Research Article

DNA fingerprinting of Vibrio cholerae and Aeromonas species by pulsed-field minigel electrophoresis

DNA molecules of Vibrio cholerae and Aeromonas species were prepared by incubating immobilized cells for 4 and 2 h, respectively, with a nonenzymatic solution that contains chemical reagents only (NDSUPlus). This method gave results as reproducible as the enzymatic one that uses proteinase K, and rendered DNA molecules suitable for fingerprinting by mini-CHEF electrophoresis. As rapid DNA separations at high electric field are achieved in mini-CHEF chamber with low heat evolution, DNA restriction fragments were separated in 5 h at 10 V/cm in a single resolution window. Then, fragment separations in three resolution windows were done in 15 h. This time is shorter than the one needed by the large CHEF chamber for resolving fragments in a single resolution window. Three windows permitted to include larger numbers of restriction fragments in the calculation of isolate similarities. Both sample preparation and mini-CHEF electrophoresis may represent an alternative for performing massive epidemiological studies of V. cholerae and Aeromonas species.

Keywords: Aeromonas / DNA fingerprinting / DNA preparation / Pulsed-field minigel electrophoresis / Vibrio cholerae

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1 Introduction

Epidemiological surveillance programs of pathogenic bacteria are based on the classification of bacterial isolates in subtypes. Subtypes are isolates of the same strain that have small genetic differences among them [1]. Molecular techniques for bacterial subtyping allow discrimination below the species level and require the characterization of DNA molecules, which usually relies on the electrophoretic separation of DNA fragments of different lengths. For instance, restriction fragments of the complete bacterial genome, resolved by PFGE, form band patterns that are regarded as DNA fingerprints of the isolates. Comparisons of fingerprints permit to calculate similarities and relatedness among isolates involved in an infectious-disease outbreak [1–3].

In the last decades, Vibrio cholerae has reemerged as the etiologic agent of many contemporary outbreaks, whereas Aeromonas spp. strains have been identified as new emerging waterborne pathogens [4–10]. DNA fingerprinting of V. cholerae and Aeromonas spp. by PFGE is a useful method to discriminate subtypes of these pathogens [10–18]. However, fragment separations in a single resolution window take 18–29 h in the CHEF chamber, and sample preparation requires long incubations with solutions that contain cell wall-disrupting enzymes and proteases [10, 16–18]. The requirements of expensive reagents and long running times are factors preventing the extensive use of PFGE in epidemiological surveillance programs. The inclusion of multiple resolution windows into fingerprinting process has improved subtype discrimination [19–21], but reagents and running time increase considerably.

In this work, V. cholerae and Aeromonas spp. immobilized DNA molecules were prepared according to the rapid nonenzymatic method reported for obtaining Pseudomonas aeruginosa DNA [22]. Additionally, as rapid DNA separations at high electric field are achieved in mini-CHEF chamber with low heat evolution [23], DNA restriction fragments were separated in 5 h at 10 V/cm in a sin-
ingle resolution window. Then, fragment separations in three resolution windows were done in 15 h. This time is 3 or 14 h shorter than the one needed by the large CHEF chamber for resolving, \( V.\) \( \text{cholerae} \) or \( \text{Aeromonas} \) spp. DNA fragments, respectively, in a single resolution window. Three windows permitted to include larger numbers of restriction fragments in the calculation of similarities among isolates.

2 Materials and methods

2.1 Bacterial strains

\( \text{Aeromonas trota} \) (At1–At9), \( \text{Aeromonas hydrophila} \) (Ah1–Ah4), \( \text{Aeromonas} \) spp. (Asp1 and Asp2), and \( \text{V.\) cholerae} \) nonO1/nonO139 (Vc1–Vc9) clinical isolates were randomly selected from the collection of the Reference Laboratory of Enteric Pathogens of the Institute of Tropical Medicine, Havana, Cuba. Isolates were identified and characterized by standard microbiological methods [24, 25].

