed into two widely separated prefectures; a patient was reported in Wakayama Prefecture in August and two other patients were reported in Kanagawa Prefecture in September (Fig. 2, DO). EHEC O157:H7 infected all three patients. Epidemiological investigation revealed that they consumed grilled beef slices (*yaki-niku*) at different steak houses (*yaki-niku* restaurant). Strains of O157:H7 were also isolated from a piece of meat unserved and left at a restaurant in Kanagawa; this meat was from the same lot of an unopened frozen stock of beef imported from the United States. The same brand of imported frozen beef was used at the restaurant in Wakayama where another patient was reported to consume *yaki-niku*. All of the isolates were of the same phage type, although it was atypical and had only the *stx*2 gene. The PFGE profiles of the isolates showed a similarity of 100% in a dendrogram analysis of the profiles conducted by GelCompar II. We confirmed indistinguishable PFGE profiles of the isolates by using three different restriction endonucleases (*Xba*I, *Bln*I, and *Spe*I).

Based on the genomic diversity of O157:H7/- isolates revealed by PFGE analysis, we suggest that four more possible diffuse outbreaks among 756 incidents occurred, whereby the isolates showed the same phage type, the same *stx* subtype, and identical PFGE profiles. Depending on the date of isolation, strains of possible diffuse outbreaks might be separately analyzed on different PFGE gels, and therefore, the percentage of similarity on the dendrogram among isolates within the same cluster was not always 100% when isolates were analyzed by the computer software. However, when we reanalyzed profiles belonging to the same cluster on the same gel, they were actually indistinguishable from one another.

The first possible diffuse outbreak (PD1) occurred between January and August 2000, and consisted of nine incidents from five dispersed prefectures; this outbreak included six sporadic cases and two outbreaks. One of these was at a house for the elderly, where four symptomatic and four asymptomatic patients were reported, and the other outbreak was at a daycare center, where three symptomatic and two asymptomatic patients were involved. Neither documentations nor isolates of the source of contamination regarding each case were reported. These isolates formed a cluster in the dendrogram of PFGE profiles. The similarity of this cluster was 92.2% and the cluster included an isolate from the outbreak at the house for the elderly; one band difference in the PFGE profile was observed. The phage type of these isolates was PT1, with the exception of the abovementioned isolate from the outbreak at the house for the elderly that was of an atypical phage type. The similarity of the profiles of these isolates was 94.7% by the exclusion of this isolate (Fig. 1, PD1).

The second possible diffuse outbreak (PD2) involved nine incidents dispersed among seven prefectures in the western part of Japan. PD2 included two outbreaks in Fukuoka Prefecture; one in a daycare center, and the other at a family barbecue party (Fig. 2, PD2). The cases were reported from August 18 to September 27. Four cases were related to the consumption of *yaki-niku* though the original source of beef was unknown. There were 15 isolates from the nine incidents that showed indistinguishable PFGE patterns, except for one isolate with a single band difference from the isolate derived from the outbreak at the family barbecue party. The phage

