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The Use of Pulsed Field Gel Electrophoresis in *Listeria monocytogenes* Sub-Typing – Harmonization at the European Union Level

Benjamin Félix, Trinh Tam Dao, Bertrand Lombard,
Adrien Asséré Anne Brisabois and Sophie Roussel
ANSES, French Agency for Food, Environmental and Occupational Health and Safety,
Maisons, Alfort Laboratory for Food Safety,
European Union Reference Laboratory for Listeria monocytogenes,
France

1. Introduction

Agarose gel electrophoresis is commonly used for separation of DNA molecules in molecular biology research and bacterial characterization in particular. It separates DNA fragments by size. It is widely used to detect PCR amplification products or determine DNA restriction genetic profiles. Consequently it is used in most of the bacteria characterization methods.

Pulsed Field Gel Electrophoresis (PFGE) has been widely applied to characterize numerous bacteria. PFGE is a form of RFLP typing in which the bacterial genome is digested with rare cutting enzymes. These restriction enzymes cut genomic DNA infrequently and thus generate a smaller number of DNA fragments (10-20 bands). These fragments of a wide range of sizes, from 20 kb to 10,000 kb (Herschleb et al., 2007), are separated using specialized electrophoresis techniques. Differences in the restriction profiles are used to carry out genetic comparisons among isolates. Computer-based analysis is simplified, enabling rapid and easy comparison on strains. Currently, PFGE is often considered the"gold standard" of molecular typing methods for bacterial foodborne pathogens such as *Salmonella*, *E.coli*, *Campylobacter*, *Yersinia*, *Vibrio* and *Listeria*.

The food-borne disease caused by *Listeria monocytogenes* (*L. monocytogenes*) is one of the main public health concerns in Europe (Allerberger & Wagner, 2010; EFSA, 2010; Goulet et al., 2008). Outbreaks and related clusters have to be detected as quickly as possible in order to improve the surveillance and control of this pathogen. Among the molecular methods used for sub-typing *L. monocytogenes*, PFGE has been widely applied to characterize food and human isolates over the last ten years (Brosch et al., 1996). Due to its high discriminating power and epidemiological relevance, this method has become the "gold standard" for *L. monocytogenes* sub-typing (Graves & Swaminathan, 2001).

One way to accelerate the recognition of clusters common to food and human isolates requires that significant number of isolates was sub-typed by laboratories involved in its surveillance. A standardized protocol was developed by the Center for Disease Control and Prevention (CDC) in Atlanta USA (PulseNet) and has been largely used at the international

level (Graves & Swaminathan, 2001). Several surveillance networks currently work throughout the world using this protocol (Gerner-Smidt et al., 2006; Pagotto et al., 2006). These networks have proven their efficiency for an early detection and a better understanding of *L. monocytogenes* outbreaks (CDC, 2010; CDC, 2011; Gilmour et al., 2010).

In Europe, in the frame of PulseNet Europe project, two PFGE sub-typing inter-laboratory trials were carried out in 2003 and 2006 (Brisabois et al., 2007; Martin et al., 2006). The resulting PFGE data demonstrated that PFGE profiles can be compared and exchanged between laboratories. However, PulseNet Europe has not been active since November 2006 due to a lack of funding (Swaminathan et al., 2006). Moreover, in the PulseNet Europe sub-typing inter-laboratory trials, only quality and interpretability of the profiles were assessed (Martin et al., 2006). Profile interpretation was not evaluated and remains difficult to standardize, in particular when dealing with a wide range of profiles including large bands, double peaks and uncertain bands.

PulseNet USA has developed standard operating procedures (SOP) for computer-assisted PFGE profile analysis using BioNumerics software (Applied Maths, Sint-Martens-Latem, Belgium) (Gerner-Smidt et al., 1998). The SOP has evolved toward an automated interpretation process. However, some steps still require the user to make critical decision during the analysis, in particular for (1) abnormal band assignment and (2) closely related profile interpretation. This crucial step is a major drawback of PFGE and its improvement remains a challenge for PFGE standardization (Gerner-Smidt et al. 2006).

In 2006, the ANSES Maisons-Alfort Laboratory for Food Safety has been designated European Union Reference Laboratory (EURL) for *L. monocytogenes*. It coordinates a network of 29 National Reference Laboratories (NRLs) representing 27 Member States as well as Norway. Most of them are in charge, amongst other tasks, of typing food, environmental and veterinary *L. monocytogenes* strains isolated at national level.

One of the EURL objectives was to harmonize PFGE protocols used by the European food NRLs. This article first describes, the principle of PFGE applied to *L. monocytogenes* and the relationship between a PFGE profile and bacteria's genetic make-up. It then explains the EURL SOP for interpreting PFGE profiles, based on PulseNet USA SOP. Finally, it focuses on the work undertaken by the EURL to stimulate NRLs to perform PFGE with a standardized protocol including an SOP for profile interpretation.

2. Principles of PFGE - Relatedness between PFGE profiles and genetic reality

The PFGE method starts with the extraction of the bacterial chromosomes without damaging to the DNA, by mean of a very gentle extraction procedure. The chromosomes are then restricted using a rare cutting enzyme. For *L. monocytogenes*, the enzymes are *Apa*I or *Asc*I (Carriere et al., 1991). These restriction enzymes *Asc*I and *Apa*I generate respectively between 6 to 12 and 14 to 17 fragments in the range of separation of the PFGE. The combinations of the profiles generated by the two enzymes are used to characterize the strains. A third profile generated by *Sma*I can be added to reinforce the analysis (Carrière et al. 1991)

The restricted DNA fragments are commonly separated in a PFGE CHEF (Contour-clamp homogeneous electric field) system (Chu et al., 1986). For *L. monocytogenes*, the range of separation is between 33 and 1135 kb. The migration parameters applied depend on the bacteria species. For *L. monocytogenes* the established parameters are a pulse angle of 120°

The Use of Pulsed Field Gel Electrophoresis in
Listeria monocytogenes Sub-Typing – Harmonization at the European Union Level
243

and a linear switch-time ramp of 4 to 40 s. These migration parameters have been standardized in the PulseNet USA protocol. The difference in the restriction profiles enables genetic comparisons among *L. monocytogenes* strains. Profiles are specific to each strain and are used as characterization data to identify them (Graves & Swaminathan, 2001). However the restriction profiles are merely an image of the genome structure and must be interpreted as such (Tenover et al. 1995).

