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Original Article

Cost-effective procedure for *Streptococcus pyogenes* immobilized DNA preparation and miniPFGE subtyping

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Abstract

Introduction: Group A streptococci (GAS) is responsible of several human diseases ranging from mild infection to severe invasive toxin-mediated disease and post-infectious sequelae. Accordingly, a GAS surveillance program based on molecular techniques is advisable for its epidemiological control. Pulsed-field gel electrophoresis (PFGE) is the gold standard for GAS molecular subtyping, but a major disadvantage is the length of the procedure, which takes 1–3days of work, minimum. The aim of this study was to develop a rapid and cost-effective procedure for PFGE subtyping of GAS isolates.

Methodology: Different incubation times of GAS, immobilized in agarose miniplugs, in solutions containing lysozyme and/or mutanolysine followed by solutions with urea instead of proteinase K, were assayed. DNA was restricted with *SmaI* and the fingerprints were obtained in clamped homogeneous electric field (CHEF) chambers and minichambers. The modified procedure was used to subtype 22 GAS isolates.

Results: Intact DNA molecules of GAS immobilized in agarose miniplugs were prepared incubating the cells, *in situ*, with a solution containing lysozyme for 4hours, followed by the incubation in a non-enzymatic solution with urea for 2hours. *SmaI*DNA macrorestriction fragments were well resolved in 5hours and 14minutes by electrophoresis in a CHEF minichamber at 10V/cm. This procedure for GAS DNA preparation was useful for fingerprinting GAS strains in the format of CHEFMapper (BioRad).

Conclusions: The procedure took 13 hours for GAS strains subtyping. Both sample preparation and electrophoresis in CHEF minichamber represent an economic alternative for performing massive epidemiological studies of this human pathogen.

Key words: GAS DNA; miniPFGE; bacterial subtyping; non-enzymatic methods.

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Introduction

Streptococcus pyogenes (group A streptococci, GAS) causes a variety of human diseases, ranging from mild skin and mucosal surface infection to severe invasive toxin-mediated disease and post-infectious sequelae, such as acute rheumatic fever, glomerulonephritis, and Sydenham's chorea [1,2]. GAS is responsible for over 600 million new infections every year worldwide, including seasonal outbreaks and nosocomial infections, and it is the cause of half a million deaths annually [3]. Consequently, it is convenient to keep an epidemiological surveillance program of this microorganism to monitor the clones highly virulent to humans, assess their clonality, and track infection sources and the spread of antibiotic resistance.

Epidemiological surveillance programs of pathogenic bacteria are based on the classification of isolates into subtypes. Molecular subtyping methods based on DNA analysis allow for differentiating the isolates that appear identical by conventional methods, such as microbial susceptibility testing or serotyping. Pulsed-field gel electrophoresis (PFGE) is a highly reproducible and discriminating tool for the molecular subtyping of bacteria, which is often recommended as a reference method in outbreak investigations. It has been successfully applied to a broad range of different Gram-negative and Gram-positive bacteria [4-11]. Bacterial subtyping by PFGE relies on the

determination of the relationship between different isolates by comparing their DNA macrorestriction patterns [12-15].

Several factors affect the discrimination power and reproducibility of the results yielded by the PFGE technique. DNA molecules are resolved by PFGE in patterns that depend on the system, DNA preparation, plug thickness, buffer, gel concentration, electric field, temperature, pulse time, and running time [15]. For that reason, efforts to standardize and validate PFGE protocols for bacterial subtyping have been performed. The use of such protocols has yielded PFGE results with acceptable intra- and inter-laboratory reproducibility [16]. Nevertheless, PFGE is still a time-consuming and technically demanding procedure. The time consumed by the original procedure for GAS DNA preparation was up to two to three days [17]. In recent years, optimized PFGE protocols for bacterial subtyping have reduced the bacterial DNA preparation time to 2–4 hours, but they still involve the use of solutions that contain cell-wall-disrupting enzymes and proteases or huge amounts of restriction enzymes, and some of them do not generate reproducible results of good quality, probably due to inefficient bacterial lysis [18-24]. As well, the separation of DNA macrorestriction fragments takes between 18 and 24 hours in the CHEF chamber [18-24]. Procedures for GAS subtyping have been reported based on these optimized protocols, but in general, they have the same mentioned drawbacks in relation to the DNA preparation and the long electrophoresis times [25-30].

Immobilized DNA of *Saccharomyces cerevisiae*, *Pseudomonas aeruginosa*, *Vibrio cholerae*, *Aeromonas*, *Salmonella*, and *Streptococcus pneumoniae* have been successfully prepared by non-enzymatic methods [31-35]. *S. cerevisia*e, *E. histolytica*, *P. aeruginosa*, *V. cholerae*, *Salmonella*, and *Aeromonas* genomes have been analyzed by CHEF electrophoresis in minichambers, reducing the running time at least twofold compared to the time needed in the CHEF chamber [32-33,35-39]. In this paper, we present a rapid and cost-effective PFGE procedure for subtyping GAS strains in approximately 13 hours after cell harvesting.

