EU Reference Laboratory for *E. coli*

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Istituto Superiore di Sanità

Report of the 4th proficiency test (PT) for pulsed field gel electrophoresis (PFGE) typing of Verocytotoxin-producing *E.coli* (VTEC) strains (PT-PFGE4) – 2015

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1. INTRODUCTION

In 2012, the European Commission (EC) DG SANCO decided to organize the collection of typing data for isolates from food and animals, to improve the surveillance and trace-back of food-borne infections at the national, European and international level, as well as the preparedness to face foodborne outbreaks. The strategy of this molecular surveillance is described in the DG SANCO document "*Vision paper on the development of data bases for molecular testing of foodborne pathogens in view of outbreak preparedness*", available at the url: http://ec.europa.eu/food/food/biosafety/salmonella/docs/vision-paper_en.pdf.

The collection of data was focused on a restricted number of pathogens, namely Salmonella, *Listeria monocytogenes* and Verocytotoxin-producing *E. coli* (VTEC), and in 2012 the European Centre for Disease Prevention and Control (ECDC) begun a pilot collection of pulsed field gel electrophoresis (PFGE) profiles of strains isolated from human infections caused by these pathogens, within the framework of the Foodborne and waterborne diseases (FWD) surveillance network.

In January 2013, the European Food Safety Authority (EFSA) received a mandate from the EC to provide technical support for the collection of molecular typing results of food/animal isolates of Salmonella, *L. monocytogenes* and VTEC. According to the mandate, EFSA was requested to set-up and manage a database for molecular typing data from food and animal isolates of food-borne pathogens in collaboration with the relevant European Union Reference Laboratories (EU-RLs). The data would be submitted to EFSA by the Member States' National Reference Laboratories (NRLs) and the EU-RLs would act as curators of the data and would contribute to the data analyses.

To contribute to the preparedness of the NRLs for *E. coli* to submit VTEC PFGE profiles to the upcoming EFSA database, the EU-RL VTEC developed a laboratory training program on PFGE, organized a basic course on the use of the *BioNumerics* Software (Applied Maths NV, Sint-Martens-Latem, Belgium) for the analysis of PFGE profiles and implemented an external quality assessment (EQA) program to verify the capability of the NRLs to perform PFGE and the quality of the profiles produced. This EQA program was initiated in 2013 and PFGE typing was included in the proficiency-testing (PT) rounds on strain identification and typing organized since then by the EU-RL: PT10 (carried out jointly with the ECDC-FWD network), PT11 and PT13. The reports of the previous PFGE typing inter-laboratory studies (PT-PFGE1, PT-PFGE2 and PT-PFGE3) are available in the EU-RL website (http://www.iss.it/vtec), section Proficiency Tests. The results of the 4th study (PT-PFGE4) are reported and evaluated in this document.

2. OBJECTIVES AND DESIGN OF THE STUDY

To best accomplish the duty of the *E. coli* NRL network of contributing PFGE profiles of VTEC strains of food and animal origin to the upcoming European database managed by EFSA, the main purposes of this PT were:

- A further assessment of the level of preparedness of the NRL network with respect to the production of high quality PFGE profiles of *E. coli* strains, suitable for the inclusion in the upcoming EFSA database of molecular typing data.
- To identify the aspects of the process of molecular data production and analysis that still need improvement.

In addition, the PT allowed a first evaluation of the capability of the NRLs to carry out the band assignment on of their PFGE profiles using the *BioNumerics* software.

The study was conducted according to the International Standard ISO/IEC 17043:2010 "Conformity assessment – General requirements for proficiency testing".

3. PARTICIPANTS

A total of 28 laboratories, including 24 NRLs representing 22 EU Member States and the NRLs of Macedonia, Norway, Russia and Switzerland, joined the study. Each NRL received its own individual laboratory numerical code, which is reported in the result tables.

The NRLs participating in the study were:

- Austria, *Österreichische Agentur für Gesundheit und Ernährungssicherheit* GmbH
- Belgium, Scientific Institute of Public Health (also representing Luxembourg)
- Cyprus, Laboratory for the Control of Food of Animal Origin (LCFAO)
- Czech Republic, Veterinary Research Institute
- Denmark, National Food Institute, Technical University of Denmark
- Finland, Finnish Food Safety Authority Evira, Helsinki
- Finland, Finnish Food Safety Authority Evira, Kuopio
- France, *VetAgro Sup Campus Vétérinaire de* Lyon
- Germany, Federal Institute for Risk Assessment (BfR)
- Greece, National School of Public Health & Central Laboratory of Public Health
- Hungary, Food and Feed Safety Directorate, National Food Microbiological Reference Laboratory
- Ireland, Central Veterinary Research Laboratory, Department of Agriculture, Food and the Marine