Strains 569B (O1, biotype classical), C6706 (O1, biotype El Tor), CRC266 (O139), and C7258 (O1, biotype El Tor) of \( \text{V.\) cholerae} \) were a kind gift of Dr. R. Filkenstein. Strains 638 and 81 are derived from the C7258 strain [26, 27].

2.2 Enzymatic and nonenzymatic immobilized DNA preparations

\( \text{Aeromonas Asp2} \) or \( \text{V.\) cholerae} \) bacterial cells were grown in Luria–Bertani broth (LB: 1% w/v tryptone, 0.5% w/v yeast extract, 1% w/v NaCl) at 37°C with aeration and vigorous shaking until the late log phase was reached. Cells were harvested, washed with 0.15 M NaCl plus 0.01 M EDTA solution (pH 8.0) and mixed with 1.5% low-melting point agarose dissolved in the washing solution. Miniplugs of \( 3 \text{ mm} \times 3 \text{ mm} \times 0.7 \text{ mm} \) (length \( \times \) height \( \times \) thickness) containing \( 1 \times 10^9, 3 \times 10^9, \) or \( 5 \times 10^9 \) colony forming units (cfu)/mL were cast in the miniplug forming mold of Guefast 04 accessory set (Neuronic SA, Cuba). Groups of miniplugs were incubated with single nonenzymatic solution (NDSU-Plus) (0.01 M Tris, 0.1 M EDTA, 1% sarcosyl, 1% Nonidet P-40, 4 M urea, pH 9.5) for 2, 4, or 16 h, and finally washed in distilled water, followed by TE-100 (0.01 M Tris-HCl, 0.1 M EDTA, pH 8.0) (Table 1). As controls, another group of miniplugs containing \( 3 \times 10^9 \) cfu/mL was incubated sequentially, as described previously [22, 28], in two enzymatic solutions. The first one is the lysis solution (0.01 M Tris-HCl, 0.1 M EDTA, 1% Nonidet P-40, 1% sarcosyl, pH 8.0) containing lysozyme at 1 mg/mL, and the second one is NDS (0.01 M Tris, 0.5 M EDTA, 1% sarcosyl, pH 9.5) containing proteinase K at 1 mg/mL (Table 1). Miniplugs were stored at 4°C in TE-100. Three independent DNA preparations were done under each experimental condition.

2.3 Restriction enzyme digestion of immobilized DNA

Each miniplug was washed three times in 1 mL of TE-0.5 (0.01 M Tris-HCl, 0.005 M EDTA, pH 8.0) at 4°C for 10 min, and further incubated with 200 \( \mu \)L of the digestion buffer recommended by the manufacturer (Amersham Bioscience, UK) at 4°C for 10 min. Then, each miniplug was transferred to 100 \( \mu \)L of fresh digestion buffer. \( \text{V.\) cholerae} \) DNA was digested with \( \text{Sfi}\) or \( \text{Not}\) restriction enzyme, whereas XbaI was the enzyme used with \( \text{Aeromonas} \) spp. DNA samples (Table 1). Digestions were stopped by replacing the reaction buffer with 1 mL of TE-100.

| Table 1. Experimental conditions for the preparation of \( \text{Aeromonas} \) spp. and \( \text{V.\) cholerae} \) immobilized DNA fragments |
|-------------------|-----------------------------|-----------------------------|
| Experimental conditions | Methods of immobilized DNA preparation (20 miniplugs/mL) | |
| | Enzymatic | Nonenzymatic |
| Cell-wall disruption buffer | Lysis solution containing lysozyme. Incubation overnight at 37°C | NDSU-Plus. Incubation times from 2 to 16 h were assayed at 50°C |
| Buffer for DNA deproteinization | NDS containing proteinase K. Incubation overnight at 50°C | |
| Miniplug washes | Six at 50°C (twice with distilled water for 10 min each and four times with TE-100 for 15 min each) | Four at 50°C for 15 min each (twice with distilled water and twice with TE-100) |
| Restriction enzyme | 30 U of XbaI at 37°C for 2 h \( \text{Aeromonas} \) spp. 30 U of NotI at 37°C for 2 h 30 U of Sfi at 50°C for 3h \( \text{Aeromonas} \) spp. 10 U of XbaI at 37°C for 2 h \( \text{V.\) cholerae} \) 5 U of NotI at 37°C for 2 h 10 U of Sfi at 50°C for 3 h \( \text{V.\) cholerae} \) |
2.4 Pulsed-field minigel electrophoresis of DNA macrorestriction fragments of Aeromonas spp. and V. cholerae