Methodology

Bacterial strains growth and immobilization

Reference strain *S. pyogenes* ATCC 12384 was used to develop the economic PFGE subtyping procedure, whereas *S. pyogenes* isolates and the reference strains 10270, NZ131, and 6180 were used to assess the reproducibility of this procedure.

Twenty-two GAS clinical isolates, coded as SP1 to SP22, were received from the clinical microbiology laboratory of Juan M. Márquez Pediatric Hospital in Havana City. Isolates were identified and characterized by standard microbiological methods [40]. The sources of the specimens included throat and skin swabs collected from hospitalized patients and from the outpatients of specialty clinics.

Reference strains and clinical isolates were grown on heart-brain broth (13.2g/L protease peptone, 9.1g/L brain extract, 2.8g/L heart extract, 2g/L dextrose, 3.3g/L disodium phosphate, 6.6g/L sodium chloride, pH 7.4) at 37°C until the log phase was reached. Bacterial cells were harvested by centrifugation and washed with washing solution (0.15 M NaCl plus 0.01 M EDTA; pH 8.0). Cells were mixed with 2% low melting point agarose (A3054, Sigma-Aldrich, St. Louis, USA) dissolved in washing solution at a ratio of 1, 2, or 3 $\times 10^9$ colony-forming units (CFU)/mL and poured into a mold to form miniplugs of 3×3×0.7 mm (length x height x thickness; Neuronic SA, Havana, Cuba).

Cell lysis and DNA deproteinization

Immobilized DNA molecules were prepared following a reported protocol based on an enzymatic method [17] or by the procedure with a single lytic enzyme and protease-free solution developed in this work.

The protocol used to prepare immobilized DNA in agarose miniplugs by the enzymatic protocol was as follows. Agarose miniplugs containing 2×10^9 CFU/mL were incubated for 16 hours at 37°C in a lysis solution (LS) containing 0.01 M Tris, 0.1 M EDTA, 1% sarcosyl, 1% Nonidet P-40 [pH 8.0] plus 2 mg/mL of lysozyme (L7651, Sigma-Aldrich) and 10µg/mL of mutanolysin (M9901, Sigma-Aldrich). Then, miniplugs were incubated in a deproteinization solution (NDS) containing 0.01 M Tris, 0.5 M EDTA, 1% sarcosyl [pH 9.5], and 1 mg/mL of proteinase K (P2308, Sigma-Aldrich) for 16 hours at 50°C. Finally, the miniplugs were washed three times with TE-100 (0.01 M Tris, 0.1 M EDTA; pH 8.0) at room temperature for 15 minutes each. The ratio of miniplug/solution volume was 20 miniplugs/mL in all steps.

For developing the single lytic enzyme and protease-free procedure, the lysis step of GAS immobilized in agarose miniplugs was assayed in the lysis solution containing a single (lysozyme or mutanolysin) or both lytic enzymes followed by the deproteinization step with NDSUPlus solution (0.01 M

Tris, 0.1 M EDTA, 1% sarcosyl, 1% Nonidet P-40, and 4 M urea; pH 9.5) instead of proteinase K for 16 hours of incubation each at 37°C and 45°C, respectively. The incubation of the miniplugs with lysozyme was assayed for 2, 4, 8, or 16 hours at 37°C, followed by NDSUPlus for 2, 4, 8, or 16 hours at 45°C. Additionally, the miniplugs were incubated in NDSUPlus alone for 16hours at 45°C.

In all cases, after the deproteinization step, miniplugs were washed three times for 15 minutes with TE-100 (0.01 M Tris, 0.1 M EDTA; pH 8.0). Immobilized DNA in miniplugs were then subjected to endonuclease digestion or stored at 4°C in fresh TE-100.

Restriction enzyme digestion of immobilized DNA

Each miniplug was washed three times in 1mL of TE-0.5 (0.01 M Tris-HCl, 0.0005 M EDTA; pH 8.0) at 4°C for 10 minutes each. After pre-incubation of each miniplug in 200 µL of restriction enzyme buffer SA 1×(10× buffer SA contained 330 mM Tris-acetate [pH 7.9], 100 mM Mg acetate, 660 mM potassium acetate, and 5 mM dithiothreitol [B7531, Sigma-Aldrich]) at 4°C for 10 minutes, the DNA was digested with 10U of *SmaI* (R4503, Sigma-Aldrich) in 100 µL of 1×fresh restriction enzyme buffer SA at 37°C for 2 hours. DNA prepared by fully enzymatic protocol was digested with 20U of *SmaI* at 37°C for 4hours. Digestion was stopped by replacing the reaction buffer with 1 mL of TE-100.