- Italy, *Istituto Superiore di Sanità*
- Lithuania, National Food and Veterinary Risk Assessment Institute
- Macedonia, Faculty of Veterinary Medicine, Food Institute
- Netherlands, Food and Consumer Product Safety Authority (NVWA)
- Norway, Norwegian Veterinary Institute
- Poland, National Institute of Public Health-National Institute of Hygiene
- Poland, National Veterinary Research Institute
- Portugal, *Instituto Nacional de Investigação Agrária e Veterinária* (INIAV)
- Romania, *Institutul de Igiena si Sanatate Publica Veterinara*
- Russia, International State Research Center for Microbiology and Biotechnology, Obolensk
- Slovakia, NRC of Environmental Microbiology, Public Health Authority of SR
- Slovakia, Department of Food Hygiene, State Veterinary and Food Institute
- Slovenia, Veterinary Faculty/ National Veterinary Institute
- Spain, *Laboratorio Central de Veterinaria de* Algete (MAGRAMA)
- Sweden, National Veterinary Institute (SVA)
- Switzerland, Institute for Food Safety and Hygiene, University of Zurich

4. MATERIALS AND METHODS

4.1. Sample preparation

The test materials sent to the NRLs were constituted by 10 *E. coli* strains (samples 1 to 10). The isolates were the same that were sent for PFGE typing to the network of public health NRLs for VTEC referring to the Program for Food- and Waterborne Diseases and Zoonoses (FWD) surveillance of the ECDC, in the framework of their 6th external quality assessment (EQA-6) round for typing of VTEC, organized by the Statens Serum Institut (SSI), Copenhagen.

As for the stability of the samples, previous experiences supported the assumption that the time range between the preparation of the specimens and the deadline for submission of results was short enough to assure the stability of the PFGE profiles.

The test samples were prepared between 8 and 9 April. They consisted of freshly prepared bacterial cultures seeded into soft (0.3 %) nutrient agar in 2 ml glass vials. The cultures were incubated 18 hours at 37 °C ± 1 °C and labeled with randomly generated numerical codes (3 or 4 digits), different for each NRL. The homogeneity of the test strains was assessed on 10 April 2015 by testing two randomly selected sets of strains for the presence

of known microbiologic characteristics. The test samples were stored at room temperature until 20 April, when the samples were sent to the participating laboratories by courier.

The PFGE profiles of the test strains are shown in Figure 1 and were considered as reference profiles to evaluate the acceptability of those submitted by the NRLs.