3. Relationship between PFGE profiles and genetic reality

The PFGE profiles are composed of DNA fragments separated along the PFGE migration range. Bands actually consists a huge copy number of the same DNA fragment flanked by two restriction sites. However it often happens that one band is composed of several fragments of the same size but coming from different parts of the bacterial chromosome (Singer et al. 2003). This explains why band intensity can vary along the profile depending of the number of superposed fragments in the same band.

The numbers and positions of the bands on the gel determine which bands are different or identical between different strain profiles. The first interpretation procedure defined by Tenover et al. (1995) showed that the interpretation of the number of band differences between a pair of isolates is based on the minimum number of genetic mutational events that would result in the observed number of band differences. For example, two isolates that differ by two to three bands would be considered as closely related since a single genetic event can explain this difference. More recently, researchers of the USA CDC proposed that the "Tenover" criteria were not generally applicable for investigation of all foodborne outbreaks. Genetic transfer, superposition of bands and other artifacts which might affect the relatedness of the profiles and the interpretation must be taken into account when interpreting profiles. According to the new criteria adopted for *L. monocytogenes* one band of difference is considered to be significant for distinguishing between two profiles (Barrett et al., 2006). However, in practice, in spite of these criteria, the interpretation requires many subjective decisions. This subjectivity increases the variability of the profiles and, consequently, affects the way in which results are interpreted (Gerner-Smidt et al. 1998).

The burden imposed by PFGE implies the application of a highly standardized protocol for the performance, interpretation and exchange of PFGE profile between centers in Europe.

4. Standardized method developed by the EURL for PFGE sub-typing

4.1 PFGE protocol

The EURL PFGE protocol developed by EURL and standardized between NRLs is similar to the newly updated PulseNet USA (PN USA) PFGE standardized protocol (Halpin et al., 2009) with minor modifications. In the PN USA extraction protocol, the cell density per plug is lower than in the EURL protocol (0.9-1.0 OD 610 PN USA against 1.6-1.8 OD 600 EURL) and consequently in proportion to the cell density, proteinase K, lysozyme, Sodium Dodecyl Sulfate and other lysis buffer reagents are used at lower concentration. The lysozyme is prepared in a TE buffer (PN USA) instead of sterile water (EURL). Lysozyme incubation is undertaken at 56°C (PN USA) instead of 37°C (EURL). The amount of restriction enzyme is higher in the PN USA protocol than in the EURL one, for *Asc*I 0.125 U/μL (PN USA) instead of 0.100 U/μL (EURL), and for *Apa*I 0.250 U/μL (PN USA) instead of 0.100 U/μL (EURL).

The reproducibility of this protocol between European NRLs has been assessed already two times at the occasion of two inter-laboratory proficiency testing trials (PT trials) in 2009 and 2010. The results obtained were satisfactory. At this time 14 NRLs, representing 14 member states, have been assessed competent by the EURL for *L. monocytogenes* PFGE sub-typing.

4.2 Standard operating procedure for PFGE profile interpretation

This method is based on the interpretation method developed by Barrett et al. (2006) and the PulseNet USA PFGE profile interpretation SOP. It includes one band of difference as the limit to consider two PFGE profiles as indistinguishable as recommended in Barrett et al (2006). It includes a down limit for band interpretation at 33kbp, established according to EURL own experience and the conclusion drawn from the PT trial organized on *Salmonella* by Peters et al. (2006), and a new profile identification strategy based on database library organization (explained in detail below). This new strategy aims to reduce any artificial diversity generated by the operator's interpretation of the profile. Prior to any analysis of PFGE profiles the quality of the gel should be checked. This involves two steps: an assessment of the overall quality of the gel and an interpretation of the PFGE profiles. These two steps will be developed below.

4.2.1 Assessment of the overall quality of the gel

4.2.1.1 Visual interpretation

The gel should not contain background or debris which impedes interpretation of the image. Nevertheless, if only part of the image is degraded, the intact part of the gel can be analysed normally. PFGE profiles must be fully visible in order to be analysed. Gels with small spots can be interpreted if the image is first processed using image processing software to remove spots from the image. Gels must enable good contrast and should not contain any fuzzy fields that could impede the analysis. The gel should not exhibit any grossly incomplete restriction bands (figure 1). The expected number of bands must not exceed the range given in table 1. The most frequent problem with PFGE profiles is by far the apparition or disappearance of bands due to incomplete restriction of the DNA, as shown by Martin et al. (2006). This problem is most likely related to incomplete DNA restriction, which is often due to poor DNA quality. If this occurs, the contamination of reagents, buffers or purified water used during the extraction step, are the primary suspects.

Enzyme	Number of bands expected
*Asc*I	6 – 12
*Apa*I	14 - 17

Table 1. Number of bands expected for a PFGE profile of *L .monocytogenes* (Carrière et al., 1991)