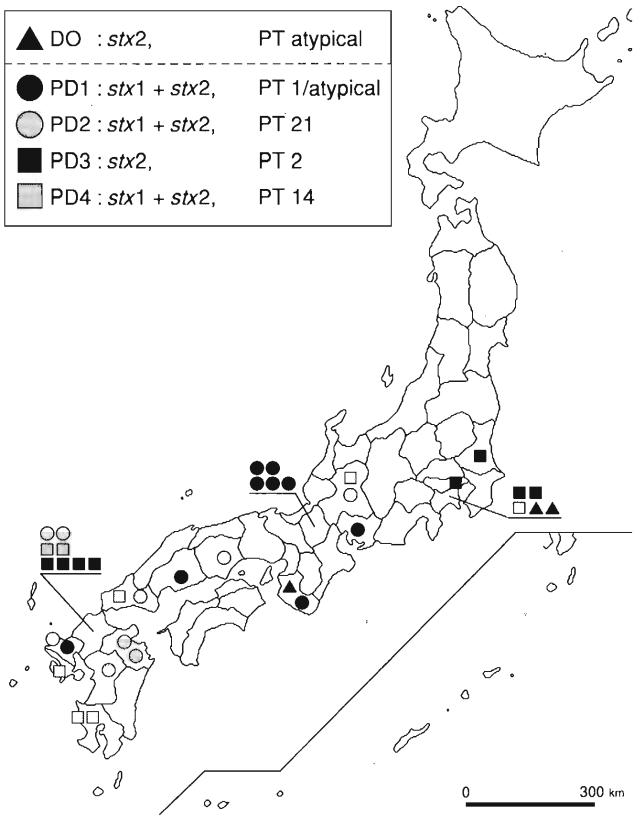

Fig. 2. A map of Japanese prefectures showing the distribution of a diffuse outbreak and several other diffuse outbreaks in 2000. Sporadic cases and outbreaks comprising a diffuse outbreak are represented by the same symbol. Isolates from the same incident showed indistinguishable PFGE profiles. *stx*1 and *stx*2 represent Shiga toxins 1 and 2, respectively. Atypical indicates an unclassified lysis pattern. PT, phage type.

type of these isolates was PT21. The isolates clustered within a similarity of 96.3% in the dendrogram.

The third outbreak (PD3) involved eight incidents in the eastern and western parts of Japan: Tokyo Metropolitan City, Kanagawa Prefecture, Ibaragi Prefecture, and Fukuoka Prefecture (Fig. 2, PD3). PD3 included two outbreaks in Kanagawa and one in Fukuoka. One of the outbreaks was reported in June in a house for the elderly who were seen at the hospital within the facility in Kanagawa. We received 11 isolates from the patients as well as from asymptomatic patients; a specific contaminated food was never identified in this outbreak. Another outbreak in Kanagawa involved two patients who had eaten *yaki-niku* at *yaki-niku* restaurant in August. All six patients involved in another outbreak in Fukuoka had traveled to Korea on the same tour for four days in the middle of September and had eaten an ethnic dish of beef in common in Korea. Isolates derived from sporadic cases from the end of July to the middle of September were clustered with a similarity of 90.8% in the dendrogram. The relative lack of similarity was probably due to the various PFGE runs needed to identify the larger number of isolates. The phage type of all of these isolates was PT2.

The fourth outbreak (PD4) involved eight independent incidents. PD4 included a *yaki-niku* associated outbreak in Kanagawa as well as other sporadic cases; all of these cases were reported in the western part of Japan, except for one in Gifu Prefecture, from the end of June to the end of September (Fig. 2, PD4). All ten isolates from these cases showed a

similarity of 95.0% in the dendrogram, and all were of phage type 14.

There appears to be a relatively high number of diffuse outbreaks depicted in Figure 1, apart from the four abovementioned outbreaks, because a few clusters with relatively high similarity in the dendrogram were observed. It is possible that the isolates belonging to the clusters with relatively high similarity in the dendrogram may represent a diffuse outbreak, since they differed by only one or two bands in the PFGE profiles and also because they were of the same phage type and *stx* subtype. However, we did not include these cases among the diffuse outbreaks due to a lack of thorough documentation from the epidemiological investigations.

We established in this report that the EHEC O157 strains had a high genomic diversity. The present findings suggest that it is important to make note of close epidemiological linkage among cases showing high values of similarity in dendrogram of PFGE profiles. The system described here has the capacity to find diffuse outbreaks. It is suggested that more prompt recognition of such cases be accomplished by reducing the time required to obtain decisive results after an initial case report. An international collaborative effort should be made to block the dissemination of pathogen-contaminated food products across national borders to prevent foodborne diseases.

ACKNOWLEDGMENTS

We thank all of the investigators from a total of more than 80 prefectural and municipal public health institutes and public health centers in Japan for providing us with samples and information. We also thank Mrs. Sumiyo Asahara and Mr. Takahiro Takei for their help in conducting the PFGE analysis.