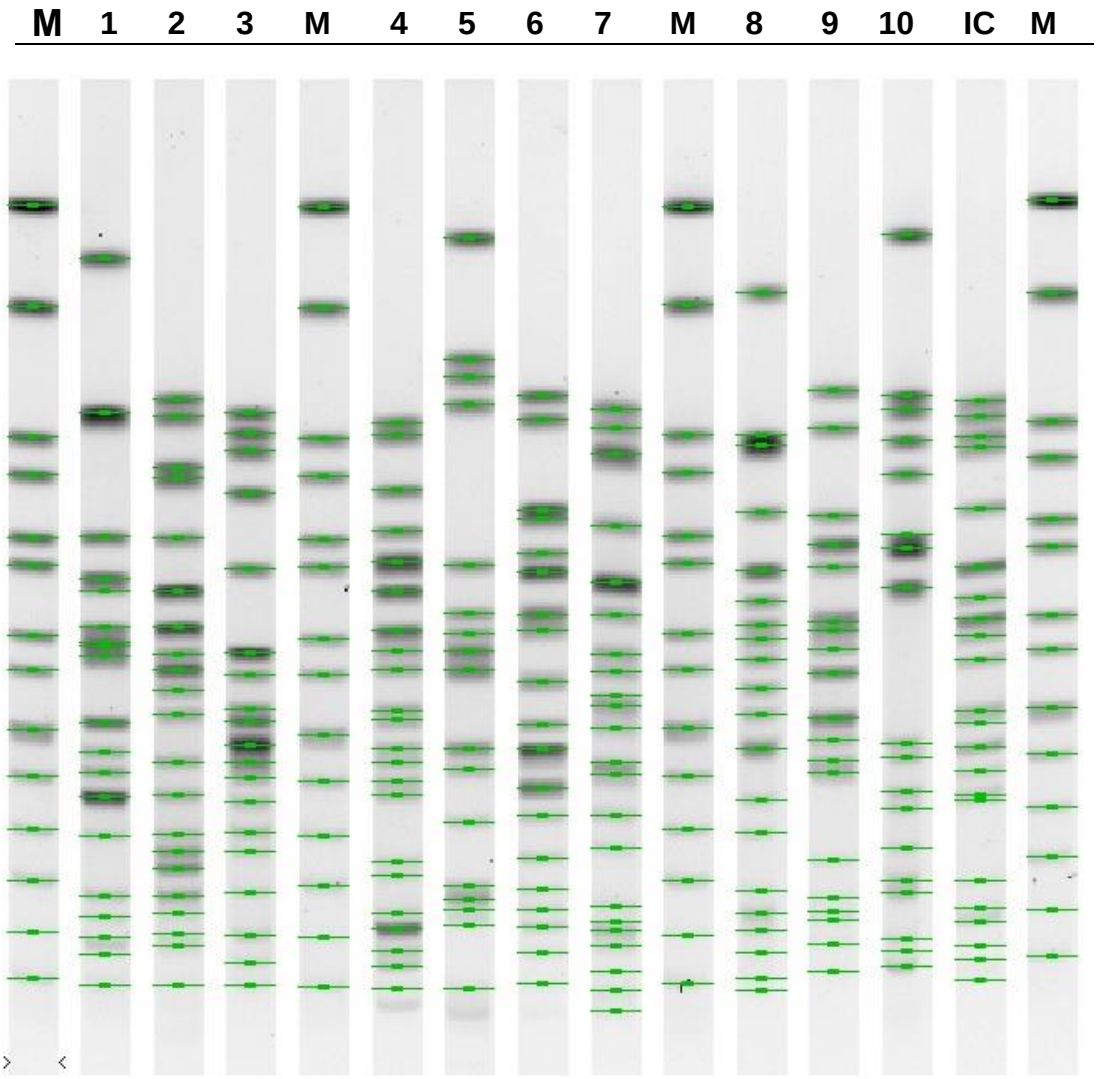


Figure 1: PFGE profiles of the *E. coli* strains included in the study. M: *S. braenderup* H9812 molecular weight standard.

4.2. Methods

4.2.1. Laboratory method for PFGE

The NRLs were requested to use the standard operating procedure for the production of PFGE profiles of VTEC recently published by EFSA (http://www.efsa.europa.eu/

en/supporting/doc/704e.pdf), which consists in an adaptation of the Standard PulseNet PFGE protocol for *Escherichia coli*, Salmonella, *Shigella sonnei* and *Shigella flexneri*, code: PNL05 (PulseNet International, 2013), with the electrophoresis conditions that are those specified for *E. coli* O157:H7 and *Shigella sonnei* strains, regardless the serotype of the test strains.

4.2.2. BioNumerics software analysis

The NRLs that were available to carry out this part of the study were requested to use the standard operating procedure for the profiles interpretation and curation published by EFSA (http://www.efsa.europa.eu/en/supporting/doc/704e.pdf).

4.3. Submission of the results

A detailed procedure for submitting the results was sent to the NRLs (**Annex 1).**

Briefly, the NRLs that did not make the *BioNumerics* analysis were requested to submit the PFGE gel images as non-compressed TIFF format files, together with a scheme of sample loading, using the Restricted Area of the Proficiency Test Section of the EU-RL website.

The NRLs that made the *BioNumerics* analysis were requested to submit the pictures of the PFGE gel images as non-compressed TIFF format files as well as the XML export files, prepared with the *BioNumerics* software and including normalization and band assignment.

4.4. Analysis of the submitted results: visual assessment of the gel images

The gel images submitted by all the NRLs were visually inspected to evaluate their suitability for the further computer-assisted analysis. The parameters used for this evaluation were:

- The position of the gel in the image: the gel should fill the entire window screen, without cutting off wells or lower bands, and the end of the gel must be visible.
- A correct identification of the samples, through the matching with the sample codes assigned to the each NRL.
- A correct positioning of the *S. braenderup* H9812 standard, which had to be loaded in lanes 1, 5, 10 when using 10-well gels or in wells 1, 5, 10, 15 when using 15-well gels. The correct position of the standard is of the utmost importance, because it allows the comparison of PFGE profiles from different gels.
- The focus of the gel image, with no over-exposure of the bands.
- The position of the lowest band of the standard, at 1-1.5 cm from the bottom of gel.
- The intensity of the bands, which should be approximately the same in each lane.

- The absence of unrestricted DNA.
- The bands should be clear and distinct all the way to the bottom of the gel; some band distortion can be accepted, but it should not interfere with the analysis.
- The gel background, which should be clear.
- DNA degradation, which should not be present.
- The resolution of the image file, which must be at least 8 bit in the color depth properties.

