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Technical improvement in DNA preparation for pulsed–field gel electrophoresis

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ABSTRACT We report a rapid pulsed-field electrophoresis (PFGE) protocol for subtyping *Salmonella* isolates. Modifications were made to reduce the number of steps and duration of proteolysis in our original protocol for the preparation of intact, genomic DNA for PFGE analysis. Comparison of PFGE patterns by independent researchers showed that subtyping results obtained using this modified, rapid protocol were highly reproducible.

ABSTRAK Kami melaporkan sesuatu protokol elektroforesis gel elektrik pulse (PFGE) yang cepat untuk menjeniskan isolat *Salmonella*. Pengubahsuaian telah dilakukan untuk mengurangkan langkah-langkah dan tempoh proses proteolisis dalam protokol asal dalam penyediaan DNA genom yang baik untuk analisis PFGE. Perbandingan corak-corak PFGE oleh penyelidik-penyelidik berlainan menunjukkan bahawa protokol cepat yang terubahsuai adalah berupaya ulangan.

(pulsed-field gel electrophoresis, rapid method)

INTRODUCTION

Salmonellosis is the most common bacterial cause of diarrhoeal illness in Malaysia. The incidence of *Salmonella* infections varied from 9.1% to 14.5% between the years 1997 to 2001 (Ministry of Health, Malaysia). The majority of *Salmonella* infections occur as sporadic disease as well as epidemic outbreaks. Outbreaks are commonly attributed to contaminated foods or water. For example, a recent outbreak of typhoid fever in Bachok, Kelantan in April 2003 where 304 people were reportedly affected and the contaminating source was from well-water (New Straits Times, April, 2003). This underscores the need for continued vigilance for early detection of clusters of *Salmonella* infections, so that potential outbreaks may be recognized as quickly as possible. One way to accelerate the recognition of potential common source clusters of salmonellosis is to subtype all the clinical isolates of *Salmonella enterica* as they are reported to the public health laboratories. Early detection will facilitate removal of contaminating sources, hence reducing additional morbidity and mortality as well as financial losses due to outbreaks.

Molecular typing methods based on the analysis of chromosomal DNA have broad applications in public health bacteriology and when used alone or in combination with standard, well-established methods, are useful tools for epidemiological purposes. For any subtyping methods to be broadly useful, it must meet the following criteria: typeability, reproducibility, discriminatory power and practicality [1]. The last criterion implies that the method should be versatile, relatively rapid, inexpensive, technically simple and provide readily interpretable results [2]. Among the many molecular typing methods, pulsed-field gel electrophoresis (PFGE) fulfils most of the criteria and is applicable to most of the commonly encountered foodborne bacterial pathogens [3].

The principle of PFGE is to use an alternating electric current (pulsed-field) to separate chromosomal DNA fragments generated by infrequent cutters. This involves embedding bacteria in agarose plugs where they are lysed *in-situ* to release the chromosomal DNA. The DNA is then digested with restriction endonucleases that have relatively few restriction sites on the genomic DNA to produce 10 to 20 DNA fragments. One crucial requirement is the need

for intact DNA. Hence, DNA isolation involved the entrapment of bacteria in agarose plugs prior to cell lysis with detergent and proteinase K, followed by washing or dialysis to remove the cell debris [4].

One of the main criticisms of this method is the long processing required time. Improvements in PFGE are needed to achieve the fast turnaround time required for laboratory-based surveillance for timely detection of clusters of *Salmonella* infections. The original protocol that we used took about five to six days to prepare DNA [4 and 5]. We modified the original protocol and reduced the preparation time to three to four days [6]. Here, we report a further improvement in the protocol and reduction of the DNA preparation time to one day. The method was adapted from the CDC PulseNet protocol (Dr Bala Swaminathan, CDC, personal communication) for our work on enteric pathogens, in particular *Salmonella* spp. The robustness and reproducibility of the results obtained with this protocol were demonstrated by repeated analysis by different researchers.