PFGE electrophoresis

DNA agarose miniplugs prepared by the standard enzymatic protocol and the modified procedure developed here were restricted and loaded into 1.5% agarose (A2929, Sigma-Aldrich) CHEF gel and minigel in 0.5X Tris-borate-EDTA running buffer (44.5mM Tris, 44.5mM boric acid, 1mM EDTA; pH 8.3). The separations were performed in the CHEF minichamber (miniCHEF) of a Guefast-06 system [41] (Neuronic SA) and in a homemade CHEF chamber [38]. The homemade CHEF chamber has a separation between electrodes of opposite polarities of 28.7 cm, whereas Guefast miniCHEF has a separation of 14.5 cm [38-39]. DNA fragments were separated by applying 5 V/cm and switching time intervals from 50 to 3 seconds during 21 hours in the homemade CHEF chamber and 10 V/cm in the miniCHEF, keeping the running buffer temperature at 20°C. The switching times applied in the miniCHEF were predicted using the equations that describe the DNA migration in the CHEF incorporated in the Guefast Simulator software [41-42].

The modified procedure based on a single lytic enzyme and protease-free solutions developed in this work was used to prepare immobilized DNA of three GAS reference strains and analyze them in the CHEFMapper (BioRad, Richmond, USA). *SmaI* DNA fragments were separated in the CHEFMapper, applying 6 V/cm and switching time intervals from 1 to 5 seconds for 2hours followed by 5 to 40 seconds over 22 hours at 14°C.

The gels were stained in ethidium bromide solution and photographed. DNA band patterns in the digital images of the gels were scanned by means of GuefastScan software (Neuronic SA), and the densitometric profile was obtained [32].

Statistical analysis

The efficiency of each modification included in the different assays was estimated by calculating the percentage R (%). R was defined as the amount of DNA suitable for PFGE and was estimated from the densitometry profiles of *SmaI*DNA band patterns of *S. pyogenes* using the method described by López-Cánovas *et al*. [32]. To estimate R(%), the following formula was used:

(1- [S/T])×100, in which T was the total peak areas (proportional to DNA/miniplug) and S the area of the peak at slot (proportional to DNA unable to enter to the gel).

Mean R values were calculated from at least three independent replicas and compared through one-way analysis of variance (ANOVA) followed by Duncan's multiple range test. Transformation of data was $\sin^{-1}\sqrt{0.01\times R}$ to achieve normality of sample distributions. Homocedasticity of the data was verified by the Levene test. The relative standard deviation (RSD) of R(T-S) was also calculated.

Resolutions between consecutive bands of the patterns were compared through student's *t*-test for independent samples.

All the statistical tests were performed at a significance level of 0.05 by the STATISTICA 8 program package (StatSoft, Inc, 2007. STATISTICA, data analysis software system, version 8.0. www.statsoft.com).

GAS isolates DNA band-based dendrograms were generated with GuefastScan (Neuronic SA) by means of the calculation of Dice coefficient and the unweighted pair group method using arithmetic averages (UPGMA) analysis and a position tolerance setting of 1%.

Results

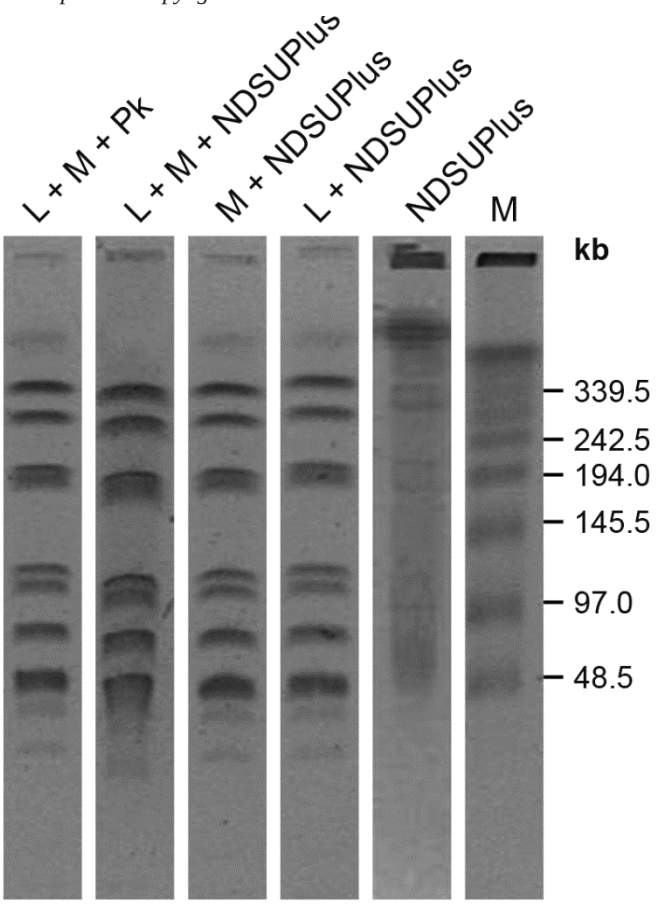
DNA preparation with a single lytic enzyme and protease-free solutions

Agarose miniplugs containing 2x10^{9}CFU/mL of *S. pyogenes* ATCC 12384 rendered band patterns of adequate intensities in miniCHEF minigels after *SmaI* restriction of the immobilized DNA molecules (Figure 1). When miniplugs were cast at concentrations of 3×10^{9}CFU/mL or 10^{9}CFU/mL, overloaded or faint DNA bands were obtained in the minigels, respectively (results not shown).

