Pulsed Field Gel Electrophoresis of group A streptococci

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Running head: "PFGE of *Streptococcus pyogenes*"

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Summary

Here we describe the protocols to perform PFGE analysis of chromosomal DNA from the bacterial species *Streptococcus pyogenes* (group A streptococcus, GAS) after digestion with the restriction enzyme *Sma*I. Large parts of the procedures are suitable for application to DNA digested with other restriction enzymes as well. We have put an effort to present extensions to solve possible limitations to the discriminatory power of the method in the specific case of *S. pyogenes*.

Key Words: *Streptococcus pyogenes*, group A streptococcus, GAS, PFGE, *SmaI*, chromosomal DNA, macrorestriction.

1. Introduction

Pulsed-field gel electrophoresis (PFGE) of chromosomal DNA extracted from *Streptococcus pyogenes* is a molecular typing method considered among the 'gold standard' tools used to investigate the clonal relationships between clinical Group A streptococci (GAS) isolates during an epidemic survey or to study outbreaks. This technique is also considered useful and suitable in large studies aimed at investigating the global clonal expansion and evolution of this human pathogen. Several studies have reported the use of PFGE, but none of them have addressed the methodological issues of the technique in the sole and specific case of *S. pyogenes*. This chapter is mainly aimed at filling this gap. Actually, the use of PFGE for *S. pyogenes* typing, albeit extensively applied, has not gained the same significance as the method has in the case of the study of other important human pathogens like *Staphylococcus aureus*. As for the latter organism, the worldwide spread of major staphylococcal clones has given the opportunity of exploiting PFGE to follow its diffusion *(1, 2)*.

Nevertheless, macrorestriction profiling by PFGE of *S. pyogenes* has shown a high degree of correlation with other typing methods. This is the case of *emm*-typing, which is the determination of the 5'-end sequence of the gene coding for the major virulence factor M-protein. The method consists of a classification scheme comprising more than 200 variants. Several molecular epidemiology studies have demonstrated a good level of correlation between *emm*-typing and PFGE analysis by using *Sma*I restriction enzyme to digest genomic DNA *(3,4,5)*. Moreover, other traits used to characterize GAS clinical strains have been found to correlate well with PFGE, such as MLST and, even at a lower degree, genetic determinants

of erythromycin resistance *(4)*. In addition, the virulence genes' content also correlates well with PFGE clustering analysis *(6)*. This may be due to the fact that several virulence genes are carried by large genetic mobile elements, such as prophages, which, due to their average number per genome and size, greatly influence the physical structure of the genome *(7)*.

PFGE is a simple but powerful technique to assess inter- and intra-species bacterial diversity. It is based on the separation of large fragments of DNA, resulting from the specific endonucleolytic activity of rare cutting restriction enzymes on the bacterial genome or large plasmids. Basically, differences among strains depend on the presence/absence of the specific restriction endonuclease recognition sites and distance separating restriction sites. Comparisons of electrophoretic banding patterns allow determination of the relatedness of the corresponding genomic DNAs. A correlation degree might also be computed depending on the number of differences between patterns *(8)*. Collected data can then be used to generate correlation trees based on UPMGA (Unweighted Pair Group Method with Arithmetic Mean).

2. Materials

Prepare all solutions using ultrapure water (prepared by purifying deionized water to attain a sensitivity of 18 M Ω cm at 25°C) and analytical grade reagents. Follow all waste disposal regulations when disposing of waste materials.

2.1 Equipments and general materials

heating stirrer

water bath

spectrophotometer

pH-meter

incubator

vortex

multivolumentric pipettes

disposable plug molds

PFGE chamber and comb

PFGE apparatus

Todd Hewitt broth

Columbia agar plates (+5% sheep blood)

*Sma*I and 10X Restriction buffer

CHEF DNA size standard lambda ladder

Ethidium bromide stock solution (10 mg/mL)