Electrophoresis runs were performed in a 7 cm \times 5 cm \times 0.5 cm (width \times length \times thickness) minigel of the mini-CHEF chamber (11.6 cm of opposite electrode separation) of Guefast 04 System (Neuronic SA) filled with 250 mL of 0.5 \textit{M} Tris-borate-EDTA buffer (TBE) buffer (44.5 mM Tris, 44.5 mM boric acid, 1 mM EDTA, pH 8.3) at 20°C [22]. DNA fragments were separated at 10 V/cm for 5 h in 1.5% agarose gels (Amersham Biosciences) cast in electrophoresis buffer. Minigels were stained for 30 min in 0.5 \textit{mg/mL} ethidium bromide solution, UV transilluminated, and photographed with a digital camera (Sony DSC707). Sizes of the restriction fragments (in kilobase pairs: kb) were calculated by comparing their band positions with comigrating \lambda-ladders and midrange I pulsed-field gel markers (Biolabs) in the minigels.

2.5 Mini-CHEF running conditions to obtain multiple resolution windows for DNA restriction fragments of Aeromonas spp. and V. cholerae

DNA restriction fragments were resolved at a ramp (Ramp I) formed by the following switching times and number of pulses: 25 s for 25 min (30 pulses), 20 s for 23 min (35 pulses), 15 s for 22 min (44 pulses), 10 s for 43 min (130 pulses), 5 s for 2 h 47 min (1000 pulses), and 3 s for 20 min (200 pulses). The interval of switching time in ramp I was similar to the ramp interval previously described [29]. Fragment resolutions in various molecular weight ranges were assayed by setting distinct ramps. Switching times and number of pulses of ramps were selected from the results given by the simulator of Guefast 04 system [30], which uses equations [31] that describe reorientation times, migration velocities, and migrated distances of linear DNA molecules in CHEF electrophoresis. Simulator was fed with the restriction fragment DNA sizes, electric field intensity, and experimental temperature. Band sharing frequency (Bsf) was calculated when required. It was

\[ \text{Bsf} = 2 \times \text{nbm} / (n1 + n2) \]  

(1)

where “nbm” is the number of band matches, “n1” and “n2” are the total numbers of bands identified in the patterns of isolates 1 and 2, respectively [32].

2.6 Estimation of the amounts of immobilized DNA per miniplug suitable for PFGE

DNA amounts suitable for restriction and PFGE analyses were estimated from the densitometry profiles recorded from band patterns of \textit{V. cholerae} 638 and \textit{Aeromonas} Asp2 DNA samples prepared by the enzymatic and the nonenzymatic methods and all incubation times. Digital images were set to 256 pixels resolution from top to bottom of the minigel, and each lane was a matrix of “n” columns (n depends on the miniplug width) and 256 rows. Pixel intensity \((y_i\text{; for } i = 0 . . . 255)\) on the densitometry profiles was the average intensity of the \(n\) pixels per row. Peaks were identified and their areas in pixels were calculated by integration. Background was recorded in the inter lane space and subtracted from signals. Percentage \((R)\) of DNA suitable for PFGE was

\[ R(\%) = \left[1-\left(\frac{S}{T}\right)\right] \times 100 \]  