MATERIALS AND METHODS

Bacterial strains

A set of six strains of *Salmonella typhimurium* provided by the Foodborne and Diarrheal Disease Division, Centers for Disease Control (CDC) and Prevention, Atlanta, USA were analysed to evaluate our modified method. *Salmonella* ser. Braenderup H9812 is a universal standard bacteria strain which serves as the DNA fragment size standard. To determine the reproducibility of the method, the DNA preparation and digestion was repeated by three independent researchers to perform the PFGE analysis following the rapid one-day protocol as described below. All the strains were purity-checked before being used for DNA preparation.

Modified DNA preparation protocol

Using a sterile cotton swab, bacteria cells were removed and suspended directly into a small labeled tube containing 100 mM Tris, 100mM EDTA, pH 8 (cell suspension buffer). The cell density of the suspension was adjusted to optical density of 0.8 at OD_{610} (range 0.8-1.0) with a spectrophometer. An aliquot of 150 µl adjusted cell suspension was transferred to a labeled 1.5 ml microcentrifuge tube. An equal volume of 1.5% Low Melt Agarose solution was added, mixed well and immediately transferred to the wells of plug mold (BioRad). The agarose plugs were allowed to set for ten minutes in ice. The solidified plugs were then transferred to appropriately labeled 10 ml tubes containing cell lysis buffer (50 mM Tris, 50 mM EDTA, pH8, 1 % Sarcosine and 1mg/ml proteinase K). The tubes were then incubated at 54°C for two hours. After lysis was completed, the buffer was aspirated out. The plugs were washed twice with 10 ml of preheated (50°C) sterile distilled water for ten minutes each, followed by four washes with preheated (50°C) sterile TE buffer (10mM Tris, 1 mM EDTA, pH8) for 15 minutes each. After the final TE wash, the plugs were sliced to 2-2.5 mm slices and prepared for restriction digestion or stored at 4°C until ready for restriction.

Digestion of genomic DNA in agarose plug slices

Intact, high-molecular–weight *Salmonella* DNA in 2-2.5 mm plug slice was digested with 20 units of *Xba*I (Promega) in buffer solutions according to the manufacturer's instruction for at least two hours at 37°C.

Electrophoresis, staining and destaining agarose gel

The DNA restriction fragments in plugs were separated by electrophoresis through 1% Sigma Low EEO Sigma agarose gel in 0.5X solution of Tris-borate-EDTA buffer at 14°C in CHEF DR II or III PFGE apparatus (Bio-Rad). The electrophoretic parameters used were as follows: 2.2 s to 63.8 s at 200V for 22H, and linear ramping. After electrophoresis, the gels were stained in 15-20 min in a 0.5 µg/ml ethidium bromide and destained by three washes of 20 min using 500 ml deionised water.

Computer analysis of PFGE patterns

The PFGE patterns were analyzed using the GelCompar II Version 2.0 software (Applied Maths, Kortrijk, Belgium). The TIFF images were normalized by aligning the peaks of the size standard strains (*Salmonella* Braenderup H and PFG lambda DNA molecular standard (New England Biolabs). Matching and dendrogram UPGMA analyses of the PFGE patterns was performed using the Dice coefficient with a 1.0-1.5% tolerance window.

RESULTS AND DISCUSSION

Assessment of the protocol

Figure 1 shows the *Xba*I PFGE profiles of the control strains from CDC. The marker strain, *Salmonella* Braenderup prepared according to our protocol was indistinguishable from the DNA prepared by CDC, Atlanta, USA (Figure 1, lanes 1, 3, 11). In addition, well-separated fragments were obtained from all the quality control strains from the CDC (Figure 1, lanes four to nine). This indicates that the rapid protocol that we developed was workable and comparable to the DNA prepared with the standardized protocol for DNA preparation used at CDC. It was noted that the thickness of the DNA bands from the plugs prepared in our laboratory was slightly bigger. However, this did not obscure comparison. The thicker DNA band is an indication of the higher cell density and this can be improved by further optimization of cell density prior to mixing with the low melt agarose.

The first step in the validation process of the protocol was to assess its reproducibility. This was accomplished by repeated analysis by at least three different independent researchers from the Biomedical Science Laboratory. The PFGE profiles of the quality control *Salmonella* strains prepared by three different individuals were indistinguishable indicating the reproducibility of the protocol (Figure 2). Again, minor variations in the concentration of the DNA was observed. The quantitative similarity/difference among the DNAs prepared by three different individuals are depicted in the dendrogram (Figure 3). No difference in the PFGE patterns was observed between different experiments.