2.2. Preparation of agarose-embedded chromosomal DNA and restriction enzyme digestion.

1. Low melting agarose solution (1.6%): add 0.160 g of low melting agarose to 10 mL of water in a flask, close with a cap or aluminium foil and bring to the boil using a heating stirrer avoiding over-heating. Transfer the flask into a water bath at 50-55°C.
2. TEN buffer: 10 mM Tris-HCl pH8.0, 1 M NaCl, 10 mM EDTA pH 8.0. To prepare 1 L, in a beaker containing 500 mL water, mix 10 mL of 1 M Tris-HCl pH 7.5, 58.4 g NaCl, 20 mL of 0.5M EDTA pH 8.0. Dissolve completely the salt and make up to 1 L with water. Store atroom temperature (20-25°C). This solution may be prepared ahead of time. It is stable for up to 1 year.
3. Lysis solution (EC): 6 mM Tris pH 7.6, 1 M NaCl, 100 mM EDTA, 0.2% sodium-deoxycholate, 0.5% sodium lauroylsarcosine, 0.5% Brij 58 in water. To prepare 1 L, mix in a beaker containing 500 mL water, 6 mL of 1M Tris-HCl pH 7.6, 58.4 g NaCl, 200 mL of 0.5M EDTA pH8.0, 2 g sodium-deoxycholate, 5 g sodium lauroylsarcosine, 5 g Brij 58. Dissolve completely the salts and make up to 1 L with water. Store at room temperature (20-25°C). This solution may be prepared ahead of time. It is stable for up to 1 year. Before use check the bottom of the storing vessel for possible precipitation of detergent. If a precipitate is present, the solution can be heated up to 40°C until complete dissolution. Immediately prior to use add 1mg/mL lysozyme and 50 μg/mL RNase A.
4. Digestion solution (ES): 0.5 M EDTA pH 8.0, 1 % sodium lauroyl-sarcosine in water. Mix 186 g EDTA and 700 mL of water and adjust to pH 8.0 with10 N NaOH. Add 10 g sodium lauroylsarcosine and make up to 1 L with water. Store at room temperature (20-25°C). This solution may be prepared ahead of time It is stable for up to 1 year. Before use check the bottom of the storing vessel for possible precipitation of detergent. If a precipitate is present, the solution can be heated up to 40°C until complete dissolution.

Immediately prior to use add 0.5 mg/mL proteinase K.

5. TE: 10mM Tris pH 8.0, 1 mM EDTA pH 8.0 in water. To prepare 1 L by mixing in a cylinder 10 mL of 1 M Tris pH 8.0, 2 mL of 0.5 M EDTA pH 8.0 and adjusting the volume to 1 L with water.

2.3. Preparation of gel and electrophoretic run.

1. 10xTBE: 1.3 M TRIS, 450 mM boric acid and 25 mM EDTA in water. Weigh 157.48 g Tris base, 27.82 g boric acid, 9.3 g EDTA and dissolve in 500 mL water. Finally adjust the volume to 1L with water. Check the pH: it should be 8.3 without requiring adjustment. Store at room temperature (20-25°C) Dilute 20 times with water (final concentration: 0.5x) prior to use.
2. 1% agarose gel. To prepare 100 mL of 1.0% agarose gel: add 1 g of agarose to 100 mL of 0.5x TBE in a flask, close with a cap or aluminium foil and bring to boil using a heating stirrer avoiding over-heating. Before pouring, cool to 45-50°C under stirring.

3. Methods

3.1. Preparation of high molecular weight DNA

The following protocol describes the procedure for a single strain. All the microbiological procedures are performed in sterile conditions.

1. Streak the selected *S. pyogenes* strain on blood agar plate and incubate at 37°C with 5% CO_2 for 18-24 h. Transfer 3-5 colonies from the agar plate into 5 mL of sterile Todd-Hewitt broth and grow them overnight at 37°C with 5% CO_2. After growth, check the optical density (wavelength: 600 nm) of the bacterial suspension (*see* **Note 1**).
2. Prepare 0.5 mL of low melting agarose per sample (*see* **Note 2**) and keep it in a water bath at 55°C.
3. Centrifuge a suitable volume of overnight bacterial suspension containing 1-2 x 10^9 cells at 10,000×g for 3 min (*see* **Note 3**). Discard the supernatant and resuspend the pellet in 1 mL of TEN buffer (*see* **Note 4**). Centrifuge again for 2-3 min at 10,000×g, discard the supernatant and resuspend the pellet in 0.5 mL of TEN buffer.
4. Add 0.5 mL of the low-melting agarose prepared at point 3.1.2. Vortex immediately for a few seconds and distribute 100 μL into each well of the disposable plug mold strip (*see* **Note 5**). Let it solidify at 4°C for 15 min.
5. Remove the tape present at the bottom of the plug mold strip. Push the plugs out from the mold by using a spatula and put them into a 15 mL sterile tube containing 2 mL of lysis solution (EC). Incubate at 37°C for 16 h. Pour off the lysis solution paying attention not to lose the plugs.