It is sometimes difficult to detect incomplete restriction bands. They are detected when, in the upper part of the profile, the bands do not follow a descending order of intensity with respect to their molecular weight (figure 2). However an exception to this rule does not mean that the profile must be systematically rejected. Some incomplete restriction bands can be tolerated in a PFGE profile and criteria have been established for validating a profile carrying slightly incomplete restriction bands (figure 1 right).
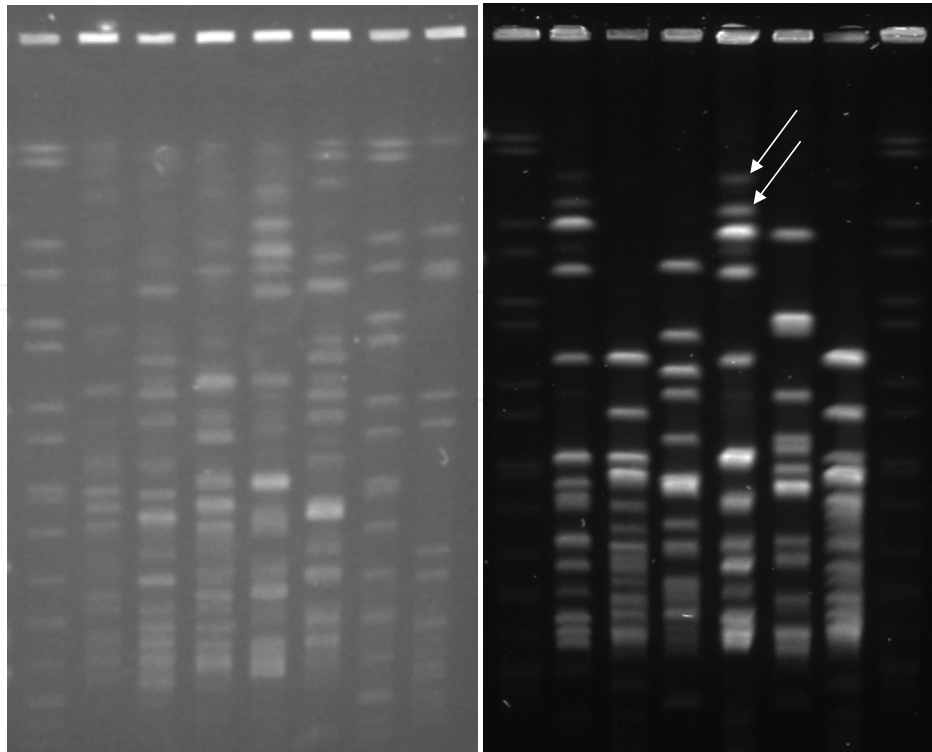
The Use of Pulsed Field Gel Electrophoresis in
Listeria monocytogenes Sub-Typing – Harmonization at the European Union Level

245

Fig. 1. Left side: *Apa*I restriction profile of *L. monocytogenes* with numerous incomplete restriction bands. Right side: *Apa*I restriction profile of *L.monocytogenes* with a few incomplete restriction bands (the white arrows indicate the doubtful bands)
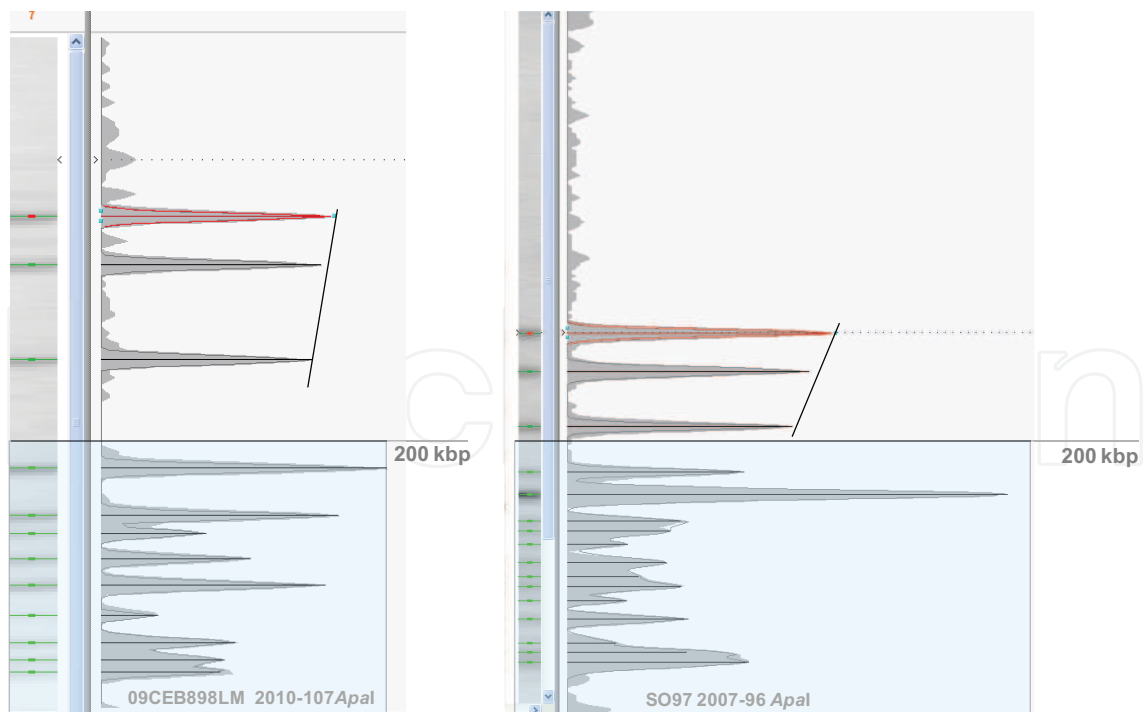

Fig. 2. Densitometric curves obtained from DNA migration profiles by mean of image processing software. The image shows two *Apa*I restriction profiles of *L. monocytogenes*. The bands in the upper part of the gel meet the criteria for arranging the upper bands by descending order of intensity.

4.2.1.2 Protocol for validation of doubtful bands in a PFGE profile

The PFGE profile should be analysed in two parts, separated by considering, first the upper part of the profile that contains the most intense bands, composed of long DNA fragments (between 200 and 1000 kb) and then the lower part of the gel which has smaller fragments (between 33 and 200 kb). In the upper part there is a low probability of bands overlapping since the bands observed in this area have a high molecular weight and are few in number. In the lower part of the gel band overlapping is more likely because there are more fragments and they have of low molecular weight. The validation protocol is only applied to the upper part of the profile. A 200 kb separation limit was decided upon for separating the upper part and lower parts of the profile. This limit was defined empirically according to the EURL database (1500 *Asc*I and *Apa*I *L. monocytogenes* PFGE profiles).

