Rapid Pulsed-Field Gel Electrophoresis Protocol for Typing of *Escherichia coli* O157:H7 and Other Gram-Negative Organisms in 1 Day

ROMESH K. GAUTOM*


Received 23 April 1997/Returned for modification 30 June 1997/Accepted 28 July 1997

Genomic DNA patterns generated by pulsed-field gel electrophoresis are highly specific for different strains of an organism and have significant value in epidemiologic investigations of infectious-disease outbreaks. Unfortunately, time-consuming and tedious specimen processing is an inherent problem which limits the use of this powerful technology as a real-time epidemic investigational tool. Here, I describe a rapid method to improve the response time and provide specific bacterial strain identification for the typing of *Escherichia coli* O157:H7 and other gram-negative organisms in a single day.

The ability to characterize and determine relatedness among bacterial isolates involved in an infectious-disease outbreak is a prerequisite for epidemiologic investigations. Several markers have been used to distinguish isolates of a given bacterial genus or species involved in such outbreaks (1, 21). Historically, clinical and public health laboratories have used conventional epidemiologic typing systems, usually ones based on specific phenotypic characterizations (1, 21). Unfortunately, as a result of inconsistently expressed phenotypic traits, these classical typing approaches are often not able to discriminate between related outbreak strains. The rapidity with which people and goods move within and between countries has increased the threat of epidemic spread of infectious diseases. As such, there is a need for rapid and accurate typing techniques to assist public health authorities in the detection and tracking of these diseases.

Since the advent of molecular fingerprinting, there has been a great deal of effort directed toward developing molecular methods suitable for use in clinical and public health laboratories. Pulsed-field gel electrophoresis (PFGE) is one such approach that identifies organisms by their molecular genotypes (4, 18, 25, 26). It involves the use of rare-cutter restriction enzymes to generate a limited number (10 to 20) of high-molecular-weight restriction fragments. These fragments are then separated by agarose gel electrophoresis with programmed variations in both the direction and the duration of the electric field (the pulsed field). The resulting electrophoretic patterns are highly specific for strains from a variety of organisms and also provide an opportunity to examine multiple variations throughout the genome of the organism so as to identify specific strains and accurately link them with disease outbreaks.

PFGE has great value in epidemiological analysis, in the differentiation of pathogenic strains, and in the monitoring of their spread among communities. The technique has been successfully employed in tracking diseases caused by a number of different bacterial pathogens, including *Escherichia coli* O157:H7 (3, 5), *Neisseria meningitidis* (27, 28), *Pseudomonas aeruginosa* (16), *Listeria monocytogenes* (6), *Bordetella pertussis* (11), *Legionella pneumophila* (15), *Vibrio cholerae* (7), *Staphylococcus aureus* (23), and enterococcal isolates (20). PFGE has also permitted the characterization of isolates indistinguishable by restriction enzyme analysis, phage typing, ribotyping, plasmid analysis, and randomly amplified polymorphic DNA analysis (2, 3, 16, 22, 24, 25).

PFGE has become a standard technique among public health agencies due to its accuracy and reproducibility between different laboratories. However, current PFGE protocols involve time-consuming, tedious procedures for the purification of intact genomic DNA trapped in agarose and lengthy restriction enzyme digestes, and extended electrophoresis times (12, 18). These time-consuming steps preclude the use of current PFGE protocols in monitoring the often rapid evolution of events during ongoing outbreaks (12, 18). However, recent events such as disease epidemics associated with the ingestion of food contaminated with either *E. coli* O157:H7 or *Salmonella* spp. in several western states, along with outbreaks of meningococcal disease (8–10, 14), underscore the pressing need to develop an alternative protocol for PFGE that would improve the response time and allow this powerful molecular typing system to become a more versatile epidemiologic tool. In this paper, I describe such a rapid method for PFGE analysis and demonstrate that it provides specific identification of bacterial strains in 1 day with an accuracy and a reproducibility equivalent to those of conventional multiday procedures.

**Bacterial strains and culture conditions.** Isolates of *E. coli* O157:H7 were submitted to the Washington State Department of Health Public Health Laboratories for confirmation of strain identification or for subtyping by standard PFGE. Other bacterial isolates were obtained from clinical specimens submitted to the Public Health Laboratories for other purposes. The isolates included in this study are listed in Table 1. The isolates were subcultured on appropriate growth media and incubated at 37°C overnight or longer (in the cases of *Bordetella* and *Legionella*).

