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Short Communication

Evaluation of Pulsed-Field Gel Electrophoresis Analysis Performed at Selected Prefectural Institutes of Public Health for Use in PulseNet Japan

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SUMMARY: In order to evaluate the reliability of pulsed-field gel electrophoresis (PFGE) analysis performed at different prefectural public health institutes (PHIs) for use in the PulseNet Japan surveillance system to detect enterohemorrhagic *Escherichia coli* O157, we compared the results of PFGE-typing of 14 selected strains of O157 performed at 8 selected PHIs to evaluate the reliability of different experimental protocols used in these PHIs. PFGE was performed for 14 strains for which there were 14 PFGE types in 3 PHIs, and 13 PFGE types in 5 PHIs by using their own protocols and/or those of the National Institute of Infectious Diseases (NIID). PFGE fingerprints from 5 out of the 8 PHIs were successfully genotyped for all of the 14 strains. A PFGE fingerprint from one PHI was successfully genotyped when the NIID pulsing protocol was used, but was not genotyped when the PHI's own protocols were used. PFGE fingerprints from 2 PHIs failed to be genotyped for one each of the strains. The cause of this genotyping failure was considered to be inappropriate PFGE pulsing protocols or inadequate digestion of chromosomal DNA. These results suggest that PFGE protocols should be standardized for the establishment of PulseNet Japan.

Following the large outbreak of food-borne enteritis by enterohemorrhagic *Escherichia coli* O157 in Sakai City, Osaka, Japan in 1996 (1), O157 has become one of the most serious food-borne related public health concerns in Japan. This is because the pathogen has been isolated frequently from diarrheal patients in local food-borne outbreaks and from sporadic gastroenteritis cases. Diffuse outbreaks of O157 gastroenteritis have been reported in recent years in Japan (2-5). These outbreaks occurred in several different prefectures at almost the same time. In the United States (U. S.), the Centers for Disease Control and Prevention (CDC) has organized the PulseNet surveillance system in order to quickly detect diffuse outbreaks of several food-borne bacterial diseases and to prevent their spread. The PulseNet is a network system connecting the CDC and public health laboratories throughout the U. S. Standardization of pulsed-field gel electrophoresis (PFGE) protocols has been reported to be necessary for obtaining interpretable PFGE fingerprint patterns and for reliable performance of the PulseNet (available from: http://www.cdc.gov/pulsenet/index.htm) (6).

In Japan, the National Institute of Infectious Diseases (NIID) and prefectural public health institutes (PHIs) have made plans to set up a PFGE-based national network (PulseNet Japan) for monitoring the cross-prefectural spread of O157 strains and detecting diffuse outbreaks in Japan (5). However, the protocols for performing PFGE for O157 have not standardized in Japan, and different laboratories continue to use their own protocols for local PFGE analyses.

In order to examine the degree of standardization necessary for the PFGE protocols in PulseNet Japan, we performed a preliminary study by matching PFGE fingerprints generated by different PHIs using O157 strains which were pre-genotyped by the NIID. Fourteen selected strains of O157 having 14 PFGE types were used for PFGE typing by 8 prefectural PHIs randomly selected from 8 different districts of Japan (Hokkaido, Tohoku, Kanto Ko-Shin-Sei, Tokai Hokuriku, Kansai, Chugoku Shikoku, Kyushu, and Okinawa). These PHIs used their own and/or the NIID PFGE protocols. The 14 strains of O157 were pre-typed as PFGE types Ia to VII (namely, Ia, Ib, Ic, IIa, IIb, IIe, IIj, IIIa, IIIb, IIIc, IV, Va, VI, and VII) by the Department of Bacteriology, NIID (7) (Fig. 1).

In the preparation of chromosomal DNA for these O157 strains, the NIID procedure was slightly different from the previously reported procedure (8). In the chromosomal DNA preparation, bacterial cells on an agar medium were directly embedded in low-melting-temperature agarose (Chromosomal Agarose; Bio-Rad Laboratories, Richmond, Calif., USA). An overnight incubation at 37°C in a lysozyme

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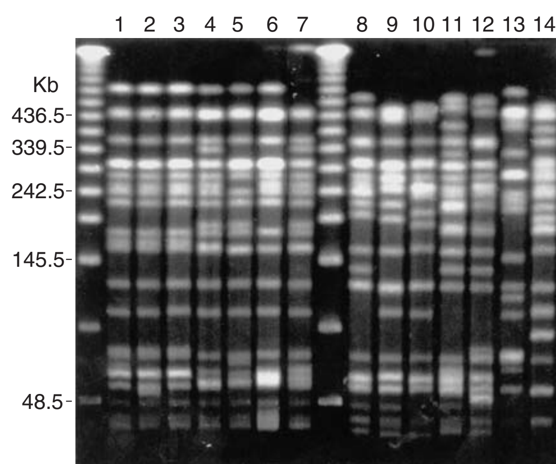