The validation protocol is based on an assessment of incomplete restriction bands relative to the average intensity of the profile's bands. Indeed, applied to the upper part of the profile, suspect bands (figures 3 left grey arrows) may be accepted if their intensity is less than 30% of the average intensity of the profile (figure 3 left) or above the average intensity value (figure 3 right). These limits were based on observation made on EURL PFGE profile database. Band below 30% of the average intensity resulted in a negligible incomplete restriction deviation and are tolerated. Band up to the average band intensity are not related to incomplete restriction and are accepted. The average intensity can be calculated from the band intensity values of the densitometric curve. Band intensity values can be calculated by any image processing software which features densitometric curve calculation from DNA migration profiles (e.i. see BioNumerics user manual). Rejected profiles are shown in figure 4.
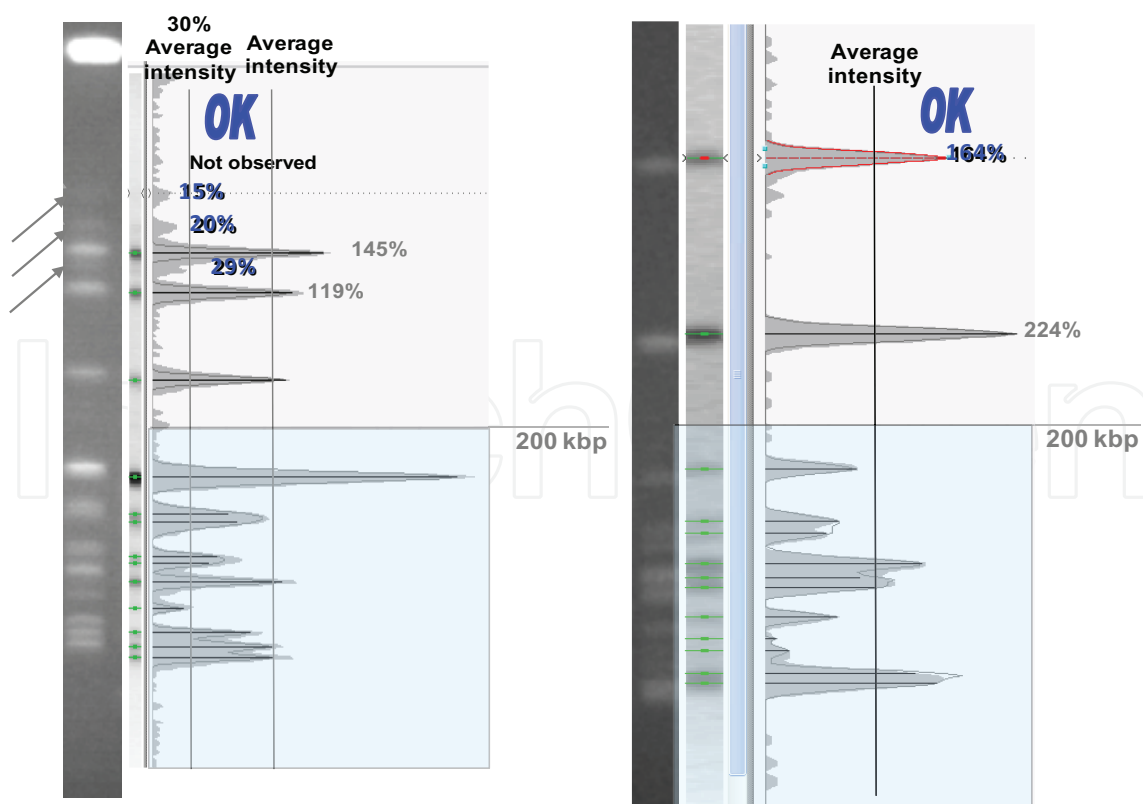

Fig. 3. *Apa*I restriction profiles of *L. monocytogenes*, carrying doubtfull bands, but which have nevertheless been accepted according to the validation protocol.
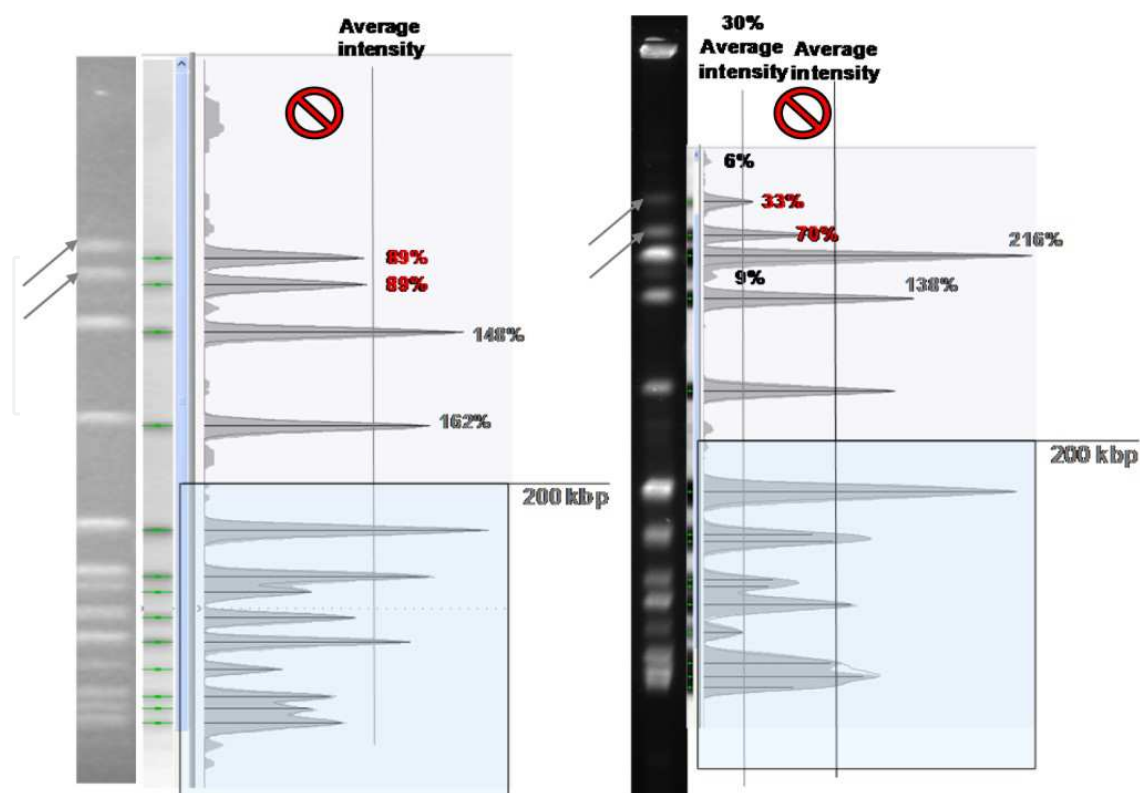
Fig. 4. *Apa*I restriction profiles of *L. monocytogenes*, carrying doubtfull bands, rejected according to the validation protocol