The incubation of the miniplugs in NDSUPlus alone gave smearing background due to poor DNA deproteinization, insufficient DNA release, and/or degradation of DNA sample by residual cellular endonucleases, even when overnight incubations were assayed (Figure 1, lane NDSUPlus).

Replacement of proteinase K with NDSUPlus solution, even in the absence of one disrupting cell wall enzyme (lysozyme or mutanolysin), caused no qualitative changes in the banding pattern with respect to standard protocol (Figure 1). In all cases, the miniCHEF chamber resolved identical numbers of bands between 20 and 400 kb when DNA molecules were digested with *SmaI* (Figure 1). However, the efficiency of the treatments assayed differed based on the comparison of DNA suitable for PFGE in each pattern, calculated as R(%) mean ($F[3,9] = 24.4$, $p < 0.001$). R(%) mean was 99.1% when the samples were prepared using the standard enzymatic protocol (Table 1), while it differed statistically from the R value obtained when the samples were prepared replacing proteinase K with NDSUPlus solution (L + M + NDSUPlus) or when a single lytic enzyme followed by NDSUPlus solution was used (L + NDSUPlus or M + NDSUPlus) ($\alpha = 0.05$, Duncan's multiple range test; Table 1). Although these results indicated most efficient DNA release in *S. pyogenes* ATCC 12384 DNA samples prepared by the standard enzymatic protocol (L+M+Pk), the rest of the variants yielded enough DNA quantity and good-quality *SmaI*DNA

Figure 1. MiniCHEF analysis of *SmaI*DNA from *Streptococcus pyogenes* ATCC 12384



DNA was prepared by the standard enzymatic or the modified procedure using a single lytic enzyme and protease-free solutions after the immobilization of 2×10^{9}CFU/mL. Lane M: ladder size marker; L: lysozyme; M: mutanolysin; Pk: proteinase K; NDSUPlus: 0.01 M Tris, 0.1 M EDTA, 1% sarcosyl, 1% Nonidet P-40, and 4 M Urea (pH 9.5). Running conditions: 1.5% agarose gels, 0.5X TBE buffer. Switching times were ramped from 25 to 3 seconds at 10V/cm and 20°C over 5hours and 14minutes in the miniCHEF chamber.

Table 1. Percentages of immobilized DNA suitable for restriction and PFGE analyses obtained from *Streptococcus pyogenes* ATCC 12384 samples prepared by the standard enzymatic protocol or the modified procedure developed in this work

Treatment	Areas (DO/mm^2) Mean ± MSE			R (%)	RSD of (T-S)
	n	Area at slot peak S (pixels)	Total peak areas T (pixels)		
L + M + Pk	4	0.2 ± 0.1	20.35 ± 1.25	99.1 ± 0.4^A	11.7
L + M+ NDSUPlus	3	0.45 ± 0.09	18.25 ± 1.24	97.6 ± 0.31^C	11.2
L+ NDSUPlus	3	1.57 ± 0.12	22.13 ± 1.02	92.9 ± 0.56^B	8.5
M + NDSPlus	3	0.25 ± 0.04	8.5 ± 0.52	97 ± 0.48^C	9.1

L: lysozyme; M: mutanolysin; Pk: proteinase K; NDSUPlus solution; DO: optical density. T was the total peak area (proportional to DNA/miniplug) and S the area of the peak at slot (proportional to DNA unable to enter into the gel). Percentage of DNA suitable for PFGE was R(%)=[1-(S/T)] x 100. MSE: mean standard error; RSD: relative standard deviation. Mean R with the same letter are not significantly different by Duncan´s multiple range test at a significant level of 0.05.