(2)

in which “T” was total peak areas (proportional to DNA/miniplug) and “S” the area of the peak at slot (proportional to DNA unable to enter into the gel). Mean \(R\) values were calculated from at least three independent replicas and compared through Student’s t-test or one-way breakdown ANOVA variance analysis, followed by Duncan’s multiple range test. Transformation of data was \(\text{sin}^{-1}\left(\sqrt{0.01 \cdot T/R}\right)\) to achieve normality of sample distributions, whereas homocedasticity of samples was attained with the logarithmic transformation of normally transformed data. RSD of the DNA amount suitable for PFGE was calculated from \((T - S)\) data. The statistical tests were performed by the STATISTICA program package for Windows (Release 4.5F, 1993). Lane densitometry profiles and calculations were done with the aid of a computer program that was written in Delphi-5 for Windows operating system.

3 Results

3.1 Aeromonas and V. cholerae nonenzymatic intact DNA preparation and validation against the enzymatic method

Optimal cell concentration of \textit{Aeromonas Asp2} or \textit{V. cholerae} 638 in miniplugs was \(3 \times 10^6 \text{ cfu/mL}\), which rendered the amount of DNA molecules that gave bands of the adequate intensities in the minigels of the mini-CHEF chambers (Figs. 1, 2). Casting miniplugs at \(5 \times 10^9\) or \(1 \times 10^9 \text{ cfu/mL}\) gave overloaded gels or undetectable DNA bands, respectively (results not shown).

Miniplugs containing XbaI DNA fragments of \textit{Aeromonas Asp2} isolate gave the same percentage \((R = 93\%)\) of immobilized DNA suitable for PFGE in samples incubated with NDSUPIlus for 2, 4, or 16 h (Table 2, \(R^2\) in Duncan’s multiple range test). Even samples incubated for 2 h with NDSUPIlus and digested with 10 U of XbaI gave sufficient DNA amount to appreciate band resolutions and identify bands in the patterns (Fig. 1). Validation experiments
Figure 1. Three mini-CHEF resolution windows of XbaI-digested Aeromonas DNA. Lanes: 1, Asp1; 2, Asp2; 3, common band pattern of At1–At9; 4, common band pattern of Ah1–Ah4; L, λ-ladder size marker. CZ: compression zone. Electrophoresis conditions: 1.5% agarose, 10 V/cm, 20°C, ramp I (A), II (B), and III (C). Miniplugs containing 3 x 10^9 cfu/mL were incubated with NDSUPlus for 2 h.

Figure 2. Composite fingerprint of NotI-digested V. cholerae DNA separated in two mini-CHEF resolution windows. (A), fragments larger than 194 kb at 25–3 s switching time interval (ramp I). (B), fragments smaller than 194 kb at 5–1 s switching time interval (ramp II). Lanes: 1, 638; 2, C7258; 3, C6706; 4, 81; 5, Vc4 isolate; 6, pattern of Vc1–Vc3 and Vc5–Vc9 isolates; 7, 569B; 8, CRC266. (*) and (d) indicate distinct bands in the 638, C7258, and 81 patterns. Electrophoresis conditions: 1.5% agarose, 10 V/cm, 20°C. Miniplugs containing 3 x 10^9 cfu/mL were incubated with NDSUPlus for 4 h.