4.2.1.3 Use of controls to validate the different stages of the analysis

4.2.1.3.1 Reference system used

The *Salmonella Braenderup* (*S. Braenderup*) H9812 reference system was established by the USA CDC for PFGE of *L. monocytogene*s (figure 5 left) (Hunter et al., 2005). The former reference system, *L. monocytogenes* H2446 (figure 5 right), is still being used for extraction control by EURL, but only digested with *Asc*I see Table 2. In both cases the reference profiles have to be visible and conducive to interpretation (figure 5), i.e., it must be possible to position all their bands precisely and the intensity of the peaks should not be at the background level. The *Salmonella Braenderup* H9812 *Xba*I digestion product must frame the analyzed profile to allow an efficient normalization process and must be run in every six lanes. *L. monocytogenes* H2446 *Asc*I digestion product is loaded at the extreme left and extreme right of the gel. Moreover all set of controls must be applied to validate the reference systems as shown in Table 2.

4.2.1.3.2 Migration distortion analysis

Migration in the gel should not be distorted excessively in comparison to the standard reference system associated with the experiment in the normalization software (Here BioNumerics v6.5 Applied Maths, Sint-Martens-Latem, Belgium). For this purpose, the "Distortion bar" option of the BioNumerics software can be used (BioNumerics v6.0 user manual). Distortions are shown as colored bars (figure 6). Light colors (sky blue or yellow) indicate a lack of distortion with respect to the experiment's internal reference. Darker colors (red or bright blue) indicate a stronger distortion which may, however, be

compensated by the software. Black coloring indicates distortion which is too great to be counteracted by the software.
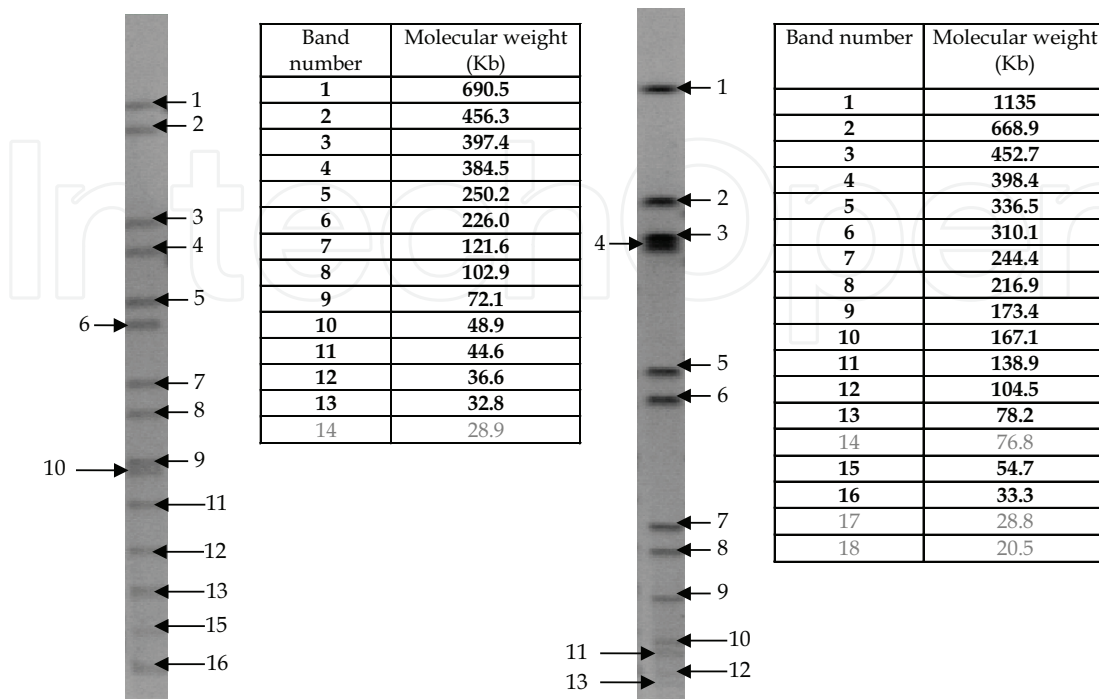

Band number	Molecular weight (Kb)
1	690.5
2	456.3
3	397.4
4	384.5
5	250.2
6	226.0
7	121.6
8	102.9
9	72.1
10	48.9
11	44.6
12	36.6
13	32.8
14	28.9

Band number	Molecular weight (Kb)
1	1135
2	668.9
3	452.7
4	398.4
5	336.5
6	310.1
7	244.4
8	216.9
9	173.4
10	167.1
11	138.9
12	104.5
13	78.2
14	76.8
15	54.7
16	33.3
17	28.8
18	20.5

Fig. 5. On the left, number of bands and molecular weight obtained for *S. Braenderup* H9812 after digestion with the *Xba*I enzyme. On the right number of bands and molecular weight obtained for *L. monocytogenes* H2446 after digestion with the *Asc*I enzyme. The black arrows show the bands taken into account for the analysis.