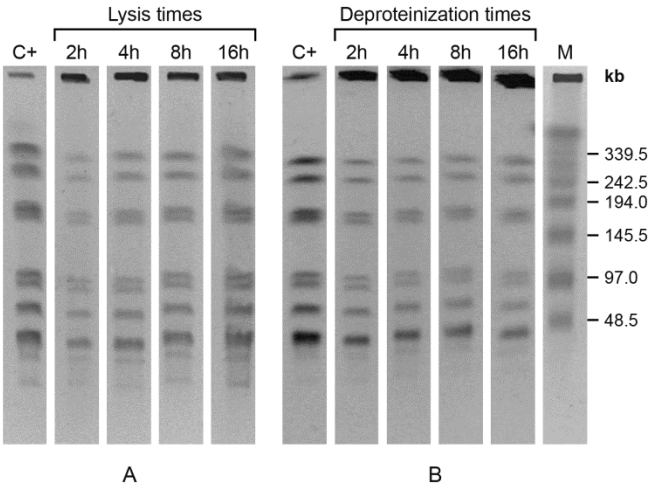
patterns at visual inspection, as well as consistent amounts of DNA suitable for electrophoresis (Figure 1, Table 1). RSD of R values ranged from 8.5 to 11.7% (Table 1), which demonstrated the reproducibility of these assays. The incubation with lysozyme or mutanolysin followed by NDSUPlus gave patterns reaching R values over 90%, but mutanolysin was a more efficient cell wall disrupting agent than was lysozyme ($\alpha = 0.05$, Duncan's multiple range test; Table 1). However, as the variant lysozyme/NDSUPlus also gave good-quality patterns and was the most economical option, the cell wall lysis and deproteinization times were optimized using these reagents. Miniplugs containing cells of *S. pyogenes* ATCC 12384 strain were treated at 2, 4, 8, or 16 hours with lysozyme followed by a NDSUPlus overnight incubation (16 hours, non-enzymatic deproteinization) (Figure 2A). Notwithstanding the procedure used to prepare the samples, the miniCHEF resolved an identical number of bands when DNA molecules were digested with *SmaI*, and the patterns had similar quality by visual judgement. However, in all cases, the patterns showed less intense bands than did the ones resolved from the miniplugs subjected to the standard enzymatic treatment (Figure 2A). R mean values did not differ between the patterns obtained from samples incubated with lysozyme for different times ($F[3, 13] = 1.06$, $p = 0.399$).

Four hours was the minimum duration of the incubation with lysozyme in the cell lysis step (Figure 2A). Different NDSUPlus incubations times (2, 4, 8, and 16 hours) were assessed. Regardless of the incubation time of the miniplugs in NDSUPlus, the miniCHEF resolved an identical number of bands when the DNA molecules were digested with *SmaI* (Figure 2B). In addition, there were no differences between R values obtained for samples incubated 4 hours with lysozyme followed by 2, 4, or 16 hours with NDSUPlus ($F[2,13] = 0.087$, $p = 0.92$). Based on these results, the developed procedure was finally established by an initial step of lysis with lysozyme over 4 hours followed by the deproteinization with NDSUPlus solution for 2 hours.

MiniCHEF and CHEF electrophoresis

*SmaI*DNA samples of *S. pyogenes* ATCC 12384 were loaded into the CHEF and miniCHEF gels. In the miniCHEF chamber, the *S. pyogenes* fingerprints were obtained at 10 V/cm and 20°C in 5hours and 14minutes (Figure 3A, miniCHEF) by applying a discontinuous ramp (25 seconds and 35 pulses, 20 seconds and 45 pulses, 15 seconds and 60 pulses, 10 seconds and 200 pulses, 5 seconds and 900 pulses, and 3 seconds and 80 pulses; switching times and number of pulses, respectively), while in the homemade CHEF chamber, the switching times were ramped from 50 to 3 seconds at 5 V/cm for 21 hours.

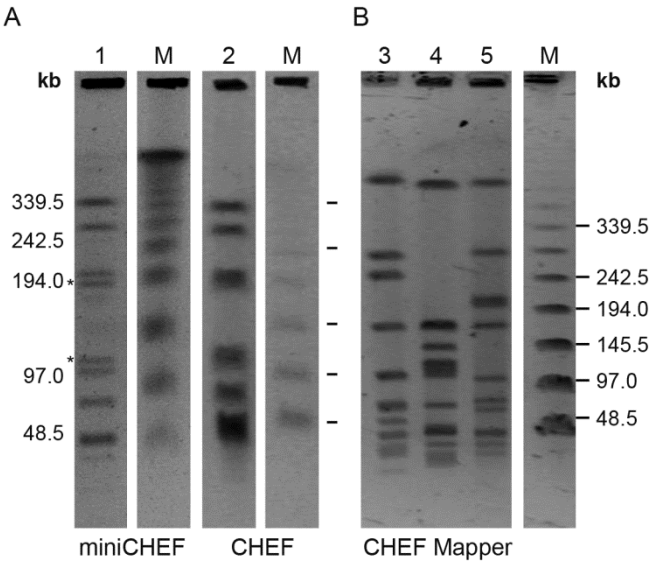
Figure 2. Cell lysis and deproteinization times optimization



*SmaI*DNA band patterns of *S. pyogenes* ATCC 12384 using the standard enzymatic or the modified procedure using a single lytic enzyme and protease-free solutions to prepare the samples. M: molecular weight marker; C+: enzymatic treatment: lysozyme + mutanolysin + proteinase K. **A:** The lysis step was performed in a solution containing lysozyme for 2, 4, 8, or 16hours of incubation time at 37°C followed by the deproteinization with NDSUPlus solution over 16hours. **B:** The deproteinization step was done in NDSUPlus solution for 2, 4, 8, or 16hours of incubation time at 45°C after 4 hours of incubation with lysozyme. DNA molecules prepared by the modified procedure were digested with 10 U of *SmaI* for 2 hours. Running conditions: 1.5% agarose gels, 0.5X TBE buffer. Switching times were ramped from 25 to 3 seconds at 10V/cm and 20°C for 5hours and 14minutes in the miniCHEF chamber.

