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Short Communication

An Improved Protocol for the Preparation and Restriction Enzyme Digestion of Pulsed-Field Gel Electrophoresis Agarose Plugs for the Analysis of *Legionella* Isolates

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SUMMARY: Pulsed-field gel electrophoresis (PFGE), which determines the genomic relatedness of isolates, is currently used for the epidemiological investigation of infectious agents such as bacteria. In particular, this method has been used for the epidemiological investigation of *Legionella* outbreaks. However, it takes 4 days to complete a *Legionella*-PFGE analysis. Due to partial digestion and DNA damage, the reproducibility of the obtained fragment digestion patterns is poor for this pathogen. In this study, we report an improved protocol that takes only 2 days to complete and that allows clear discrimination of the restriction profile with higher reproducibility than that previously achieved.

Each bacterial strain has genomic diversity in its nucleotide sequence. Restriction enzyme digestion of genomic DNA produces a distinctive restriction profile that is useful for epidemiological analyses. The standard gel electrophoresis protocol was improved by introducing an alternating voltage gradient to obtain better resolution of larger DNA fragments; this technique is known as pulsed-field gel electrophoresis (PFGE) (1). The PFGE method is very useful for discriminating between bacterial genomes.

Legionella are ubiquitous inhabitants of aquatic environments and moist soil, and they replicate as intracellular parasites of protozoa (2,3). Hot springs, public baths, and cooling towers are the most common sources of legionellosis in humans. PFGE has been used to identify sources and routes of *Legionella* infections (4,5). The conventional PFGE protocol was modified by De Zoysa and Harrison; according to this modification, no lysozyme treatment was performed, and the concentrations of proteinase K (2 mg/ml) and *Sfi*I (60 U/plug) were increased (6). However, that protocol required at least 4 days to yield results, and reproducibility remained poor (4,6). Given these disadvantages associated with this method, we conducted the present study to improve the PFGE protocol.

A total of 16 *Legionella* strains belonging to 6 species were used in this study (Table 1). We collected 10 *L. pneumophila* strains isolated from patients, bath water, and cooling tower water, and these strains belonged to serogroups 1, 5, 6, and 7. Strain K6 possessed a Km^r Laz^+ cassette gene on the chromosome of strain Philadelphia-1 (8). Five non-*L. pneumophila* strains isolated from patients were also collected, and these belonged to different *Legionella* spp. (Table 1). All strains were grown at 37°C on buffered charcoal yeast extract (BCYE) agar (Becton Dickinson, Sparks, Md., USA). After a 2-day incubation period, *Legionella* cells were suspended in sterile water at an optical density at 600 nm (OD_{600}) of 0.5. The suspensions were then mixed with the same volume of 1% SeaKem® Gold Agarose (Cambrex Bio Science Rockland, Maine, USA) and were used for casting plugs. A conventional protocol (4,9) and the improved protocol discussed below were both used to process the agarose plugs for PFGE.

Table 1. Summary of *Legionella* strains used in the study

Strains no.	Species	Serogroup	Strain, source or reference
80-045	*L. pneumophila*	1	Clinical isolate (7)
K6	*L. pneumophila*	1	(8)
Philadelphila-1	*L. pneumophila*	1	ATCC33152[1)], Clinical isolate
NIIB0733[2)]	*L. pneumophila*	1	Bathtub
NIIB0805	*L. pneumophila*	1	Bathtub
NIIB0744	*L. pneumophila*	1	Cooling tower
NIIB0802	*L. pneumophila*	1	Cooling tower
NIIB0784	*L. pneumophila*	5	Bathtub
NIIB0792	*L. pneumophila*	6	Bathtub
NIIB0794	*L. pneumophila*	7	Cooling tower
NIIB0806	*L. pneumophila*	7	Cooling tower
NIIB0008	*L. micdadei*	–	ATCC33218, Clinical isolate
NIIB0009	*L. bozemanii*	1	ATCC33217, Clinical isolate
NIIB0010	*L. dumoffii*	–	ATCC33343, Clinical isolate
NIIB0012	*L. longbeachae*	2	ATCC33484, Clinical isolate
NIIB0052	*L. feeleii*	2	ATCC35849, Clinical isolate

[1)]: ATCC, American Type Culture Collection.
[2)]: NIIB, National Institute of Infectious Diseases, Department of Bacteriology.