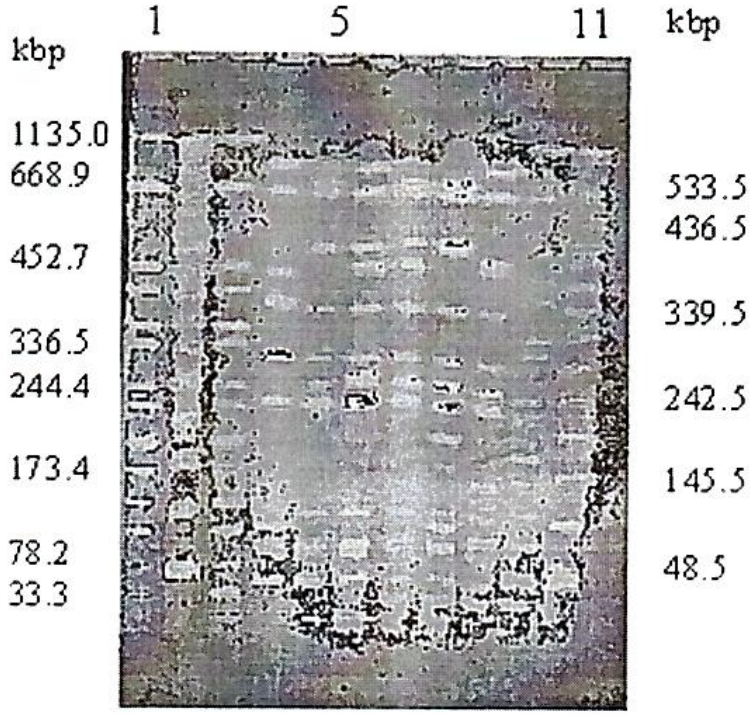

Figure 1. Gel showing the PFGE patterns of the control strains using our laboratory protocol. Lanes 2 & 11: lambda PFG marker, lanes 1,3: *S.* Braenderup (prepared by lab protocol), lanes 4-9: *S.* Typhimurium isolates, lane 10: *S.* Braenderup (prepared by CDC, Atlanta).

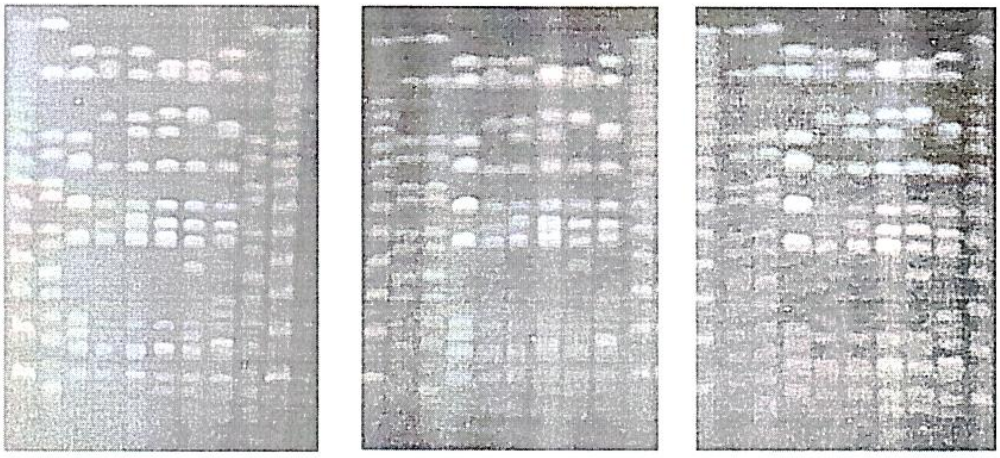

Figure 2. PFGE profiles of quality control strains generated by 3 different researchers (A-C). **Gels A & B:** lanes 1,10: lambda DNA concatemers, lanes 2,3: *S.* Braenderup marker strain, lanes 4-9: quality control strains. **Gel C:** lanes 1,10: lambda DNA concatemers, lanes 2,9: *S.* Braenderup marker strain, lanes three to nine, quality control strains.