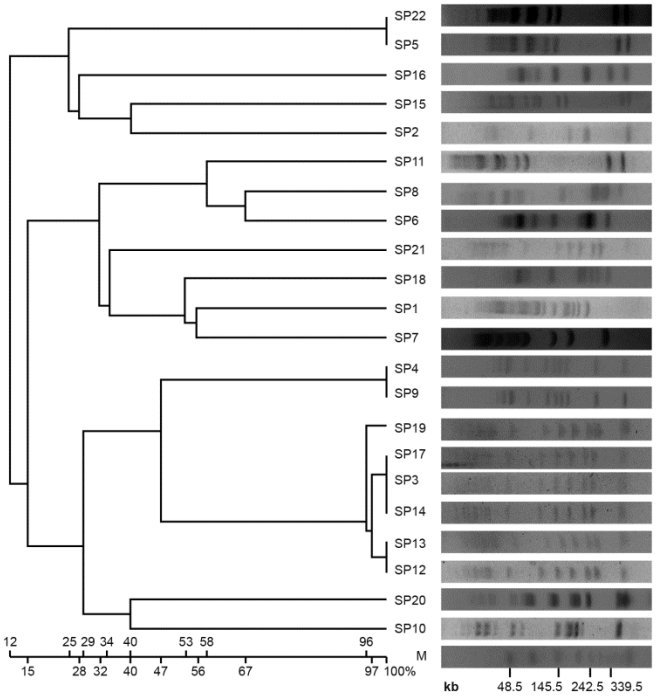
The simple enlarging of the photograph of the miniCHEF minigel reproduced the CHEF DNA band patterns, which had approximately 10 fragments between 20 and 400 kb (Figure 3A). The comparison of the resolutions between the consecutive pairs of bands in the two patterns showed that only the resolution between the bands at approximately 120 and 194 kb (Figure 3A, bands tagged with *) was different between them ($t=6.76$, $df=5$, $p < 0.05$). This result indicated that the miniCHEF was able to provide the same level of discrimination among restriction fragments of *S. pyogenes* DNA as was the conventional CHEF if the equivalent electric field strength, pulse times, and number of pulses were applied.

Figure 3. Band patterns of *SmaI*DNA restriction fragments of *Streptococcus pyogenes* in CHEF and miniCHEF



A: DNA from strain ATCC 12384 was prepared by the modified (lane 1) or enzymatic procedure (lane 2) and resolved in the miniCHEF and in the homemade conventional CHEF chamber, respectively. M: molecular weight marker. Switching times were ramped from 50 to 3 seconds at 5V/cm and 20°C over 21 hours in CHEF chamber or from 25 to 3 seconds at 10V/cm and 20°C for 5hours and 14minutes in the miniCHEF. **B:** DNA molecules from strains ATCC 10270 (lane 3), NZ131 (lane 4), and 6180 (lane 5) were prepared with the modified procedure (lysozyme and NDSUPlus), digested with 10 U of *SmaI* for 2 hours, and resolved in CHEFMapper chamber (BioRad). M: Molecular weight marker. Switching times were ramped from 1 to 5seconds for 2hours and from 5 to 40 seconds for 22 hours and 14°C. Lanes M, ladder size markers. Running conditions: 1.5% agarose gels in 0.5X TBE buffer. * Tags the pair of bands with different resolution in the CHEF and miniCHEF

Figure 4. PFGE analysis of group A streptococci isolates



Dendrogram and the corresponding *SmaI*DNA band patterns illustrating the genetic diversity of all GAS isolates (SP1 to SP22) fingerprinted. DNAs were prepared by the modified procedure. M: molecular weight marker. Running conditions: 1.5% agarose gels, 0.5X TBE buffer. Switching times were ramped from 25 to 3 seconds at 10V/cm and 20°C over 5 hours and 14 minutes in the miniCHEF chamber.