4.5. Computer-assisted analysis of the PFGE profiles: migration distortion analysis

The gel images that passed the visual inspection entered the instrumental analysis, which was carried out with the *BioNumerics* software, according to the standard operating procedures for PFGE profiles interpretation and curation recently published by EFSA (http://www.efsa.europa.eu/en/supporting/doc/704e.pdf).

The first parameter considered was the migration distortion analysis, which takes into consideration the distortion of the gel in comparison to the standard reference system associated with the experiment. When the 'Distortion bar' option of the *BioNumerics* software is applied, the level of stretching of each lane is translated into colored bars, as shown in Figure 2. Light colors (sky blue or yellow) indicate an acceptable level of distortion (Figure 2, panel A). Darker colors (red or bright blue) indicate a stronger distortion, which may, however, be compensated by the software (Figure 2, panel B). Black coloring indicates distortions too strong to be compensated by the software (Figure 2, panel C). The submitted profiles were considered as suitable for cluster analysis when the normalization distortion bars displayed clear colors (Figure 2, panels A and B) while those with black colors were rejected (Figure 2, panel C).

For the NRLs that submitted only the pictures of the PFGE gels as TIFF files, this analysis was carried out by the EU-RL.

For the NRLs that made the *BioNumerics* analysis, the EU-RL checked directly the XML files submitted. When problems (*i.e.* image area selection, assignment of the *S. braenderup* H9812 standard bands, background subtraction) were observed, the analysis was repeated by the EU-RL.

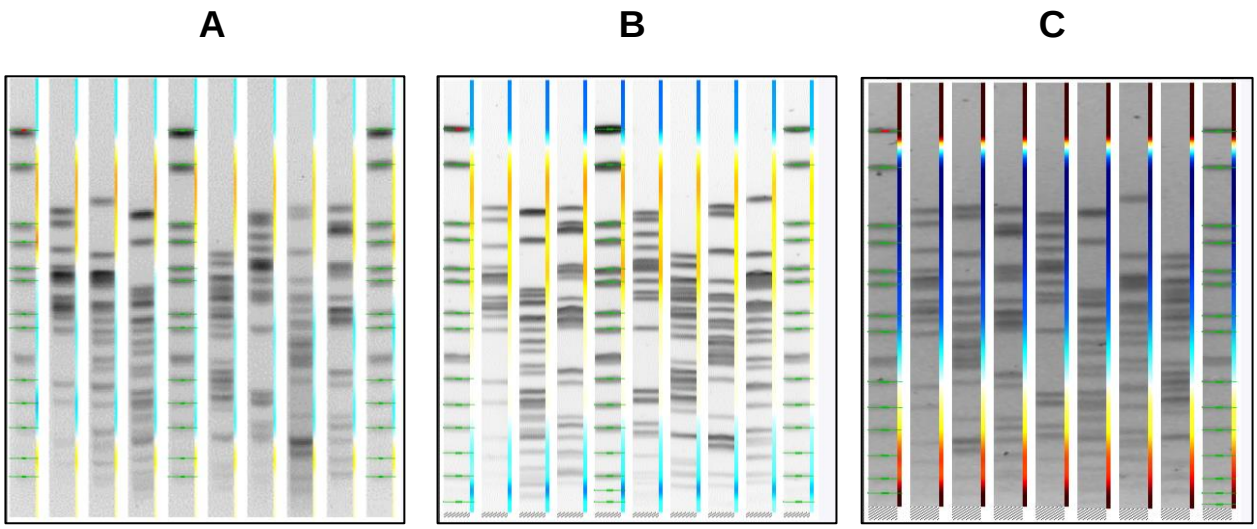


Figure 2. Examples of migration distortion analysis. Panel A: light colors, indicating an acceptable level of distortion. Panel B: darker colors, indicating a stronger distortion, which may be still compensated by the software. Panel C: black coloring indicates that distortions are too strong to be compensated by the software.

4.6. Computer-assisted analysis of the PFGE profiles: cluster analysis

The submitted images that passed the migration distortion analysis step entered the band assignment step, then the cluster analysis with the related reference PFGE profiles produced by the EU-RL was performed (Figure 1). The similarity between a submitted profile and the corresponding reference profile was calculated using the Dice coefficient, which depends on the number of bands that are common to both profiles, with tolerance and optimization parameters set at 1.5 %. A single profile was considered as acceptable for inclusion in a database when the cluster analysis returned a 97 % similarity and above. Profiles showing a similarity rate lower than 97 % were considered as “not accepted”.