**Standard PFGE procedure.** This procedure was designed by the Centers for Disease Control and Prevention for use in the tracing of *E. coli* O157:H7 outbreaks (12). Briefly, a single colony of the isolate to be tested was inoculated in 3 ml of standard Trypticase soy broth (Becton Dickinson and Co., Cockeysville, Md.) and incubated at 37°C on a roller drum for 16 to 18 h for further growth. The cells were washed in SE (75 mM NaCl, pH 8.0; 25 mM EDTA, pH 8.0) by centrifugation, and the optical density of the cells at a wavelength of 610 nm was adjusted to 1.40. Chromosomal-grade agarose (Bio-Rad

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* Phone: (206) 361-2885. Fax: (206) 361-2814.
TABLE 1. Bacterial isolates analyzed by rapid PFGE

<table>
<thead>
<tr>
<th>Organism</th>
<th>No. of isolates analyzed</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Escherichia coli</em> O157:H7 patient isolates</td>
<td>40</td>
</tr>
<tr>
<td><em>Escherichia coli</em> O157:H7 food isolates</td>
<td>2</td>
</tr>
<tr>
<td><em>Escherichia coli</em> non-O157:H7 isolates</td>
<td>1</td>
</tr>
<tr>
<td><em>Salmonella enteritidis</em></td>
<td>2</td>
</tr>
<tr>
<td><em>Salmonella saint-pauli</em></td>
<td>1</td>
</tr>
<tr>
<td><em>Shigella sonnei</em></td>
<td>2</td>
</tr>
<tr>
<td><em>Shigella flexneri</em> 1</td>
<td>1</td>
</tr>
<tr>
<td><em>Shigella flexneri</em> 2</td>
<td>2</td>
</tr>
<tr>
<td><em>Shigella flexneri</em> 4</td>
<td>1</td>
</tr>
<tr>
<td><em>Neisseria meningitidis</em></td>
<td>5</td>
</tr>
<tr>
<td><em>Stenotraphomonas maltophilia</em></td>
<td>8</td>
</tr>
<tr>
<td><em>Enterobacter aerogenes</em></td>
<td>5</td>
</tr>
<tr>
<td><em>Pseudomonas aeruginosa</em></td>
<td>2</td>
</tr>
<tr>
<td><em>Klebsiella sp.</em></td>
<td>1</td>
</tr>
<tr>
<td><em>Bordetella pertussis</em></td>
<td>3</td>
</tr>
<tr>
<td><em>Bordetella parapertussis</em></td>
<td>2</td>
</tr>
<tr>
<td><em>Bordetella bronchiseptica</em></td>
<td>1</td>
</tr>
<tr>
<td><em>Vibrio cholerae</em></td>
<td>2</td>
</tr>
<tr>
<td><em>Legionella pneumophila</em></td>
<td>3</td>
</tr>
<tr>
<td><em>Legionella sp.</em></td>
<td>3</td>
</tr>
</tbody>
</table>

Laboratories, Hercules, Calif.) was prepared in 10 mM Tris and 0.1 mM EDTA to a final concentration of 1.2% and maintained at 55°C. Plugs were formed by mixing 0.5 ml of cell suspension with 0.5 ml of agarose, and this mixture was then pipetted into plug molds (Bio-Rad Laboratories). After the plugs solidified they were transferred to lysis buffer (50 mM Tris, pH 8.0; 50 mM EDTA, pH 8.0; 1% sarcosine; 1 mg of proteinase K per ml) for 16 to 20 h of incubation in a 55°C water bath. The lysis buffer was removed, and the plugs were washed with 5 ml of sterile distilled water for 5 min and then with 3 ml of TE (10 mM Tris, pH 8.0; 1 mM EDTA, pH 8.0) for 5 min at room temperature. A final set of four washes, 30 min each, was done with 3 ml of TE per wash. Two 1-mm-thick slices of plugs were preincubated with 200 μl of 1× XbaI buffer (Boehringer Mannheim Corporation, Indianapolis, Ind.) for 30 min. The buffer was then removed and replaced with a fresh mixture containing 50 U of XbaI restriction enzyme (Boehringer Mannheim Corporation) in 1× restriction buffer and incubated at 37°C for 16 to 20 h. The plugs were then briefly soaked in standard 0.5× Tris-borate-EDTA (TBE) prior to electrophoresis or, if necessary, stored at 4°C in 0.5 ml of TE for several hours. Electrophoresis of the prepared samples was performed on the CHEF-mapper (Bio-Rad Laboratories) system by using pulsed-field-certified agarose (Bio-Rad Laboratories) with 2 liters of standard 0.5× TBE running buffer. The electrophoretic conditions used were as follows: initial switch time, 2.16 s; final switch time, 54.17 s; run time, 22 h; angle, 120°; gradient, 6.0 V/cm; temperature, 14°C; ramping factor, linear. After electrophoresis the gels were stained for 30 min in 1 liter of sterile distilled water containing 100 μl of ethidium bromide (10 mg/ml) and destained in three washes of 30 min each by using 1 liter of distilled water.