Table 2. Comparison of the solutions used and time consumed in the main steps of the fully enzymatic protocols used to prepare DNA from *Streptococcus pyogenes* suitable for PFGE and the procedure developed in this work

Main steps	Previous procedures		Modified procedure
	Standard enzymatic*	Rapid enzymatic**	Single lytic enzyme and protease-free solutions***
Cell wall disruption	*In situ* 2 mg/mL lysozyme and 10 µg/mL mutanolysine for 16 h at 37°C in lysis buffer	In suspension 0.1 mg/mL lysozyme for 10 min at 37°C in CSB	*In situ* 2 mg/mL lysozyme for 4 h at 37°C in lysis buffer
DNA deproteinization	1 mg/mL Proteinase K for 16 h at 50°C in NDS	1 mg/mL proteinase K and 1% SDS (inside the plug) plus 0.15 mg/mL proteinase K (outside the plug) for 2h at 54°C in CLB	NDSUPlus for 2 h at 45°C
Enzymatic restriction	20 U of *SmaI* at 37°C for 4 h	10 U of *SmaI* at 37°C for 2 h	10 U of *SmaI* at 37°C for 2 h
Running conditions	CHEF chamber (homemade), switching times from 50–3 s for 21h	CHEFMapper (BioRad), switching times from 4–40s, for 20 h	CHEF minichamber (Guefast-06), switching times from 25–3 s for 5 h, 14 min
Total time (approx.)	57 hours	24 hours	13 hours

*Based on the procedure described by Stanley *et al*. [17]; **Based on the procedure described by Chiou *et al*. [25]; ***Procedure developed in this work; lysis buffer: 0.01 M Tris, 0.1 M EDTA, 1% sarcosyl, 1% Nonidet P-40 (pH 8.0); NDS: 0.01 M Tris, 0.5 M EDTA, 1% sarcosyl (pH 9.5); CSB: 0.01 M Tris, 0.001 M EDTA (pH 8.0); CLB: 0.05 M Tris, 0.05 M EDTA, 1% SDS (pH 8.0); NDSUPlus: 0.01 M Tris, 0.1 M EDTA, 1% sarcosyl, 1% Nonidet P-40, 4 M urea (pH 9.5)

DNA samples of the *S. pyogenes* reference strains 10270, NZ131, and 6180 were prepared by the modified procedure developed here using the accessories of CHEFMapper (BioRad) and resolved in this equipment after *SmaI* restriction. High-quality patterns were obtained after applying 6 V/cm at 14°C and two lineal switching time ramps from 1–5seconds for 2hours and from 5–40seconds for 22hours (Figure 3B).

Repeated subtyping of the *S. pyogenes* ATCC 12384 strains using DNA samples prepared by the modified procedure yielded band patterns that were reproduced from run to run, yielding similar fingerprints in CHEF chambers and minichambers, even after five years of storage in TE-100 (results not shown).

Subtyping of clinical isolates

The modified procedure developed here and the running conditions assayed in the miniCHEF chamber were used for subtyping 22 GAS clinical isolates. *SmaI*DNA restriction fragments were clearly resolved in 9 to 12 bands at 10V/cm, 20°C, and 25-3 seconds of switching time for 5hours and 14minutes. Most of the fragments were in the size range between 20 and 500 kb (Figure 4). The analysis of the clonal relationship between the isolates identified 17 different PFGE patterns and three subtypes among the 22 isolates (Figure 4). The results suggest that the procedure developed in this work is useful to discriminate GAS isolates. From an epidemiological point of view, these results also suggest that there is an abundant clonal diversity among the isolates that circulate in the child population of Havana City. As additional result, the typeability by miniCHEF electrophoresis of 22 GAS clinical isolates prepared using a single lytic enzyme and protease-free solutions was 100% (Figure 4).

Discussion

A single incubation step with NDSUPlus solution was successfully used to prepare *P. aeruginosa, V. cholerae*, *Salmonella*, and *Aeromonas* spp. DNA suitable for PFGE fingerprinting [32-33,35]. However, an initial step of cell lysis with lytic enzymes (lysozyme or mutanolysin) was necessary for preparing GAS DNA, probably due to the different composition of the cell walls of Gram-positive and Gram-negative bacteria. In addition, the resistance to *in situ* lysis of the cell wall of Gram-positive bacteria is known [23].

The releasing of DNA suitable for PFGE was achieved more efficiently using a standard enzymatic protocol (lysozyme/mutanolysine/proteinase K). However, the lysis step with only one lytic enzyme (lysozyme or mutanolysine) followed by incubation with urea instead of proteinase K gave reproducible and good-quality DNA macrorestriction patterns on visual inspection. In addition, this procedure yielded patterns with more than 90% of DNA suitable for electrophoresis based on R(%) value calculation (Table 1, Figure 2). This result permitted the selection of the most economical option (lysozyme) in the lysis step and reduced the incubation times, helped by the small thickness of the miniplugs (0.07 cm) [33].