For the NRLs that submitted only the pictures of the PFGE gels as TIFF files, both the band assignment and cluster analysis were carried out by the EU-RL.

For the NRLs that made the *BioNumerics* analysis, the band assignment made by the NRL was directly used for the cluster analysis. For the profiles for which the cluster analysis showed errors in the band assignment, the procedure was repeated by the EU-RL before evaluating the NRL’s performance.

4.7. Evaluation of the NRL performance

4.7.1. Evaluation of the PFGE profiles

The performance of each NRL in producing PFGE profiles suitable for inclusion in a database of molecular typing data was evaluated by the rate of not accepted profiles, according to the following scheme**:**

- **Excellent:** No rejected profiles
- **Good:** < 30 % of rejected profiles
- **Fair:** between 30 % and 60 % of rejected profiles
- **Poor:** > 60 % of rejected profiles

4.7.2. Evaluation of the capacity to carry out the BioNumerics analysis

For the NRLs that submitted the XML files, the capacity to correctly perform the *BioNumerics* analysis was assessed and the laboratories were assigned to categories from A to E, according to the following criteria**:**

- **A:** No modifications of the band assignment in the XML files were needed
- **B:** Only some modifications of the band assignment were needed
- **C:** Major modifications of the band assignment or complete re-assignment were needed
- **D:** Both normalization and band assignment had to be repeated
- **E:** The XML file was not usable for the cluster analysis

4.7.3. Individual reports

Each NRL received its own individual report with the performance evaluation, the critical assessment of the gel image and suggestions on how to improve the quality of the profiles, with respect to the specific points that generated underperformance. An example of individual report is presented as **Annex 2.**

5. RESULTS

A total of 28 NRLs joined the study, but only 26 submitted results.

Ten NRLs submitted only the images of the PFGE gels as TIFF files.

Sixteen NRLs submitted both the TIFF files and the XML export files, including normalization and band assignment.

5.1. Evaluation of the PFGE profiles submitted

The 26 PFGE gel images submitted were first evaluated by visual assessment (Paragraph 4.4): all of them passed this stage. At the following evaluation step of distortion bar analysis (Paragraph 4.5), three images presented distortions too strong to be compensated by the

software and were considered as not acceptable (Figure 3). The 230 profiles resulting from the accepted 23 images were further analyzed by cluster analysis with the related reference PFGE profiles produced by the EU-RL (paragraph 4.6). At the end of the evaluation process 193 PFGE profiles of the total 260 submitted (74.2 %) returned a 97 % similarity and above with the related reference profiles and were considered suitable for inclusion in a database of molecular typing data (Figure 3).

The similarity values obtained by the cluster analysis with the related reference PFGE profiles are shown in detail in Table 1.