Type of control	Strain(s) used	Expected action
Extraction control	*L. monocytogenes* H2446 and *L. monocytogenes* H2446 strains test already extracted and validated during a former migration	Extract it with the other strains of the analysis. Verify that the quality of the profile is similar to the former extraction.
Control for restriction with the *Asc*I enzyme	*L. monocytogenes* H2446 from another extraction batch	Digest it with the extraction control to check *Asc*I restriction quality
Control for restriction with the *Xba*I enzyme	*S. Braenderup* H9812 from another extraction batch	Digest it with *S. Braenderup* H9812 chosen for the analysis to check *Xba*I restriction quality
Control for gel migration	*S. Braenderup* H9812 and *L. monocytogenes* H2446 strains validated during a former migration	Verify that all bands have migrated as in the former analysis
Control for analysis with gel processing software	*L.monocytogenes* H2446 already analysed under gel processing software	Verify that all bands can be processed as made in the former analysis

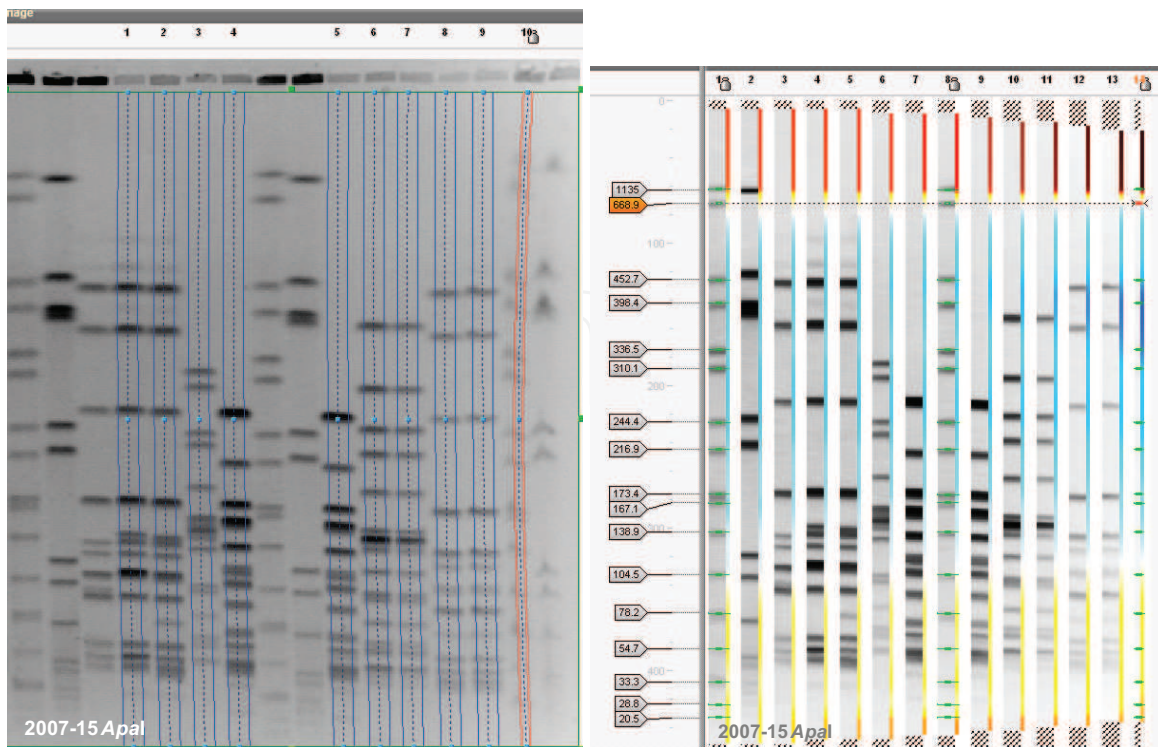Table 2. Controls used in the analysis of *L.monocytogenes* strains by PFGE.

Fig. 6. *S. Braenderup* reference system which migrated abnormally.

4.2.2 Interpretation of the PFGE profiles

4.2.2.1 Interpretation of profile saturated intensity area

If a profile contains saturation zones, it cannot be interpreted. To detect this type of anomaly, the densitometric curve of the profile's bands simply needs to be displayed via the densitometric curve calculation feature (e.i. see BioNumerics user manual). Saturated peaks are shown with their tips truncated (figure 7). No saturation can be accepted in a molecular PFGE profile.
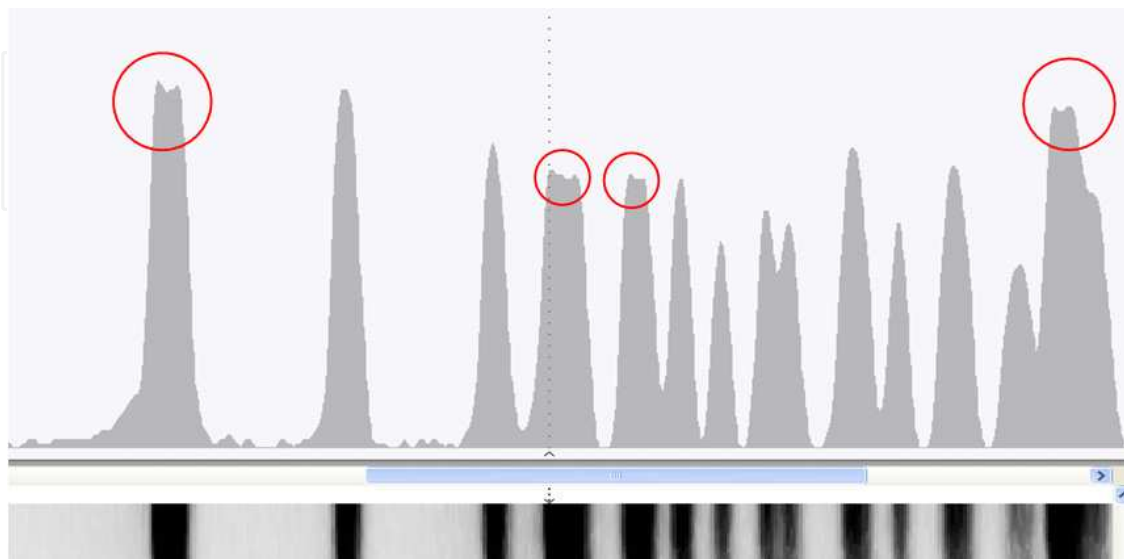
Fig. 7**.** PFGE profile with saturated bands (red circles).