revealed that mean R was 99.7% if samples were prepared with lysozyme and proteinase K. This R value differed significantly (p(F) = 0.001) from the 93% obtained from samples prepared by the nonenzymatic method (Table 2, R_D, R_E in Duncan’s multiple range test). On the other hand, optimal incubation with NDSUPlus was 4 h for miniplugs containing V. cholerae 638 (Table 2). These immobilized DNA molecules were susceptible to complete digestion with 10 U of SfiI, or 5 U of Notl per miniplug, and the restriction fragments were separated by mini-CHEF in well-resolved band patterns, as the examples of Fig. 2 show. The percentage of V. cholerae DNA suitable for PFGE analyses (R) was 90%, and it differed significantly from the 95% calculated from samples prepared by the enzymatic method (p(t) = 0.005, Table 2). These results indicated most efficient DNA release in V. cholerae or Aeromonas Asp2 DNA samples prepared by the enzymatic method. Although miniplugs prepared enzymatically needed 30 U of restriction enzyme to achieve complete DNA digestion, and samples prepared by both methods gave similarly resolved band patterns (results not shown). Finally, when Aeromonas Asp2 or V. cholerae DNA samples were prepared with NDSUPlus and these miniplugs were stored in TE-100 for 1 year, mean R values were 96.8 ± 1.2% or 90.4 ± 0.8%, respectively, and the DNA restriction fragment band patterns resolved from these miniplugs (result not shown) were as the ones presented in Figs. 1, 2. These results indicated 1 year of stability of DNA molecules prepared by the nonenzymatic method. RSD values of [T – S] ranged from 9 to 13% for samples prepared in NDSUPlus and from 8 to 14% for samples prepared with proteinase K (Table 2), indicating the results obtained by nonenzymatic method were as reproducible as those given by the enzymatic one.

3.2 Resolution windows in mini-CHEF for XbaI DNA restriction fragments of Aeromonas isolates

Restriction fragments of Aeromonas DNA were prepared from 14 clinical isolates by incubating miniplugs with NDSUPlus for 2 h and cutting DNA with 10 U of XbaI. Five hours of mini-CHEF electrophoresis at ramp I resolved the fragments in the 388–97 kb range and permitted the differentiation of isolates. For instance, Asp1 isolate gave a unique pattern that had nine well-resolved bands (Fig. 1A, lane 1), Asp2 isolate gave a distinct pattern (Fig. 1A, lane 2) that differed in two bands from the common one displayed by the nine A. trota isolates (Fig. 1A, lane 3, bands near to 97 and 194 kb markers), and the four A. hydrophila isolates displayed a common and distinctive band pattern (Fig. 1A, lane 4). According to Tenover’s criteria [3], Asp2 and A. trota isolates are closely related strains with analogous clonal identity.
Table 2. Percentages of immobilized DNA suitable for restriction and PFGE analyses obtained from samples prepared by nonenzymatic or enzymatic method

<table>
<thead>
<tr>
<th>Densitometry profile information</th>
<th>XbaI Aeromonas Asp2 DNA</th>
<th>SfiI V. cholerae 638 DNA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Nonenzymatic</td>
<td>Enzymatic</td>
</tr>
<tr>
<td>Miniplug incubation time (h) a)</td>
<td>2</td>
<td>4</td>
</tr>
<tr>
<td>Number of replicas</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>Area at slot peak (S, pixels) b)</td>
<td>16 ± 5</td>
<td>16 ± 4</td>
</tr>
<tr>
<td>Total peak areas (T, pixels) c)</td>
<td>217 ± 19</td>
<td>220 ± 28</td>
</tr>
<tr>
<td>DNA suitable for PFGE (R, %) d)</td>
<td>92.7 ± 2.3</td>
<td>92.8 ± 2.0</td>
</tr>
<tr>
<td>RSD of T - S (%) e)</td>
<td>10.7</td>
<td>13.0</td>
</tr>
</tbody>
</table>

a) Lysis and deproteinization times of miniplugs containing 3 × 10^8 cfu/mL.
b) Areas (mean ± mean standard error) calculated from peaks along the densitometry profiles.
c) R was calculated according to Eq. (2).
d) RSD.

distinct superscripts D and E indicated significant differences between means (p(F) = 0.001, for 3 and 9 degrees of freedom) in the variance analysis and Duncan’s multiple range test.

* Indicates significant differences between means (p(t) = 0.005, for 11 degrees of freedom) according to Student’s t-statistic.

