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ORIGINAL ARTICLE

A rapid PFGE protocol for typing *Legionella* isolates from fresh or frozen samples

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Abstract Within the realm of studies on nosocomial infections and epidemiology, pulsed field gel electrophoresis (PFGE) is often considered as the "gold standard" for typing of *Legionella* or other bacteria. Performing this protocol usually requires 2–5 days; this excessive time requirement, lack of a standardized procedure, and high cost have limited its use. However, recently the typing of *Legionella* with PFGE has been reduced to about 2 days, and we further shortened the procedure by reducing the time for the plug preparation and electrophoresis steps. To shorten plug preparation, we used a strong thermal shock and high-temperature washes to reduce cell lysis and DNA isolation time, and we stressed the electrophoresis to obtain comparable macrorestriction patterns among strains in 16 h. The DNA digestion phase was not altered. We also applied this protocol directly to frozen bacteria from strain collections with the aim of shortening the entire procedure. We developed a protocol that can be completed in 24 h, or less if necessary, while avoiding some typical artifacts of traditional procedures. This new protocol also provides good results when directly applied to frozen material from strain collections, thus saving bacteria growth time. Our observations indicate that PFGE tolerates a wide range of adjustments, allowing its application to fresh or frozen samples in a short amount of time.

Keywords Pulsed field gel electrophoresis (PFGE) · *Legionella* spp. · Bacteria typing

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Introduction

In epidemiological studies, accurate discrimination between isolates is important to identify sources and routes of diffusion. In cases of nosocomial infections, intraspecies-level analytical methods are necessary to discriminate among clinical and environmental isolates; this is especially true in *Legionella* epidemiology [1]. Although pulsed field gel electrophoresis (PFGE) is expensive and requires several days, it is a well-established method to type bacteria involved in nosocomial and food-borne infections [2, 3]. Further, its discriminatory power is such that it can separate *Legionella* strains showing the same ribotypes [4]. Time-consuming steps often limit the use of current PFGE protocols. It has also been recently highlighted that the lack of a standardized procedure, presence of "phantom" bands, and fragment distribution on the gel can affect interlaboratory comparison of PFGE results [5].

Orsini et al. [6] showed a preliminary comparison between a commonly used PFGE protocol for *Legionella* typing and a shortened protocol requiring 26 h to complete. One year later, Chang et al. [7] published a new protocol for analyzing *Legionella* isolates needing only 2 days to complete. In comparison to traditional protocols, it reduces the time allotted to cell lysis by proteinase K and DNA digestion by restriction enzymes. Now, in this article, we report further improvements and optimizations to the *Legionella* PFGE protocol that collectively reduce total

execution time to less than 24 h. This modified protocol applies a strong thermal shock to lyse cells, followed by high-temperature washes, and a stressed electrophoresis run. In addition to cultured bacteria, we successfully tested the protocol directly on bacteria from frozen samples; using frozen samples directly allows avoidance of time to grow cultures and hence quick comparison of isolates collected at different times. We present the modified protocol and evaluate its parameters in terms of limits, artifacts, time savings, and utilization of frozen samples.

Materials and methods

The *Legionella* strains were selected from those collected from several monitoring programs. The data presented here come from environmental sampling; none of the data are related to any clinical infection. Collection sites were private houses, hospitals, thermal plants, retirement homes, and hotels that spontaneously joined a monitoring program. At isolation, the strains showed morphological and cultural features of *Legionella pneumophila*. Genus and species assignment performed by phenotypic methods suggested by national guidelines [8] failed; thus, the identification of the samples was confirmed by 16S rDNA sequencing [9]. The final experimental set included the strains *L. anisa*, *L. taurinensis*, *Legionella* spp., and *L. pneumophila*. Once grown and identified, bacteria were stored at −80 °C in glycerol (15 %) stock [10]. The samples included in this study were stored for 9 months to 2 years.