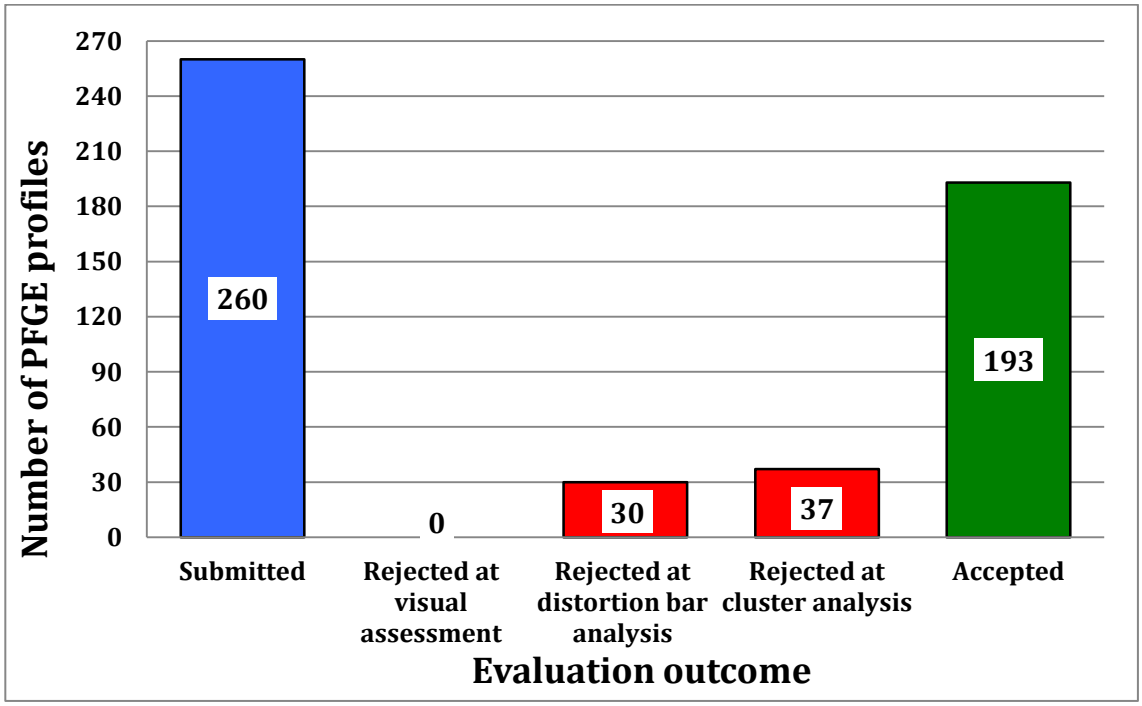


Figure 3. Three-step evaluation of the submitted PFGE profiles.

Table 1. Cluster analysis of the PFGE profiles submitted by the NRLs with the related reference PFGE profiles. Profiles were considered as acceptable when the cluster analysis returned a 97 % similarity and above. The green boxes indicate the acceptable profiles, the red boxes those considered as not acceptable. The numbers in the boxes indicate the % of similarity. NA: not analyzable (indicates that the quality of the profile was not suitable for cluster analysis).

NRL	Similarity (%) of the PFGE profile submitted by the NRL with the related reference profile for:									
	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5	Sample 6	Sample 7	Sample 8	Sample 9	Sample 10
L130	97.3	100	100	100	100	100	100	100	100	100
L140	97.3	100	100	100	100	100	100	100	100	97.1
L180	97.3	97.6	100	100	100	97.4	97.7	100	97.3	100
L204	97.4	100	100	97.8	100	97.4	97.7	97.4	97.4	97.1
L244	97.3	100	100	100	97.0	97.3	97.7	100	100	97.1
L257	94.4	100	100	100	97.0	97.3	100	97.4	97.3	97.1
L261	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
L285	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
L327	91.4	90.0	94.4	78.4	93.8	97.3	90.0	94.7	94.4	97.1
L420	100	100	100	100	100	97.4	100	97.6	100	97.3
L498	100	100	100	100	100	100	97.7	97.4	97.3	100
L528	100	100	100	100	100	100	100	100	100	100
L545	100	100	100	100	100	100	100	100	100	100
L546	94.7	100	100	100	100	97.4	97.8	97.6	100	100
L547	100	97.6	100	97.7	100	100	97.8	100	97.4	97.3
L568	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
L574	94.7	100	100	97.8	97.1	97.4	97.8	95.2	97.4	97.3
L615	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
L627	94.4	97.6	100	100	97.0	100	97.7	97.4	97.3	100
L658	100	100	100	100	100	100	97.8	100	100	100
L714	94.4	95.0	100	90.0	97.0	97.3	97.7	97.4	97.3	97.1
L782	94.7	100	100	100	100	100	97.7	97.4	100	100
L831	100	100	100	100	100	100	100	100	100	100
L849	100	100	100	100	100	100	97.7	100	100	100
L968	97.3	100	100	100	97.0	100	100	100	100	100
L997	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA

Twenty-one NRLs submitted suitable PFGE profiles, while five NRLs submitted images from which none of the profiles could be accepted. For three of them the profiles could not be evaluated by cluster analysis due to the excessive distortion (Figure 3). For L261 and L285 the cluster analysis was carried out but all the profiles returned similarity rates lower than 97 % (Table 1).

The number of suitable profiles provided by each NRL ranged from 10 to 2, with 14 NRLs having all their profiles accepted (Figure 4).