**Rapid PFGE procedure.** In working out the details of this protocol, a variety of specific reaction conditions and reagents were tested. The elements that make up the final procedure were chosen to maximize efficiency while maintaining a high level of accuracy of bacterial typing.

Individual bacterial colonies grown overnight on plates were suspended directly with cotton swabs in about 2 to 3 ml of TE buffer (100 mM Tris and 100 mM EDTA). The cell suspensions were adjusted with TE buffer to 20% transmittance by using a bioMérieux Vitek (Hazelwood, Mo.) colorimeter. Aliquots of 100 μl of the cell suspensions were transferred to 1.5-ml microcentrifuge tubes. Lysozyme (10-mg/ml stock solution) and proteinase K (20-mg/ml stock solution) were added to a final concentration of 1 mg/ml each and mixed several times by being pipetted up and down. The bacterial suspensions were incubated at 37°C for 10 to 15 min. InCert agarose (FMC BioProducts, Rockland, Maine) was prepared in water to a final concentration of 1.2% and maintained at 55°C in a water bath. Following the lysozyme-proteinase K incubation, 7 μl of 20% sodium dodecyl sulfate and 140 μl of 1.2% InCert agarose were mixed with each bacterial suspension with the help of a pipette. This bacterium-agarose mixture was immediately added to plug molds (Bio-Rad Laboratories). The plugs were allowed to solidify for 5 to 10 min at 4°C and then transferred to 2-ml round-bottom tubes containing 1.5 ml of ESP buffer (0.5 M EDTA, pH 9.0; 1% sodium lauryl sarcosine; 1 mg of proteinase K per ml). They were incubated in a water bath at 55°C for 2 h. After the completion of proteolysis, the plugs were transferred to 50-ml tubes containing 8 to 10 ml of sterile, preheated (50°C) distilled water and incubated for 10 min at 50°C with gentle mixing in a shaker water bath. Subsequently, four 50°C washes were done in a shaker water bath for 15 min each with 8 to 10 ml of preheated (50°C) TE buffer (10 mM Tris, pH 8.0; 1 mM EDTA, pH 8.0). The plugs were then cooled to room temperature in TE buffer. At this point, they could be used immediately or stored for 3 to 4 weeks at 4°C in 1 ml of TE. For restriction endonuclease digestion, two 1-mm-thick slices of each plug were incubated at 37°C for 3 h with 50 or 30 U of XbaI or SpeI enzyme, respectively, in 100 μl of the appropriate (1×) restriction enzyme buffer.

Several different kinds and concentrations of agaroses were utilized to optimize the resolution of the fingerprint profiles. SeaKem Gold agarose (FMC BioProducts) at a concentration of 1% provided the desired resolution of DNA fingerprints. The plug slices of the samples were loaded and electrophoresed in 1% SeaKem Gold agarose with 2 liters of standard 0.5× TBE running buffer. The electrophoresis was performed with the GenePath or CHEF-mapper system (Bio-Rad Laboratories) with all conditions except the run time being identical to those used in the standard procedure described above. These conditions were designed for a run time of 22 h; however, for this rapid procedure, the system was manually shut down after 14 h of electrophoresis (which corresponded to a final switch time of 35.07). Since the initial development of this procedure, several electrophoretic runs programmed to stop after 14 h were also performed. In these runs, the initial and final switch times were 2.16 and 35.07 s, respectively; all other parameters remained identical with those of the standard procedure. The results obtained from these runs were always identical to the ones obtained when the system was manually shut down after 14 h. Following electrophoresis, the gels were stained for 20 min in 500 ml of sterile distilled water containing...
50 μl of ethidium bromide (10 mg/ml) and destained in three washes of 30 min each in 1 liter of distilled water. For comparison, plucks from the same isolate, prepared in accordance with both the Centers for Disease Control and Prevention standard protocol and this rapid 1-day procedure, were electrophoresed in adjacent lanes on 1% SeaKem Gold gels. The gels were photographed under UV illumination with Polaroid film. A well-characterized strain of E. coli O157:H7 (12) was included in all runs as a control.