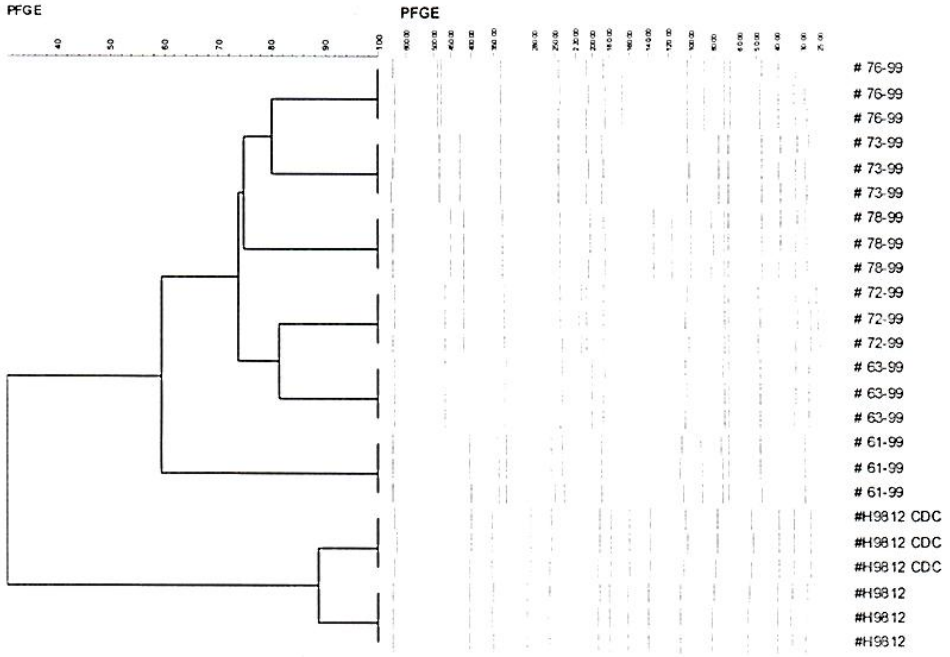

Figure 3. Dendrogram showing the results of the comparison between the PFGE patterns obtained by three independent researchers. The dendrogram shows that each researcher obtained the same pattern on matching isolates, indicating a high degree of reproducibility.

Changes to the original protocol are as follows: In the previous protocols [5, 6], a double-lysis method was used: a preincubation step with lysozyme and RNase followed by proteinase K treatment for at least 24 hours. However, in the present protocol, the preincubation step was eliminated and the cell lysis was performed at 55°C for three hours instead of an overnight incubation. The data indicated that neither extended nor double lysis is required for efficient release of DNA from *Salmonella* cells. The purification of the DNA-agarose plugs, essentially a dialysis process, was speeded up by increasing the temperature of the washing buffer. In the previous protocol, three washings were carried out using prechilled TE buffer for an hour per wash. In the present protocol, duration of the washing was shortened to an hour. Water was used for the first wash to increase the dialysis effect and subsequent washes with TE allowed for further removal of cell debris. In the original PulseNet protocol, SDS was added to the low melt agarose (LMA). However, this resulted in high background (data not shown). The presence of SDS in the plug agarose may cause immediate cell lysis resulting in the release of DNA while the agarose-cell suspension mixture is still in the liquid form. The use of SDS in the LMA suspension required technical skill of an individual who prepares the DNA plug. Because of the inconsistency between different researchers in handling SDS-LMA, we have eliminated the use of SDS in the prelysis buffer.

In conclusion, we have demonstrated the utility of a modified, rapid DNA protocol for PFGE analysis. The method is relatively rapid, easy to use, technically simple and PFGE profiles can be visually distinguished. The method is also 'universal' as it is applicable to a number of bacterial strains such as *Vibrio cholerae*, *Pseudomonas aeruginosa*, *Shigella* spp., and pathogenic *Escherichia coli*. We hoped that this modified PFGE protocol will be useful for rapid investigation of the molecular epidemiology of *Salmonella* strains and provide a fast turnover time for timely intervention of foodborne outbreaks.

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