Although Aeromonas patterns appeared distinct at ramp I, clusters of overlapped fragments appeared in the 100–10 kb size range (Fig. 1A, lanes 3, 4), introducing uncertainty in the identification of band matches at this size interval and evidencing the need of improving fragment separations for obtaining more reliable estimates of Bsf according to Eq. (1). Ramp II achieved this improvement, and it was set as follows: 5 s for 3 h 20 min (1200 pulses), 3 s for 1 h 15 min (750 pulses), and 1 s for 25 min (750 pulses). Differing from ramp I, the ramp II resolved in 12 bands the A. trota (At3) and A. hydrophila (Ah2) fragments smaller than 145 kb and seven band matches appeared between the patterns (Fig. 1B, lanes 3, 4). The Bsf of these two isolates were 0.42 at ramp I (13 and 11 fragments resolved), 0.48 at ramp II (14 and 15 fragments resolved), and 0.5 (20 and 16 fragments resolved) in both resolution windows for patterns paired at the position of the 145.5 kb marker. Bsf = 0.5 was considered the most reliable similarity estimation between isolates At3 and Ah2, because greater number of bands were identified in both resolution windows, which meant a higher probability of detecting fragment length polymorphisms. Finally, at ramp II, a group of unresolved DNA fragments appeared near to the 48 kb marker of A. hydrophila’s band patterns (Fig. 1B, lane 4). Fragments of this cluster were resolved in various bands at ramp III (3 s for 2 h 38 min (1575 pulses) and 1 s for 2 h 47 min (5025 pulses)) (Fig. 1C, lane 4), indicating that this resolution window could be useful to improve Bsf estimates and thus Aeromonas fingerprinting. The three mini-CHEF runs required 15 h of running time (5 h each), which is 14 h less than the time required [10, 16, 18] for separating bands in a single resolution window by conventional CHEF electrophoresis.

3.3 Typing V. cholerae strains in two mini-CHEF resolution windows

Restriction fragments of V. cholerae DNA were prepared from 15 different strains by incubating miniplugs with NDSUPlus for 4 h, and cutting DNA for 2 h with NotI. After 5 h of electrophoresis in mini-CHEF at ramp I, 10 V/cm and 20°C, restriction fragments of strains were resolved in 10–22 bands in the 340–15 kb range; but groups of unresolved fragments appeared ahead of the 97 kb marker, preventing common band identification in this pattern region. Ramp II (5–1 s) resolved these groups of fragments, and the patterns obtained at ramps I and II (Fig. 2, upper and lower parts, respectively) were paired at the 194 kb size marker and composite fingerprints were constructed. The composite fingerprints displayed 20–27 bands in the 340–15 kb range (Fig. 2, upper and lower panels), and revealed more reliable information about similarities among isolates. For instance, bands sharing frequencies between Vc4 and Vc1 were 0.44 (at ramp I), 0.65 (at ramp II), and 0.60 (at ramp I + II) (Table 3). Bsf = 0.6 was considered the most reliable similarity estimation between these isolates because greater numbers of band were identified in both resolution windows, implying higher probability of detecting fragment length polymorphisms.

Composite fingerprints of V. cholerae C7258, C6706, and 81 strains were indistinguishable, and they differed in three bands from the one given by the 638 strain (Fig. 2, lanes 1–4, bands indicated with * and ●). In addition, V. cholerae nonO1/nonO139 clinical isolates presented two

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distinct composite fingerprints: a unique one resolved for the Vc4 isolate (Fig. 2, lane 5) and a common one for the remaining Vc1–Vc3, Vc5–Vc9 isolates (Fig. 2, lane 6). Both patterns differed in 10 bands from the fingerprints given by the O1 or O139 strains (Fig. 2), suggesting the possibility of differentiating nonO1/nonO139, O1, and O139 V. cholerae strains based on these NotI composite fingerprints. NotI composite fingerprints of the C7258, C6706 O1 El Tor biotype strains and 569B O1 Classical biotype strain had bands within the 340–15 kb interval, but 13 common bands were detected only (Fig. 2, lanes 2, 3, and 7); then, these V. cholerae O1 strains were typed as different in agreement with their distinct biotype classification. In silico determination of NotI restriction sites of V. cholerae DNA base sequences [33] gave fragment sizes that agreed with those estimated in the two resolution windows.