Fig. 1. PFGE fingerprint patterns made by the NIID.
Lambda DNA concatemers were placed in the left side end and middle lanes. Lanes 1 to 14, PFGE types Ia, Ib, Ic, IIa, IIb, IIe, IIj, IIIa, IIIb, IIIc, IV, Va, VI, and VII.

solution (1 mg/ml in 0.5M EDTA, pH 8.0) was added to the procedure as a lysing step. All of the reagents except for the low-melting-temperature agarose and Pefabloc SC (Roche Dignostics GmbH., Mannheim, Germany) were purchased from Sigma Chemical Co. (St. Louis, Mo., USA).

The 8 participating prefectural PHIs were randomly designated as A to H. The NIID supplied the 14 strains of O157 in stab cultures to the Aichi Prefectural Institute of Public Health (AiPHI). AiPHI numbered the cultures from 1 to 14, and sent the cultures in agar stabs to the remaining 7 PHIs. Two PHIs (A and B) received the 14 strains belonging to the 14 different PFGE types. However, the remaining 5 PHIs received 14 strains of 13 different PFGE types, i.e., 2 samples with the same PFGE type IIb (no sample with type IIe) were shipped out to these 5 PHIs from AiPHI by mistake. The different protocols and apparatuses used in the 8 PHIs together with the NIID protocol are shown in Table 1.

Four PHIs (D, E, G, and H) prepared chromosomal DNA by using commercially available kits. Two (A and F) of these used the NIID protocol, and the remaining two (B and C) used both their own (procedures B and C) and the NIID protocols. As for the difference between their own procedures and the NIID, procedure B did not involve a lysis step using a lysozyme. Procedure C was different with respect to the concentration of low-melting-temperature agarose (the institute C: 1.2%; NIID: 1.0%) and the components of the lysozyme solution. In procedure C, the lysis solution (10 mM Tri-HCl, pH 8.0, 1M NaCl, 0.2% deoxycholate, 0.5% polyoxyethylene acyl ether [Brij 58], Sarkosyl [N-lauroylsarcosine]) was used. Regarding the restriction enzyme, all of the 8 PHIs used *Xba*I just as did the NIID; however, considerably different amounts of the enzyme were used in each PHI (30 units for 4 institutes, 50 units for 2 institutes, and 10 and 20 units for 1 institute each). Half of the PHIs (A, E, F, and H) used the same amount (30 U) of *Xba*I as the NIID used routinely. On top of these differences, these 8 PHIs utilized reagents prepared by themselves. With regard to the PFGE pulsing protocol, 3 PHIs (A, C, and G) used the NIID protocol; 4 (D, E, F, and H) applied their own protocols, but the protocols of 3 (E, F, and H) of these 4 PHIs were very similar to the NIID protocol. PHI B used both its own and the NIID protocol. In all of the PHIs, gels were stained with ethidium bromide solutions (0.2 - 0.5 μg/ml) after electrophoresis and photographed with a Polaroid or a charge-coupled device camera. Six PHIs sent the gel pictures to AiPHI by ordinal mail and one (G) sent the pictures as digital images by e-mail. A staff member in AiPHI, who had had experience working in the field of molecular epidemiology of bacterial pathogens using PFGE for over 10 years, compared the submitted fingerprints with ones supplied by the NIID and classified the strains into 14 PFGE types.

A total of 10 PFGE fingerprints from the 8 PHIs were visually analyzed for typing (namely, 2 each from institutes B and C generated by their own and the NIID protocols, and 1 each from the remaining 6 PHIs). Classification of theses fingerprints revealed that PFGE fingerprints from 5 PHIs (A, C, F, G, and H) correctly classified all of the 14 strains despite using different PFGE experimental protocols and apparatuses. It is noted that the pulsing protocols used in these 5 PHIs were identical or very similar to the NIID protocols. As for institute B, which used both their own and the NIID pulsing protocols, all of the 14 strains were correctly typed only when the NIID pulsing protocol was used. An interest-