The conventional protocol is shown in Fig. 1; *Legionella* cells in the agarose plugs were first lysed with lysozyme (Wako Pure Chemical Industries, Osaka, Japan). Subsequently, the plugs were treated overnight with 1 mg/ml of proteinase K (Roche Diagnostics, Mannheim, Germany) and 1% N-lauroylsarcosine (Sigma-Aldrich, St. Louis, Mo., USA) in 0.5 M EDTA (pH 8.0) at 50°C. After washing the plugs with Pefabloc SC (Roche Diagnostics), TE Buffer (10 mM Tris: 1 mM EDTA, pH 8.0), and 1×M buffer (TaKaRa Bio, Otsu, Japan), DNA in the plugs were digested overnight with

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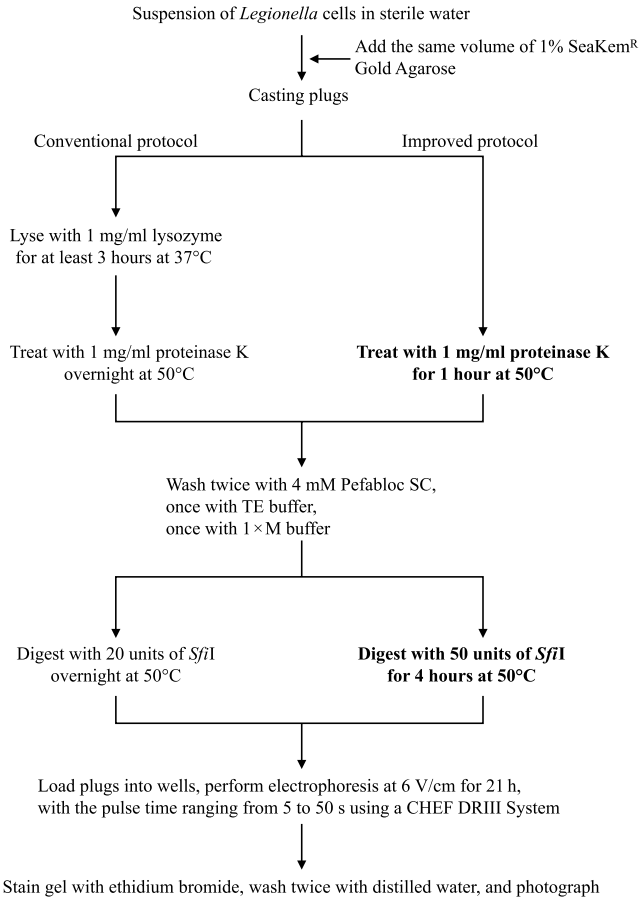

Fig. 1. Experimental overview of the conventional and improved PFGE protocols for *Legionella* strains. The main modified steps are shown in bold.

20 units of *Sfi*I (10 U/μl; TaKaRa Bio). Subsequently, electrophoresis was carried out using a CHEF DRIII System (Bio-Rad Laboratories, Richmond, Calif., USA). Thereafter, the gels were stained and photographed. This conventional PFGE protocol took 4 days to complete. The electrophoresis profiles of 16 *Legionella* strains yielded by the conventional protocol are shown in Fig. 2A. A white smeary band at the bottom of the gel (e.g., lanes 3, 5, 9, and 10) was observed, suggesting that some damage to the DNA had occurred. Furthermore, some faint bands possibly attributable to incomplete restriction enzyme digestion were visible in the profiles of all strains. As the incompletely digested bands appeared at different positions in various experiments (data not shown), it was difficult to obtain reproducible profiles and to compare sample data between gels. Similar observations have also been reported by De Zoysa and Harrison (6).

In order to overcome these disadvantages, we modified the conventional protocol for the preparation and restriction enzyme digestion of agarose plugs. To reduce plug preparation time and decrease damage to the genomic DNA, the lysozyme treatment was eliminated. Furthermore, the time required for treatment with proteinase K and N-lauroylsarcosine at 50°C was reduced from overnight to 1 h. Next, a high concentration of *Sfi*I (40 U/μl; Roche Diagnostics) of 50 units/plug was used in order to complete the digestion of *Legionella* DNA. The electrophoresis profiles of the 16 strains analyzed by this improved method are shown in Figs. 2B and 2C. With the improved protocol, no white smeary band was obtained, and the bands of digested DNA fragments were significantly clearer than those shown in Fig. 2A. Fewer digested DNA fragments from *Legionella* strains were seen as compared to the number of those from the corresponding strains presented in Fig. 2A; this difference may be attributed to the disap-