Finally, the mini-CHEF runs performed at ramp I and ramp II gave two resolution windows that permitted to detect larger numbers of differences among NotI macrorestriction fragments of V. cholerae strains and needed 10 h of total running time. It approached half of the time needed by previously reported protocol for obtaining V. cholerae NotI fingerprints in the conventional CHEF chamber [17].

### 4 Discussion

#### 4.1 Nonenzymatic DNA preparation from Aeromonas spp. and V. cholerae

Single incubation step with NDSUPlus solution rendered DNA suitable for PFGE fingerprinting (Table 2, Figs. 1, 2), indicating that the chemical reagents of NDSUPlus solution disrupted cell walls, lysed the cells, and deproteinized DNA molecules. Lyses of P. aeruginosa cells are easily accomplished in few minutes with EDTA solutions [34], a component of the NDSUPlus solution [22], but similar results have not been reported for the bacterial species studied in this work. V. cholerae and Aeromonas spp. required 4 and 2 h of incubation with NDSUPlus, respectively, instead of the 30 min needed for P. aeruginosa immobilized DNA preparation [22]. Probably, the requirements of distinct incubation times were related to the different cell wall compositions of these bacterial species. The results presented here indicated greater percentages of DNA molecules suitable for PFGE (R in Table 2) when Aeromonas Asp2 or V. cholerae 638 immobi-lized DNA samples were prepared by the enzymatic method (Table 1), which pointed out to greater efficiency of the enzymatic treatment. However, the nonenzymatic method gave sufficient DNA amount for fingerprinting, as Figs. 1, 2 show, and sample preparation was faster. Optimal number of V. cholerae or Aeromonas spp. per mini-plug was 3 × 10^5 cfu/mL for fingerprinting in mini-CHEF (Figs. 1, 2). This cell number is near to three times the number of cells used (10^6 cfu/mL) for P. aeruginosa DNA preparation [22]. An explanation may be the distinct genome sizes of these bacteria. P. aeruginosa has a single 6.26 Mb chromosome, V. cholerae has two chromosomes of 2.96 and 1.07 Mb [33], whereas genome size of A. hydrophila has been estimated at 4.5 Mb [35]. At most, 10 U was the amount of restriction enzyme needed to achieve complete digestion of V. cholerae and Aeromonas spp. DNA molecules prepared by incubations with NDSUPlus for 4 and 2 h, respectively (Figs. 1, 2). These results contrasted to the 30 U of NotI used by the fast protocol reported for preparing V. cholerae DNA by 2 h of incubation with proteinase K [17]. The high operating cost would prevent the extensive use of this protocol in epidemiological surveillance programs. Our work supported the nonenzymatic intact genomic DNA preparation as a convenient laboratory method to implement such programs. The method was flexible enough to permit intact genomic DNA preparation from V. cholerae and Aeromonas spp., in addition to P. aeruginosa [22], yeasts [36, 37] and E. histolytica [38] reported previously. It was easy to perform because it required a single miniplug incubation step with NDSUPlus. It was as reproducible as the enzymatic method (Table 2), was time saving (2–4 h of incubation with NDSUPlus and 2 h of DNA restriction), and cost-effective because NDSUPlus solution contains neither expensive chemical reagents nor cell wall-disrupting enzymes or proteases.

#### 4.2 Multiple resolution windows in mini-CHEF

Mini-CHEF and CHEF chambers give the same band pattern resolution of any set of DNA molecules if identical agarose concentration, buffer ionic strength, tempera-

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Table 3. Band sharing frequencies between NotI DNA patterns of isolates Vc1 and Vc4 of V. cholerae separated in one or two mini-CHEF resolution windows