4.2.2.2 Interpretation of molecular profiles

Because every signal is related to the presence of DNA in the gel, molecular PFGE profiles must be interpreted objectively, as shown below, with a band on every signal (the three examples in figure 8).
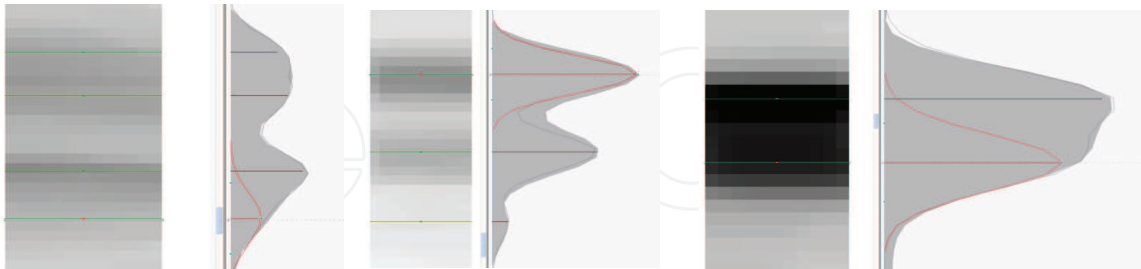
Fig. 8**.** Example of objective allocation of bands based on the densitometric curve of the profile.

4.2.2.3 Profile analysis protocol

The analysis begins with the marking of the bands found on the PFGE profiles, followed by the method developed by EURL to help operators to take band assignment decisions. As told before the profile identification strategy is based on the use of library identification. This method uses the whole database as a reference to assign profiles within a group of profiles similar at 90% (Also called library unit) and then allows the assignment of pulsotype number. A library is composed of several library units organized as follows. The percentage of similarity between two profiles within a library unit is calculated using the Dice coefficient, which depends on the number of bands that are common to both profiles. The determination of bands common to both profiles depends on two parameters, tolerance and optimization both set at 1% as recommended by PulseNet Europe (Martin et al., 2006). Profiles are grouped together according to the UPGMA (unweighted pair group method using arithmetic averages). This method allows profiles to be grouped according to their percentage of similarity. A library pools the profiles obtained with the same restriction enzyme according to the same PFGE protocol. For *L. monocytogenes* two libraries were created for *Apa*I and *Asc*I profiles.

The profile interpretation step starts following the assignment of the bands on the profile. The purpose of this step is to minimize the diversity within library unit by reducing artificial diversity generated by the operator's interpretation of the profile. The first step of the interpretation starts by the comparison of the new profile against all library units. The new profile will be included in the library unit with the nearest average profile. At this stage the operator has to respect the following library unit definition: (1) verify the homogeneity of the new profile with library unit content, (2) change the new profile to match with its assigned library unit as much as possible, (3) perform profile modification within the library unit limit (90% similarity within library unit components), (4) check that a band is always placed on a true signal and finally. The example detailed below shows how this method in applied.

In the case of the no. 17 library unit (figure 9), all the profiles have a strong signal in their central part marked by three bands (yellow rectangle). However, in some cases the shape of the signal does not enable three bands to be positioned with certainty. These bands are

called suspect signal bands (figure 10). Thus there are two distinct profile categories in this library unit, those which allow the easy positioning of these three bands (figure 11) and the other profiles carrying suspect signals. This is in this situation, that analysis by library of profile comes into play. In this example, all the suspect profiles will be marked with the same number of bands as the clearly marked profile, but only if the suspect profiles allow the positioning of three bands on their signal (see the question marks in figure 10).