The focus of the present study was to develop a rapid PFGE subtyping method for E. coli O157:H7. A total of 42 isolates of this pathogen, 40 from patients and 2 from a suspected food source, were analyzed by this new rapid procedure. Clinical isolates (Table 1) belonging to 10 other genera were also included to assess the general applicability of this rapid PFGE protocol to other gram-negative bacteria.

The consistency of the control DNA patterns confirmed the reproducibility of the procedure. All isolates included in this study were typed by this new rapid procedure within 24 h. When used to analyze E. coli O157:H7 strains, the 24-h procedure, with its maximum electrophoresis time of 14 h, was able to achieve sufficient specificity in its banding patterns (Fig. 1). Each sample of E. coli was processed by both conventional and rapid procedures, and the resulting DNA profiles were compared. In all cases, the results of the rapid electrophoresis approach compared favorably with the resolution achieved by the conventional technique (data not shown). The specific enzyme used in each case was chosen to result in a similar size range of bands for each organism analyzed on the same gel.

In order to evaluate the applicability of this procedure to different gram-negative bacteria, samples of DNA isolated by both techniques from 10 E. coli, 5 N. meningitidis, 5 Salmonella sp., 5 Shigella sp., 5 Stenotrophomonas maltophilia, 2 Enterobacter aerogenes, 2 B. pertussis, and 1 Klebsiella sp. isolates were run in adjacent wells on the same gel. The restriction patterns obtained by the standard and rapid methods were indistinguishable for each strain. Examples of this are shown in Fig. 2 and 3. Background smearing was less noticeable in the samples prepared by the rapid method, suggesting that the DNA had remained intact.

The procedure was shortened at several stages. The most notable time savings were achieved by (i) utilizing bacterial cells directly from the culture plates of clinical specimens, (ii) expediting cell lysis and proteinase K treatment, (iii) shortening the washing times by using preheated water and TE buffer, (iv) shortening the restriction digestion time, and (v) using SeaKem Gold agarose, which allows for more rapid electrophoresis. It is evident that the modified procedure provides a large amount of clean, intact chromosomal DNA that can be easily digested with a variety of restriction enzymes in a short period. From a single isolate, a comparison of the DNA fingerprints obtained from several-day-old and from fresh cultures gave indistinguishable patterns (data not shown). This suggests that in an outbreak situation it should be possible to directly process isolates coming from different laboratories.

Rapid, accurate typing is of fundamental importance for effective epidemiology and outbreak investigation. Important
public health decisions regarding the institution of preventive measures or the providing of reassurance that no true outbreak exists (10) are often based primarily on molecular typing data. It is well established that different strains of one serotype circulate in most populations. PFGE is reproducible because it looks at a stable genotype rather than at variably expressed phenotypic characteristics. Most standard PFGE procedures take 5 to 6 days, although more rapid methods have been described (13, 19). None of these rapid approaches provide results with the speed of the procedure described here, and all of them require trade-offs not encountered in the present approach. Other rapid PFGE procedures have saved time either through the elimination of proteinase K digestion or by the use of achromopeptidase. These approaches, however, have mainly been applied to gram-positive organisms without extensive investigation of their applicability to gram-negative organisms (2, 13, 17), and the turnaround time still exceeds 1 day. The procedure described in this report allows for the successful typing of a variety of gram-negative organisms within a 24-h period. This has broad implications for both epidemiologic investigations and therapeutic interventions in outbreak situations.

I thank Jon Counts and Donna Osmond for their continuous support during the development of this method. I also thank Jairam Lingappa for his excellent suggestions to improve the text of the manuscript and Kaye Campos and Bill Lawrence for their technical assistance.

REFERENCES