Before starting PFGE, bacteria from fresh or frozen samples were suspended in TE buffer [10 mmol l^{-1} Tris, 1 mmol l^{-1} ethylenediaminetetraacetic acid (EDTA)] and washed twice to remove media traces. After preliminary tests in which different bacteria concentrations were used (0.3–0.8 OD at 600 nm; data not shown), we chose a bacterial sample quantity of about 0.4 OD, which provided good results for fresh and frozen specimens. Lysis was performed by a strong thermal shock just before preparing the plugs: one volume of melted 1.8 % agarose (molecular biology grade, with a melting temperature of 93 °C, or higher) in TE buffer was added to 400 µl bacterial suspension, and the mixture was thawed in a microwave oven at 400 W for 20 s in a 2.0-ml tube with the cap pointed by a needle [11]. Proteinase K (Promega catalog #V3021) was added (final concentration: 0.4 µg μl^{-1}) immediately before dispensing melted mixture into molds (Biorad catalog #170-3622, ~100 µl). Once solidified, the plugs, which appeared opaque, were transferred to a 2-ml conical bottom tube (two plugs for each tube) containing 0.5 ml lysis buffer (10 mmol l^{-1} Tris, 1 mmol l^{-1} EDTA, 100 mmol l^{-1} NaCl, 1 % sodium lauroyl sarcosinate) and incubated in a thermomixer (Eppendorf catalog #5360000019 with thermal block for 2.0-ml tubes) set at 55 °C and 600 rev min^{-1} for at least 15 min.

The first wash was conducted in 1 ml wash buffer (10 mmol l^{-1} Tris, 1 mmol l^{-1} EDTA, 100 mmol l^{-1} NaCl, 0.2 % sodium lauroyl sarcosinate) in a thermomixer set to 80 °C for 15 min with gentle shaking (300 rev min^{-1}) to avoid damage to the agarose plugs by high temperature. The high-temperature washes and modest mixing speed during rinsing avoided, using phenylmethylsulfonyl fluoride, to inactivate proteases [4, 10]. Wash buffer was replaced by 1 ml pre-warmed H_2O and plugs were incubated at 55 °C for 15 min with gentle shaking. Plugs were then washed twice (10 min each, 55 °C 300 rev min^{-1}) with 1 ml TE 0.1× (10 mmol l^{-1} Tris, 0.1 mmol l^{-1} EDTA). Plugs not immediately used were stored at 4 °C in TE 0.1× buffer.

For DNA enzymatic digestion, one-third to one half of the plugs were preincubated with 300 µl restriction buffer (New England Biolabs catalog #B7004S; NE buffer 4, with 100 µg ml^{-1} bovine serum albumin) for 30 min at working temperature (we used *Sfi*I enzyme, which works at 50 °C). Buffer was removed and DNA was digested with the *Sfi*I enzyme. Enzyme concentrations of 10, 20, and 40 U were tested; the reaction was carried out in a final volume of 100 µl for 2 h in a thermomixer at minimum speed (300 rev min^{-1}). Finally, plugs were washed with a 0.5× TBE buffer for 5 min at minimum speed and sealed in agarose gel.

The fragments were separated in 1.0 % w/v pulsed field certified agarose gel (Biorad catalog #162-0138) prepared and run in a 0.5× TBE buffer using a BioRad Chef Mapper III apparatus (Biorad catalog #170-3695). Full-run electrophoresis parameters were 6 V cm^{-1} for 24 h, 120° included angle, and initial and final switch times of 3 and 54 s, respectively. The quick electrophoresis run parameters were 6 V cm^{-1} for 12–16 h in 0.8 % w/v agarose gel with initial and final switch time of 3 and 29 s, respectively. Gels were stained in ethidium bromide and photographed. The procedure is summarized in Fig. 1.

Results

Shortening the protocol

Figure 2a shows PFGE profiles of three *Legionella* strains (*L. anisa*, *L. anisa*, *Legionella* spp.) collected at the same time and subjected to the described quick plug preparation protocol followed by a traditional electrophoresis run. Proteinase K incubation was stopped after 15 min (plugs appeared transparent); enzymatic DNA digestion used enzyme concentrations of 10–40 U, apparently without affecting overall DNA digestion performance. However, at