Fig. 11: signal allowing an easy positioning of the bands

Fig. 10: suspect signal bands
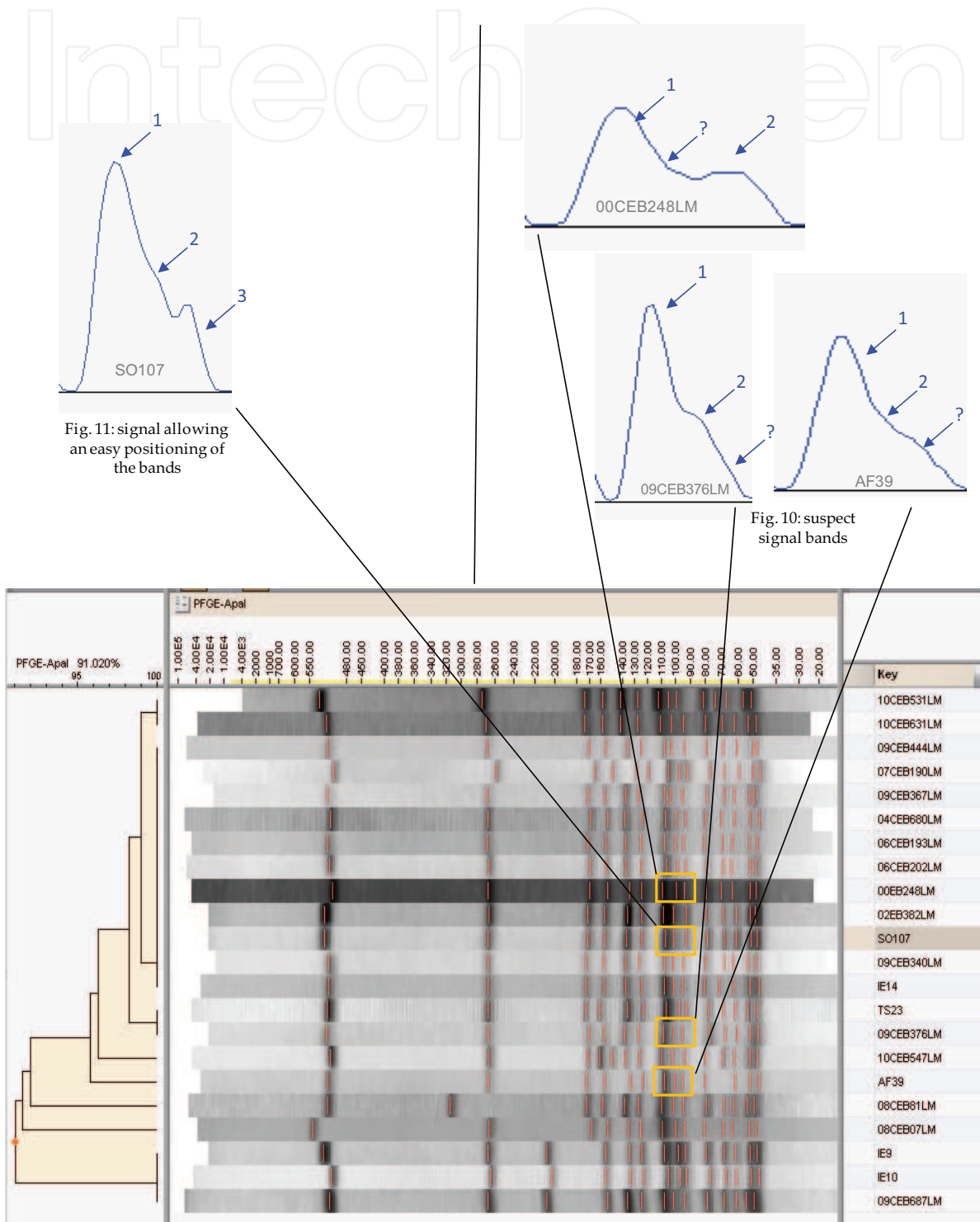
Fig. 9. *Apa*I library unit n°17 presented entirely as a PFGE profile comparison file.

If a profile has two clearly distinct bands in central position, and not three as for the other profiles of this library, these bands must be marked as they are and then depart from the library type profile. It will then be necessary to check that this profile is maintained clearly in the library and that it meets the library unit definition. In this example we focused on a part of the profile, however this method must be applied for every suspect signals.

Finally once the new profile has been included into the library unit it remains to be checked that there is no spanning between units 17 and another unit in the database (profile move from a library unit to another one). This verification can be made by marking the library unit on the global database dendrograms. This parallel organization of the database dendrograms and library unit allows the monitoring of database organization and integrity. The introduction of new profiles into the global database dendrograms can generate, time after time, changes in the organization of the UPGMA. These changes must be followed and checked on a regular basis (every three month at the EURL) to keep the library unit organisation consistent with the global database dendrograms. An automated script will be developed in collaboration with the software supplier to help the operator in this task.

5. Strengthening NRLs capacity for standardized sub-typing of *Listeria monocytogenes*

The EURL PFGE methods were dispatched to all NRLs. The laboratory has been certified by the French Accreditation Committee (COFRAC) for the PFGE methods since 2008 (accreditation no.1-2246, Section Laboratories, www.cofrac.fr). Annual workshops, including typing sessions, organized by EURL make stimulation of NRLs to perform PFGE. Since 2008, annual trainings has been organized by the EURL. Moreover, the EURL has organized PT trials in 2009 and 2010 to evaluate the ability of NRLs to perform conventional serotyping, molecular serotyping and PFGE. The PT trial would be renew on a regular basis. Next PT trial has been already paned for 2012.

6. Conclusion

The PFGE profile interpretation SOP is vital for the administration of a PFGE profile database. The published SOP deploys the process used by the curator to treat PFGE profiles. It could be followed by NRLs for their own local database organization. This SOP solves a problem caused by PFGE profile databasing, which is the introduction during the profile interpretation of an artificial diversity due to the operator in charge of the analysis. PT trials will be organized by the EURL on PFGE profile interpretation based on this SOP. The implementation of the SOP is part of the effort made by EURL to strengthen PFGE typing at European level. Once NRLs trained and evaluated on the SOP, it will be possible not only to share comparable PFGE profiles but also to share PFGE profiles normalized and marked. From the project naturally outcomes the implementation of a European PFGE database shared and filled in by the NRLs. The EURL database for *L. monocytogenes* food isolates (EURL Lm DB) was established in 2011 by EURL and is currently available for all NRLs. It enables to gather exhaustive typing and epidemiological information on *L. monocytogenes* strains circulating throughout the food chain across Europe.

7. Acknowledgements

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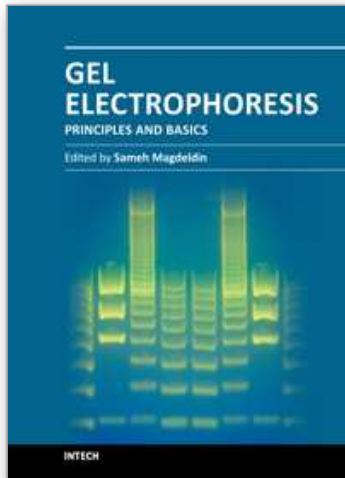
Gel Electrophoresis - Principles and Basics

Edited by Dr. Sameh Magdeldin

Most will agree that gel electrophoresis is one of the basic pillars of molecular biology. This coined terminology covers a myriad of gel-based separation approaches that rely mainly on fractionating biomolecules under electrophoretic current based mainly on the molecular weight. In this book, the authors try to present simplified fundamentals of gel-based separation together with exemplarily applications of this versatile technique. We try to keep the contents of the book crisp and comprehensive, and hope that it will receive overwhelming interest and deliver benefits and valuable information to the readers.

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