Table 1. Experimental protocols and apparatuses used in the 8 PHIs and the NIID

Institute	DNA preparation procedures	*Xba*I per sample (Units)	Run time (hr)	Voltage (V/cm)	Pulsing protocol	Models of PFGE apparatus (manufacturer)	Results of genotyping
A	NIID	30	22	200[1)]	NIID	Gene Navigator (P)	Yes
B	NIID	10	22	6	NIID	CHEF Mapper (B)	Yes
	Procedure B	10	22	6	22hr:5 - 50sec	CHEF Mapper (B)	No
C	NIID	20	22	200[1)]	NIID	Pulsaphor 2015 (P)	Yes
	Procedure C	20	22	200[1)]	NIID	Pulsaphor 2015 (P)	Yes
D	GenePath Reagent Kit	50	22	6	22hr:4 - 50sec	CHEF DR III (B)	No
E	CHEF Bacterial DNA Plug Kit	30	20	6	11hr:4 - 8sec; 9hr:8 - 50sec	CHEF DR II (B)	No
F	NIID	30	22	6	9hr:4 - 8sec; 13hr:8 - 50sec	CHEF DR III (B)	Yes
G	GenePath Reagent Kit	50	22	6	NIID	GenePath (B)	Yes
H	GenePath Reagent Kit	30	21	6	11hr:4 - 8sec; 10hr:8 - 50sec	GenePath (B)	Yes
NIID	NIID	30	22	6	12hr:4 - 8sec; 10hr:8 - 50sec	CHEF DR III (B)	
		30	22	200[1)]	6hr:4 - 8sec; 1min:8 - 4sec; 6hr:4 - 8sec; 10hr:8 - 50sec	Gene Navigator (P)	

PHIs, public health institutes; NIID, National Institute of Infectious Diseases, Japan; P, Pharmacia; B, Bio-Rad Laboratories.
Yes: all of the 14 strains were correctly genotyped. No: one strain out of the 14 strains was not correctly genotyped.
[1)]: Overall voltage.

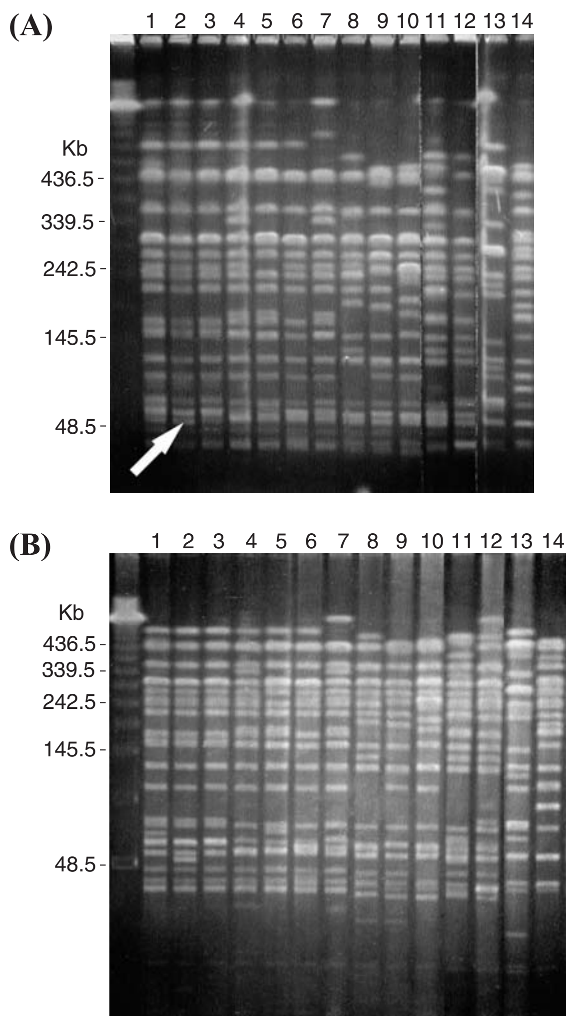

Fig. 2. (A) PFGE fingerprint patterns submitted from the institute B with their own pulsing protocol.
Lambda DNA concatemers were placed in the left side end lane. Lanes 1 to 14, PFGE types Ia, not typed (should be Ib), Ic, IIa, IIb, IIe, IIj, IIIa, IIIb, IIIc, IV, Va, VI, and VII. White arrow highlights a fragment that should separate into two fragments.
(B) PFGE fingerprint patterns submitted from the institute B with the NIID pulsing protocol.
Lambda DNA concatemers were placed in the left side end lane. Lanes 1 to 14, PFGE types Ia, Ib, Ic, IIa, IIb, IIe, IIj, IIIa, IIIb, IIIc, IV, Va, VI, and VII.

ing finding here was that the 2 PFGE fingerprints from institute C were correctly genotyped although two different DNA preparation procedures were used.

Even though most of the PFGE patterns submitted were correctly classified, 1 each out of the 14 fingerprints from 3 PHIs could not be classified into the PFGE types previously supplied by the NIID. Among these 3 unclassified patterns, 2 cases occurred in the classification of PFGE type Ib, which has 2 characteristic bands at the region of approximately 52 kb. In these 2 unclassified cases, these characteristic bands became 1 band with a slightly higher intensity of the fingerprint from institutes B (Fig. 2A, lane 2) and D. Among the 8 PHIs, only these 2 PHIs applied a single-step pulsing protocol. Judging from these results, the one-step pulsing protocols applied in these PHIs are inappropriate to have a good separation of low molecular weight bands (<100 kb). It should be noted that the pattern of fingerprints made by institute B using the NIID pulsing protocol was correctly classified into the corresponding PFGE pattern supplied by the NIID (Fig. 2B).