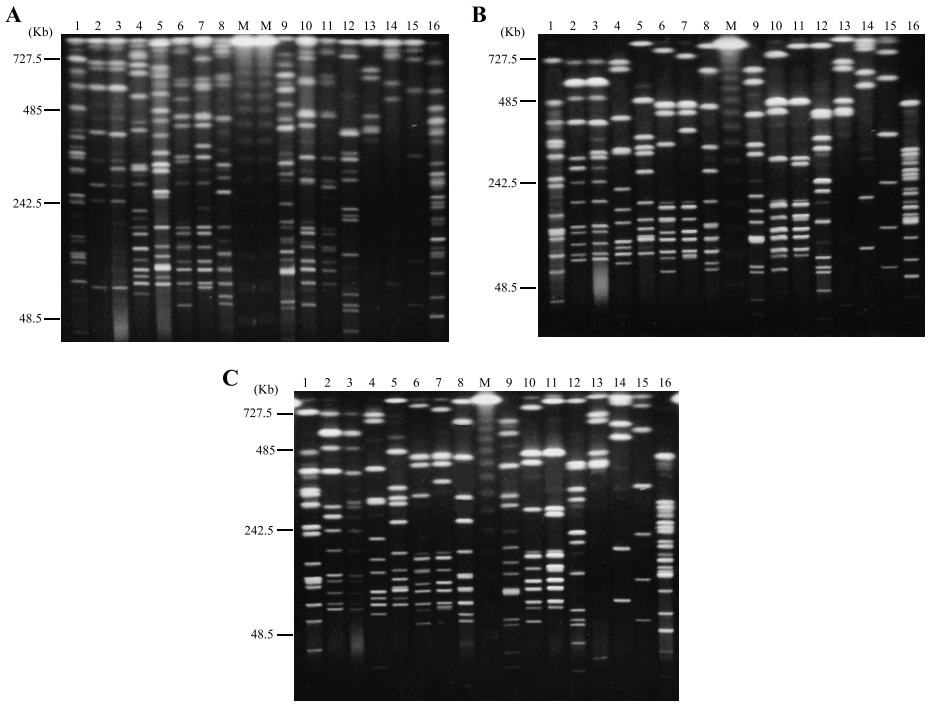

Fig. 2. PFGE profiles obtained with the conventional (A) and improved (B and C) protocols for *Legionella* strains. Lambda ladder marker (Bio-Rad) was used as a DNA size standard and molecular sizes are indicated on the left. Lane 1, 80-045; lane 2, K6; lane 3, Philadelphila-1; lane 4, NIIB0733; lane 5, NIIB0805; lane 6, NIIB0744; lane 7, NIIB0802; lane 8, NIIB0784; lane 9, NIIB0792; lane 10, NIIB0794; lane 11, NIIB0806; lane 12, NIIB0008; lane 13, NIIB0009; lane 14, NIIB0010; lane 15, NIIB0012; lane 16, NIIB0052; M: lambda ladder marker. A white smeary band at the bottom of the gel A (e.g., in lanes 3, 5, 9, and 10) indicates DNA damage.

pearance of incompletely digested fragments (Fig. 2B). The modified protocol of PFGE took only 2 days, as compared to the 4 days required to carry out the original protocol. A time saving of 2 days would exert a significant positive impact on the genotyping of *Legionella* infections in the context of medical diagnostics services.

To examine the reproducibility of the profiles obtained by the improved treatment, PFGE of these *Legionella* strains was separately performed at least 3 times. Figures 2B and 2C show two profiles of independently prepared plugs of *Legionella* isolates produced using different CHEF DRIII System machines. The profiles of the respective strains, with the exception of NIIB0010 (lane 14), were the identical between agarose gels run on different CHEF DRIII System machines. The 3 bands located at the top of lane 14 were separated in Fig. 2B, whereas only imperfect separation is shown in Fig. 2C. When the same machine was used, no differences were observed between the profiles of NIIB0010 (data not shown). Therefore, the difference noted above might be attributable to the different conditions required for the use of different machines. The present results demonstrated that reproducible profiles could be obtained with the improved PFGE method.

In this study, we attempted to overcome some of the disadvantages associated with the conventional PFGE method used for the analysis of *Legionella* strains. The improved protocol was found to be quicker and easier than the former approach. Lysozyme treatment for the preparation of PFGE plugs of Gram-negative bacteria (*Campylobacter jejuni*, *Escherichia coli* O157:H7, *Salmonella*, *Shigella*, *Vibrio cholerae*, and *Yersinia pestis*) has already been omitted from the protocols recommended by Centers for Disease Control and Prevention (CDC) (http://www.cdc.gov/PULSENET/protocols.htm). De Zoysa and Harrison also demonstrated that the use of lysozyme is not necessary for treating *Legionella* plugs (6). Therefore, lysozyme treatment was omitted in the present study as well. The profiles of the isolates examined were identical with and without lysozyme treatment (data not shown). In addition, the duration of proteinase K treatment was reduced in the present study. No differences between profiles of corresponding samples treated with proteinase K for 1, 8, and 24 h (data not shown) were observed. Treatment with 0.5 mg/ml of proteinase K for 1 h was sufficient to produce the same results (data not shown). Thus, the present results demonstrated that *Legionella* cells in plugs can be efficiently lysed by treatment with 0.5 - 1 mg/ml of proteinase K for 1 h. Overnight digestion with *Sfi*I at 50°C gave a white smeary band at the bottom of the gel (data not shown), which was suggestive of damage to the *Legionella* DNA due to extended digestion with the restriction enzyme. Use of the improved protocol described in this study rendered the fragment bands more clearly visible, and thus comparison of the profiles between different isolates was facilitated, as was analysis of the linkage between environmental isolates and the clinical isolates of *Legionella*. This new protocol will therefore be recommended for use in epidemiological investigations of *Legionella* isolates.

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