6. Add 3 mL of digestion buffer (ES + proteinase K) to the plugs. Incubate for 2 days at 50°C. Remove the buffer and add 3 mL of fresh ES (without proteinase K). Plugs can be stored at 4°C for more than one year without loss of DNA integrity.

3.2. Digestion with *SmaI* restriction enzyme

1. Take one plug using a spatula and submit it to an overnight dialysis with 20 mL of TE buffer in a 50 mL tube at room temperature (20-25°C). Change the TE buffer every 3 h for 3 times the following day.
2. Digest the plug-embedded DNA in 0.3 mL solution containing 1X restriction buffer and 20 U of *Sma*I enzyme (*see* **Note 6**). Incubate at 30°C for 24 h. To stop digestion, add 1 mL of 0.5 M EDTA pH 8.0 to the sample and incubate for 5 min at room temperature (20-25°C).

3.3. Pulsed-field Gel Electrophoresis

1. Cool 2.2 L of 0.5X TBE buffer to 4° C.
2. Cool 100 mL of 1% agarose gel to 50°C.
3. Place the plugs over the comb teeth, cut them just over the midpoint and stick them to the comb teeth with 0.005 mL of melted agarose. At this stage remember to include the plug containing the CHEF DNA size standard lambda ladder. Put the comb at 4°C for 10-15 min to let the plugs adhere firmly to the comb.
4. Assemble the gel casting tray, position the comb with samples in the upper corresponding slots of the casting stand so that the height of the comb is 2 mm above the surface of the platform. Pour in the melted agarose and wait until it solidifies (approximately 15-30 minutes). Upon pouring, it may happen that bubbles form. They need to be removed. They

can be either dragged to the side of the gel using a disposable pipette tip or made blow up by touching the bubble surface with a pin or a flamed wire loop if available.

5. Pour the 0.5X TBE buffer into the electrophoresis chamber and activate the variable speed pump at maximum speed and set the cooling module at 14°C. Let the system reach the 14°C uniformly.
6. Once the gel is solidified, remove the comb and place it on the frame located in the electrophoresis chamber.
7. Set the instrument in the two-state mode with the parameters reported in Table 1 and start the run.
8. Once the program is completed, stain the agarose gel by soaking it in 300 mL of H_2O containing 0.5 µg/mL ethidium bromide for 30 min. Rinse three times with 300 mL tap water each time, then visualize and photograph under UV light (254-360 nm). A typical result obtained applying the “standard” run condition is shown in Figure 1 (*see* **Note 7**).

4. Notes

1. It is opportune to measure optical density of the bacterial suspension as differences are obtained for different strains after the overnight culture. They need to be equalised to make the comparison of PFGE profiles easier and more reliable. Optical density of a typical *S. pyogenes* over-night culture is about 1-2 units (600 nm). Hence the bacterial suspension needs to be diluted by about 1:1 (e.g. 500 μL of bacterial suspension and 500 μL of broth medium) to obtain a reading below 1 unit.
2. The minimum volume of agarose gel to be prepared is 10 mL regardless of the number of samples.
3. In a typical bacterial suspension, equation that relates optical density (OD at a wavelength of 600 nm) and colony forming units (c.f.u.) is 1 OD_{600nm}/mL = 1-2 x 10^9 cfu/mL and it corresponds to 4-10 μg of chromosomal DNA/mL.
4. Discard the supernatant immediately and do not lose the pellet. Some strains may show a fluffy pellet and loss of a certain amount of cells is unavoidable. In that case, the pellet shows a visible size reduction. A partial solution is to increase the starting volume of bacterial culture so as to obtain an approximately constant pellet size for all the samples. It is also possible to prepare the bacterial suspension by starting from the bacterial colonies grown on solid medium supplemented with sheep blood, instead of resuspending a few colonies into specific broths *(9)*. In that case, it is necessary to resuspend the colonies into TEN buffer and check the turbidity of the bacterial suspension until it reaches approximately 1.5-2 McFarland. At this time, the bacterial suspension is ready to be mixed with low melting point agarose (ratio of 1:1).
5. In order to work as fast as possible and to avoid undesired solidification of the agarose-