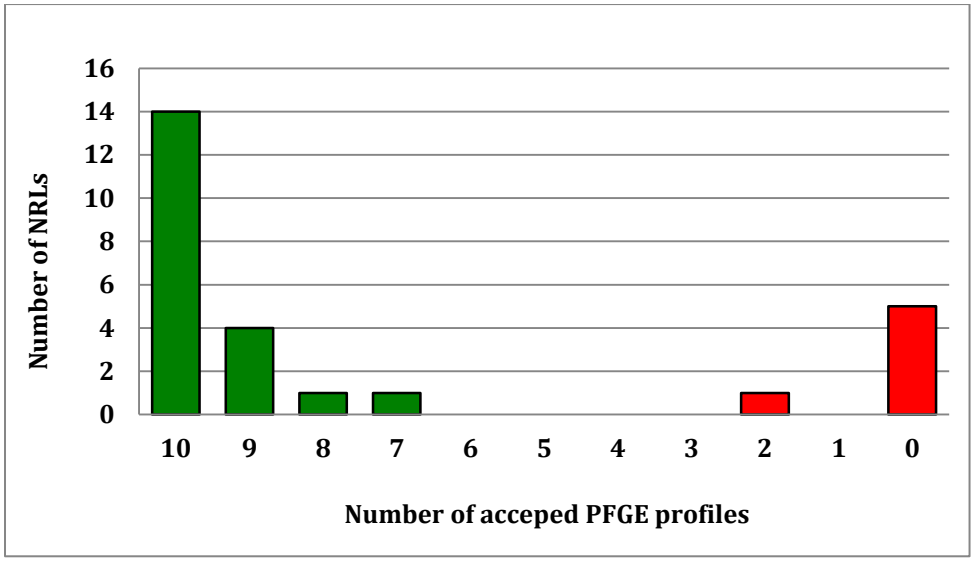


Figure 4. Number of suitable PFGE profiles, by number of NRLs. The red bars indicate the NRLs whose performance was considered as “poor”.

The performance of each NRL in producing PFGE profiles suitable for inclusion in a database of molecular typing data was evaluated according to the criteria described in section 4.7.1., on the basis of the rate of PFGE profiles considered as suitable.

Figure 5 shows the score obtained by each NRL and Figure 6 the number of NRLs grouped according to their score. The performance was classified as “poor” for 6 NRLs (23 %), as “good” for 6 NRLs (23 %) and as “excellent” for 14 NRLs (54 %).

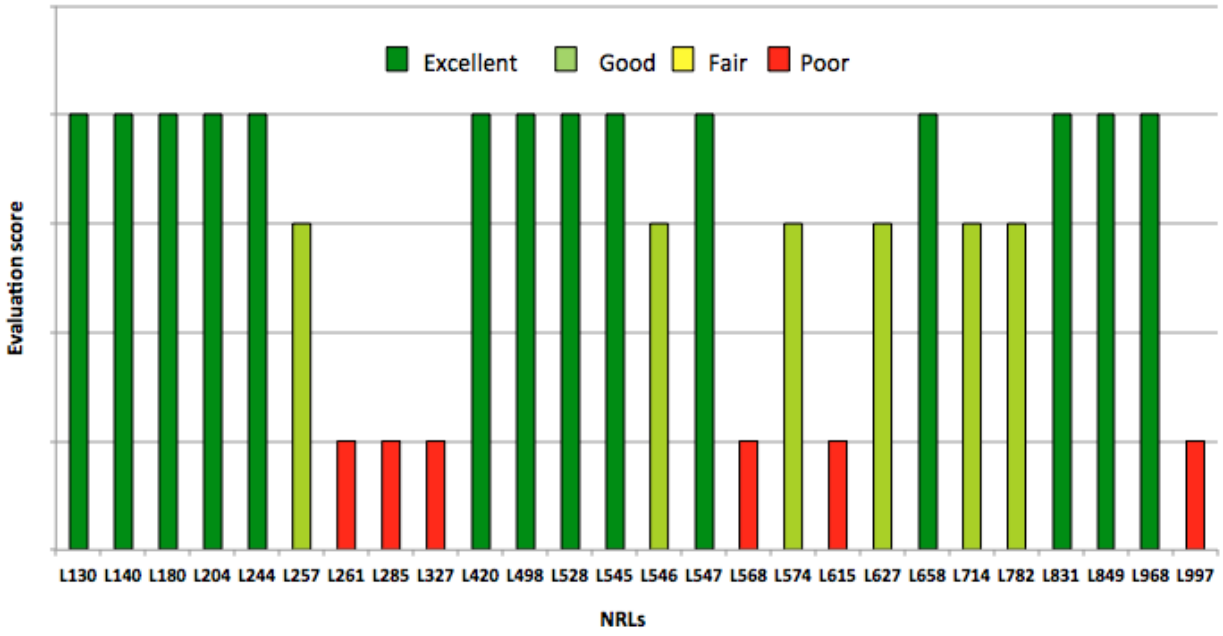


Figure 5. Evaluation of the performance of each NRL in producing PFGE profiles. The red bars indicate the NRLs whose performance was considered as “poor”.