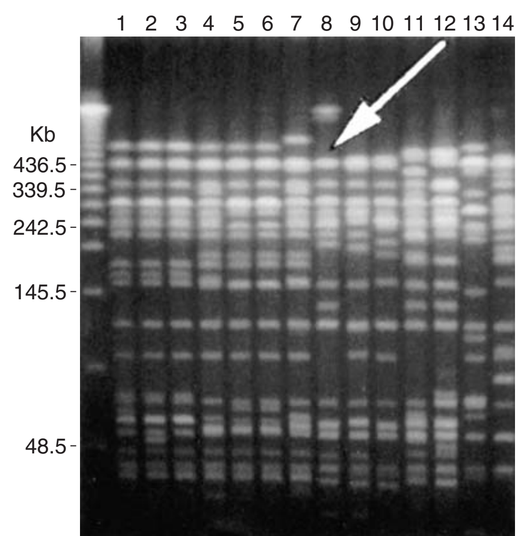

Fig. 3. PFGE fingerprint patterns submitted from the institute E.
Lambda DNA concatemers were placed in the left side end lane. Lanes 1 to 14, PFGE types Ia, Ib, Ic, IIa, IIb, IIb, IIj, not typed (should be IIIa), IIIb, IIIc, IV, Va, VI, and VII. White arrow highlights loss of a fragment that is specific to PFGE type IIIa.

In an inter-laboratory study of PFGE typing for methicillin-resistant *Staphylococcus aureus*, it was found that the pulsing protocol is one of the most important experimental protocols for obtaining interpretable PFGE fingerprint patterns and for performing reliable comparison of the PFGE patterns among different institutes. Therefore, the study concluded that the pulsing protocol should be standardized to obtain inter-laboratory reproducibility of PFGE analysis (9).

Another unclassification occurred in the classification of PFGE type IIIa, which has 1 characteristic band at approximately 530 kb (Fig. 3, lane 8). The PFGE pattern submitted from institute E, however, had 2 unexpected bands over 630 kb instead of the 530 kb band, the observation of which was considered to be caused by incomplete digestion of chromosomal DNA. The loss of the 530 kb band and acquisition of 2 bands over 630 kb may have been caused by decreased activity of the restriction enzyme despite using the appropriate amount (30 U per sample). There are two possible explanations for the decreased activity of the enzyme: (i) proteinase K, which can degrade the restriction enzyme, may have existed in the reaction solution due to insufficient addition of an inhibitor of the proteinase K (phenylmethanesulfonyl fluoride or Pefabloc SC) and (ii) the density of bacterial suspension may have been extremely high in relation to the amount of the restriction enzyme added. The precise mechanism underlying these contradictory observations, however, remains to be elucidated.

In this study, we visually analyzed the PFGE fingerprints of a limited number of only 14 strains. Moreover, the PFGE types of all of these 14 strains were previously known when determining the corresponding types of these strains based on the analysis of PFGE patterns submitted from the different PHIs. In contrast to the situation we experienced in this study, the PulseNet Japan system will need to be capable of analyzing numerous PFGE fingerprint patterns of unknown genotypes submitted from a far greater number of PHIs all around the country. Accordingly, it is reasonable to assume that certain

unexpected problems, such as un- or mis-typing of O157, will occur in PulseNet Japan. In order to overcome or lessen these problems, it is considered preferable to standardize all of the PFGE experimental protocols and apparatuses used in all of the participating institutes. On the basis of the findings observed in this study, the NIID has developed a new protocol for the establishment of PulseNet Japan. The new protocol includes the CDC pulsing protocol (6V/cm, 2.2 - 54.2 s, 19 h) and *Salmonella* Braenderup H9812 as a standard marker. The newly introduced protocol by the NIID is considered to be suitable as the PFGE standard protocol for PulseNet Japan with a potential for international application of the PFGE network. Moreover, there is no doubt that analytical software is an essential tool for the analysis of large numbers of PFGE fingerprint patterns. However, in the study presented here, we tried to match PFGE fingerprint patterns generated by different PHIs by visual analysis as a preliminary study for the establishment of PulseNet Japan. This is because none of the participating PHIs, including AiPHI, had the necessary analytical software at the time this study was performed.

It should also be remembered that we were able to obtain satisfactory results in this study mainly because we selected the participating PHIs from eight areas of Japan with a substantial amount of experience in PFGE analysis of bacterial classification. In the present study, it should be emphasized that training of staff members in PHIs, especially those having no or little experience in PFGE analysis, is important for the establishment of the PulseNet Japan system and maintenance with considerable reliability.

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