This work was supported by a grant from the Ministry of Health, Labour and Welfare of Japan.

REFERENCES

1. Watanabe, H., Wada, A., Inagaki, Y., Itoh, K. and Tamura, K. (1996): Outbreaks of enterohaemorrhagic *Escherichia coli* O157:H7 infection by two different genotype strains in Japan, 1996. Lancet, 348, 831-832.
2. National Institute of Infectious Diseases and Infectious Diseases Control Divission, Ministry of Health and Welfare (2000): The status of enterohemorrhagic *Escherichia coli* infection in Japan, 1998-March 2000. Infect. Agents Surveillance Rep., 21, 92'-93'.
3. Iyoda, S., Wada, A., Weller, J., Flood, S. J., Schreiber, E., Tucker, B. and Watanabe, H. (1999): Evaluation of AFLP, a high-resolution DNA fingerprinting method, as a tool for molecular subtyping of enterohemorrhagic *Escherichia coli* O157:H7 isolates. Microbiol. Immunol., 43, 803-806.
4. Izumiya, H., Terajima, J., Wada, A., Inagaki, Y., Itoh, K. I., Tamura, K. and Watanabe, H. (1997): Molecular typing of enterohemorrhagic *Escherichia coli* O157:H7 isolates in Japan by using pulsed-field gel electrophoresis. J. Clin. Microbiol., 35, 1675-1680.
5. Izumiya, H., Masuda, T., Ahmed, R., Khakhria, R., Wada, A., Terajima, J., Itoh, K., Johnson, W. M., Konuma, H., Shinagawa, K., Tamura, K. and Watanabe, H. (1998): Combined use of bacteriophage typing and pulsed-field gel electrophoresis in the epidemiological analysis of Japanese isolates of enterohemorrhagic *Escherichia coli* O157:H7. Microbiol. Immunol., 42, 515-519.
6. Terajima, J., Izumiya, H., Wada, A., Tamura, K. and Watanabe, H. (2000): Molecular epidemiological investigation of enterohaemorrhagic *Escherichia coli* isolates in Japan. J. Appl. Microbiol., 88, 99S-105S.
7. Terajima, J., Izumiya, H., Iyoda, S., Tamura, K. and Watanabe, H. (1999): Detection of a multi-prefectual *E. coli* O157:H7 outbreak caused by contaminated Ikura-sushi ingestion. Jpn. J. Infect. Dis., 52, 52-53.
8. Smith, D., Willshaw, G., Stanley, J. and Arnold, C. (2000): Genotyping of verocytotoxin-producing *Escherichia coli* O157: comparison of isolates of a prevalent phage type by fluorescent amplified-fragment length polymorphism and pulsed-field gel electrophoresis analyses. J. Clin. Microbiol., 38, 4616-4620.
9. Stephenson, J. (1997): New approaches for detecting and curtailing foodborne microbial infections. JAMA, 277, 1339-1340.
10. Willshaw, G. A., Smith, H. R., Cheasty, T., Wall, P. G. and Rowe, B. (1997): Vero cytotoxin-producing *Escherichia coli* O157 outbreaks in England and Wales, 1995: phenotypic methods and genotypic subtyping. Emerg. Infect. Dis., 3, 561-565.
11. Pebody, R. G., Furtado, C., Rojas, A., McCarthy, N., Nylen, G., Ruutu, P., Leino, T., Chalmers, R., de Jong, B., Donnelly, M., Fisher, I., Gilham, C., Graverson, L., Cheasty, T., Willshaw, G., Navarro, M., Salmon, R., Leinikki, P., Wall, P. and Bartlett, C. (1999): An international outbreak of Vero cytotoxin-producing *Escherichia coli* O157 infection amongst tourists; a challenge for the European infectious disease surveillance network. Epidemiol. Infect., 123, 217-223.