cells mixture, be sure to have both the micropipettes and the vortex close to you before starting this step. Moreover, it is strongly suggested to work close to a water bath set at 50°C in which the melted agarose will be kept when preparing the agarose-bacterial cells mixture in order to avoid the solidification of agarose.

6. The chromosomal DNA of some strains is not susceptible to *Sma*I macro-restriction, undermining the possibility of analyzing and comparing these strains with other strains. The reason for the resistance to digestion is methylation of the 3' cytosine of the *Sma*I recognition site due to the expression of a methylase encoded by a gene carried by chimeric genetic elements *(10)*. Two related exogenous elements have been described so far possessing the mentioned methylase, namely the bacteriophage Φ10394.4 *(11)* and the conjugative transposonTn1207.3 *(12)*. Among genes harbored by these exogenous elements there are *mef*(A) and *msr*(D) that are responsible for the low level resistance to 14- and 15- membered macrolides (e.g. erythromycin). Nowadays, the prevalence of *mef*(A)-positive erythromycin resistant *S. pyogenes* is generally low, but it used to be quite high in the past *(13)*. Moreover, among *mef*(A)-positive erythromycin resistant *S. pyogenes* only a fraction harbor the genetic elements expressing the methylase that modifies the *Sma*I recognition sites. Once the presence of *mef*(A)-positive strains with *Sma*I undigested chromosomal DNA is observed, it is necessary to use alternative restriction enzymes, such as the methylation insensitive isoschizomer of *Sma*I, Cfr9I *(14)*.

7. The *in silico* *Sma*I restriction analysis of *S. pyogenes* genomic DNA available in public databases gives an overview of the general features of the product of "macro-restriction". The average number of cutting sites per genome is 23 (C.I.95%: 22-24) with a maximum of 26 (strain NZ131, ref. *15*) and a minimum of 20 (strains MGAS1882 and MGAS15252, ref. *16*). The average fragment size is 81,293 bp (C.I. 95%: 78,645 – 83,941 bp) with a

maximum at 554,983 bp (MGAS8232, ref. *17*) and a minimum at 1,099 bp (NZ131). It is possible to plot the distribution of fragments resulting from the digestion with *Sma*I of the genomic DNA of each strain. Figure 3 shows the result of the *in silico* restriction analysis in a form resembling that obtainable by a real PFGE run. It is evident how banding is concentrated in the range of lengths between 20,000 bp and 200,000 bp. Hence, a good resolution of the PFGE would be expected for fragments within that range. It is often the case of pictures reported in published papers showing a good resolution between 50,000 and 500,000 bp of fragment lengths. Bands below 50,000 bp are not shown; either because the gel is cut or the run has been set to resolve bands above that limit. This occurrence is a limitation to the possibility of resolving differences accounted for by bands < 50,000 bp. Another weak point is the unneeded search for resolving bands above 300,000 bp, in view of their paucity in the typical *Sma*I generated banding pattern in *S. pyogenes*. Actually there have been good examples of PFGE executions where part of this issue was eventually considered *(18)*. If the epidemiological study requires a complete and deep discrimination between strains, the same DNA samples run with either condition #1 or #2 may be separated by FIGE using condition #3 (Table 1), which gives the best resolution in the range 5,000 – 50,000 Kb (Figure 2). Results can then be combined for cluster analysis.

5. References

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Figure Captions

Figure 1. Picture of a sample PFGE gel, run applying the standard condition #1 (Table 1). CHEF DNA size standard lambda 48.5 Kb ladder is loaded onto lanes 1, 8, and 15.

Figure 2. Picture of a PFGE gel run applying the FIGE condition #3 (Table 1). DNA size standard 5 Kb ladder is loaded onto lane 9.

Figure 3. *In silico* generated banding pattern of chromosomal DNA sequences from genomes available in the GeneBank database. Strain names are reported along the X-axis.