<table>
<thead>
<tr>
<th>Resolution windows</th>
<th>Bands resolved in fingerprints</th>
<th>Band matches</th>
<th>Bsfb (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Vc1 (n1)</td>
<td>Vc4 (n2)</td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>14</td>
<td>13</td>
<td>7</td>
</tr>
<tr>
<td>II</td>
<td>22</td>
<td>21</td>
<td>14</td>
</tr>
<tr>
<td>I + II</td>
<td>25</td>
<td>24</td>
<td>15</td>
</tr>
</tbody>
</table>

a) Resolution windows at ramps I, II, or I + II.
b) Bsfb was calculated according to Eq. (1). Electrophoresis conditions as for Fig. 2.
turence, switching time, and electric field are used in the two chambers; but mini-CHEF gives the results 1.5–2 times faster than CHEF because it uses thinner agarose plugs [22, 39]. In this work, as for *P. aeruginosa* DNA fingerprinting [22], band patterns of *Aeromonas* spp. or *V. cholerae* restriction fragments were separated at 10 V/cm in 5 h in a single resolution window (Figs. 1, 2). If the time needed for immobilized DNA sample preparation and restriction are considered too, 12–14 h, after cell harvesting, were required for fingerprinting, which is one-fifth or one half of the time needed when *Aeromonas* spp. or *V. cholerae* fingerprints are obtained according to previously reported protocols, respectively [10, 16–18].

The appearance of unresolved fragments has led to propose the use of multiple PFGE resolution windows [19–21], but various lengthy runs in CHEF chamber (29 or 18 h each for *Aeromonas* or *V. cholerae*, respectively) would make impractical outbreak investigations. In contrast, rapid DNA separations attained in mini-CHEF chamber permitted the design of at least three resolution windows for fingerprinting. Differentiations of the *Aeromonas* or *V. cholerae* isolates, analyzed in our work, were not improved by resolving DNA fragments in multiple resolution windows, but these windows permitted to detect greater numbers of bands (n1 and n2), and obtain more reliable estimates of band sharing frequencies (Table 3). Dendrograms and isolate relatedness calculations rely on Bsfl estimates [1].

Some interesting findings in this work were *A. hydrophila* and *A. trota* DNA had different band patterns in the resolutions windows (Fig. 1, lanes 3–4), suggesting the possibility of differentiating these species under the experimental conditions assayed in the mini-CHEF. Other findings were that strains of *V. cholerae* O1 El Tor and Classical biotypes were differentiated by *NotI* fingerprints (Fig. 2, lanes 1–4, 7), in agreement with results reported for other strains [19]. In addition, nonO1/non0139, O1, and O139 *V. cholerae* strains were differentiated too (Fig. 2). Unfortunately, the analyses of greater number of isolates are necessary to confirm these findings. Interesting, C7258 and 81 strains displayed identical *NotI* fragments (Fig. 2, lanes 2, 4), although the 81 strain was constructed by deleting the CTXφ prophage from the chromosome I of the C7258 [26, 27], and is unable to assemble the cell surface mannosensitive hemaglutinine [40]. On the contrary, the 638 strain, which is derived from *V. cholerae* 81 [26, 27] and was constructed inactivating the hemaglutinin protease gene of the 81 strain by inserting the endoglucanase A gene of *Clostridium thermocellum* [41], had a set of distinct *NotI* fragments (Fig. 2, lanes 1, 2 and 4, bands indicated with * and ○). However, these fragment differences are unrelated to the genetic manipulations. *V. cholerae* 638 is a candidate vaccine strain [42], and the identified polymorphism might be useful during vaccination campaigns.

In summary, the results presented here supported single-step incubation with NDSUPlus solution as a suitable protocol for rapid and nonexpensive preparation of immobilized DNA molecules from *Aeromonas* spp. and *V. cholerae* strains. Results also supported mini-CHEF as chamber suitable for resolving rapidly the restriction fragments band patterns and allowing the incorporation of multiple resolutions windows into fingerprints. The simplicity of sample preparation, the achievement of fast separation, and the possibility of bacterial subtyping in multiple resolution windows could make our fingerprinting procedure a useful tool in the control of infectious outbreaks caused by these pathogenic bacteria.

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5 References

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