There are other rapid PFGE protocols reported for subtyping Gram-positive bacteria [19,23-25,34,43-45]. Matushek *et al.* reported a rapid procedure for DNA preparation of Gram-positive bacteria for pulsed field, reducing the incubation times to 4 or 5 hours in some cases. However, they included lytic enzymes, DNase-free RNase, and proteinase K in their protocol [45]. Benson and Ferrieri used elevated enzyme concentrations (10 mg/mL of lysozyme and 20 mg/mL of proteinase K) and high incubation temperatures (55°C) for DNA sample preparation, and they increased the running buffer temperature from 10°C to 14°C in order to reduce the complete procedure to 39 hours [44]. Immobilized DNA of Gram-positive cocci have been prepared without proteinase K, although lysostaphin was required in the incubation solutions [43]. Only the protocol developed by McEllistrem *et al*. eliminated the cell lysis step and performed DNA release and deproteinization without the addition of complex buffers or enzymes [34]. However, this procedure is only applicable to *S. pneumoniae*, due to the unique ability of this bacterium to undergo autolysis [46-47] On the basis of PulseNet's *Listeria monocytogenes* PFGE protocol established in 1998, Chiou *et al.* fingerprinted 179 *S. pyogenes* clinical isolates in approximately 24hours by incubating the cell suspension with lysozyme, treating the immobilized bacteria with proteinase K, and applying 20hours of electrophoresis for separating the DNA restriction fragments [25] (Table 2). Unlike to this rapid enzymatic protocol to prepare GAS DNA, where the lysis time is only 10 minutes, our procedure required more time because all the treatments were performed *in situ* (Table 2). However, the GAS DNA preparation *in situ* with a single lytic enzyme, protease-free solutions, and the miniCHEF run required a total time of approximately 13hours, which is 7hours less than the time required for only separating bands by conventional CHEF electrophoresis.

On the other hand, in our previous experience, the rapid enzymatic protocol for *S. pyogenes* has the same reproducibility problems as the original PulseNet protocol for *L. monocytogenes* reported by Halpin *et al*. [23]. The modifications developed in 2010 by Halpin *et al.* [23] to the PulseNet protocol for *L. monocytogenes*, which mainly were increasing the lysis temperature to 56°C, may also improve the reproducibility of *S. pyogenes* DNA preparation for PFGE. However, in our opinion, the reproducibility problems of these rapid protocols are most related to the cell lysis in suspension *per se* rather than the lysis temperature used. Lysozyme hydrolizes the bacterial cell walls mainly of Gram-positive bacteria. The presence of hypothonic solutions, such as water or Tris-EDTA buffers at low concentrations, could provokes lysis, which is not necessarily preceded by formation of protoplast [48]. In consequence, the breakdown of cell walls exposes the genomic bacterial DNA to the mechanical shearing during the pipetting for casting the plugs. The lysis incubation time should be carefully monitored to guarantee a homogeneous protoplast formation and to avoid an extensive bacterial lysis before the immobilization, which could be a factor hindering the reproducibility of the protocols. Another factor conspiring against the reproducibility of the lysis step could be the different sensitivities to lysozyme of the cell walls of the diverse bacterial genus and species [48]. For that reason, the lysis step with lysozyme *in situ*, though it requires more time, could give more consistent and reproducible results. Further optimization of the procedure reported here could include increasing the lysozyme incubation temperature of immobilized bacteria in order to reduce even more the total procedure time.

The DNA size range reported for *S. pyogenes* DNA restricted with *SmaI* is between 20 and 500 kb, and these fragments are resolved in approximately 10 to 20 bands in the CHEF (BioRad) chamber [27-28,30]. In our experiments, the *SmaI* DNA from *S. pyogenes* resolved in the miniCHEF showed band patterns similar to those previously reported [27,30], but they were obtained 3.8–4.5 times faster due to the fact that the miniCHEF uses greater electric field strength. We were able to reproduce in the miniCHEF the patterns obtained in CHEF conventional chambers by applying equivalent running conditions as had previously been reported [32-33,35]. These results confirm that equations that describe the DNA migration in CHEF [42] were also able to bring the suitable running conditions for rapid DNA fingerprinting of *S. pyogenes* by miniCHEF electrophoresis.

The procedure reported here was as reproducible as the standard enzymatic protocol (Table 1), and was time saving (DNA preparation in only 6hours, DNA restriction in 2hours and electrophoresis in 5hours and 14minutes) and cost-effective compared with any other previous reported protocol because NDSUPlus solution contains neither expensive chemical reagents nor proteases (Table 2).

We used the procedure reported here to prepare intact DNA and fingerprinting by miniCHEF electrophoresis 22 GAS isolates, which gave reproducible results and easily interpretable banding patterns. The results suggest that this procedure should allow more feasible evaluation of large numbers of isolates in shorter periods of time.

Conclusions

The resources saved and the achievement of fast separation could make our fingerprinting procedure a useful tool in the control of infectious outbreaks caused by GAS. It is noticeably shorter than the one-to three-day minimum previously reported [25-30]. The procedure reported here was also useful to prepare, in 6hours, immobilized DNA from *S. pyogenes* in the sample format of the CHEFMapper (BioRad) and to obtain well-resolved patterns. Finally, further multi-laboratory validation of this procedure and/or additional optimizations are also advisable.

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