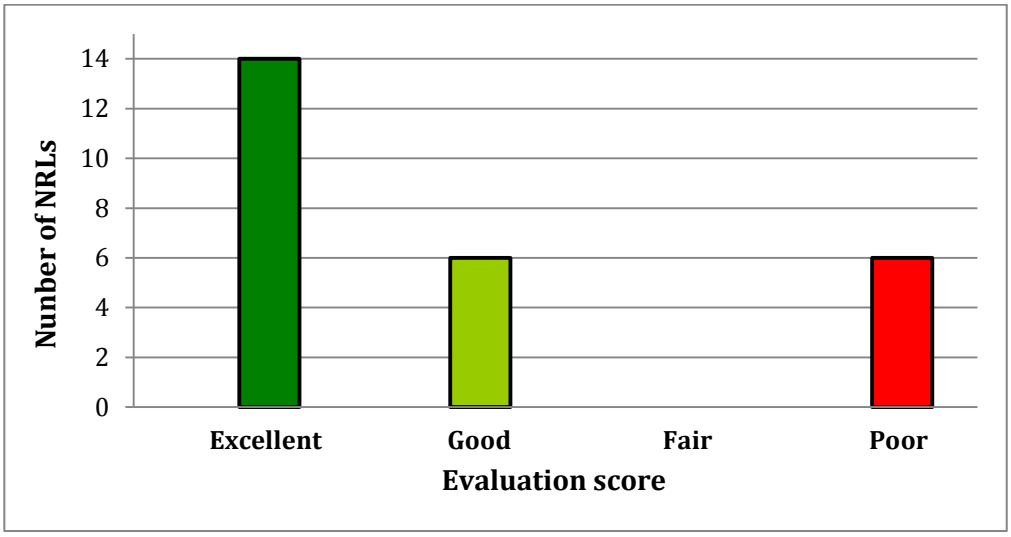


Figure 6. Evaluation of the NRL performance in producing PFGE profiles. The red bars indicate the NRLs whose performance was considered as "poor".

The problems most commonly encountered by the NRLs in producing PFGE profiles are shown in Figure 7.

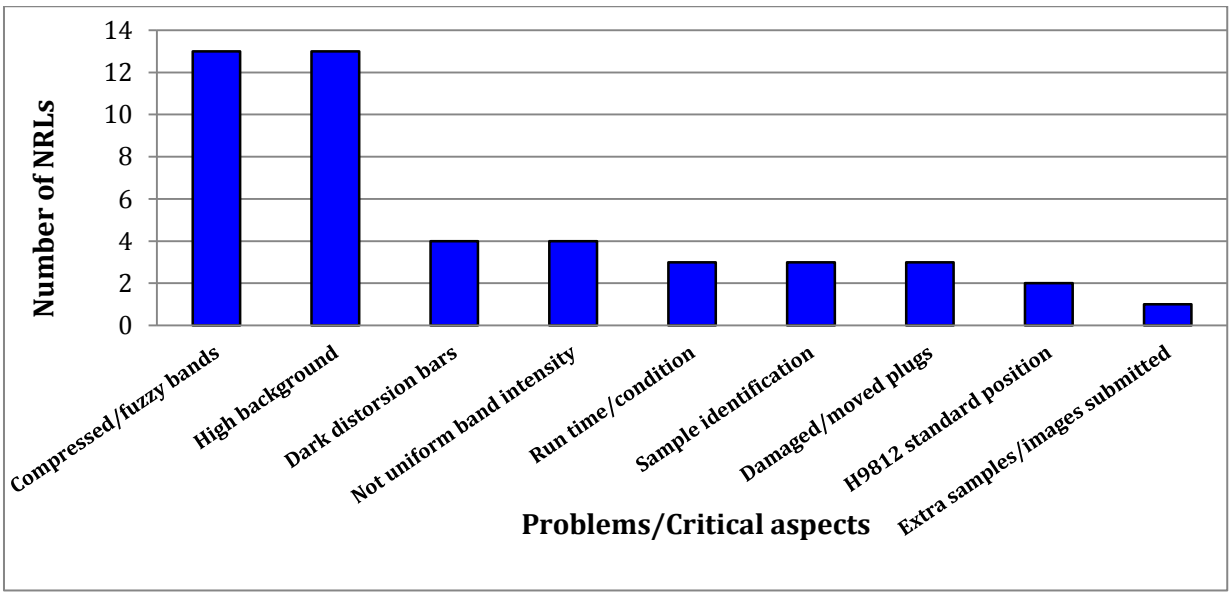


Figure 7. Technical problems most frequently encountered by the NRLs in PFGE profile production.

The most frequent problems were related with the gel background and the presence of bands that appeared fuzzy and/or compressed in the central part of the lanes. Each NRL received proper advice to overcome its specific problems in the individual report (**Annex 2**).

The rate of suitable profiles varied with the *E. coli* strain tested and ranged from 100 % of strains 6 and 10 to 67 % of strain 1. Figure 8 shows the rate of accepted profiles by single strains, carried out considering the profiles submitted to cluster analysis.

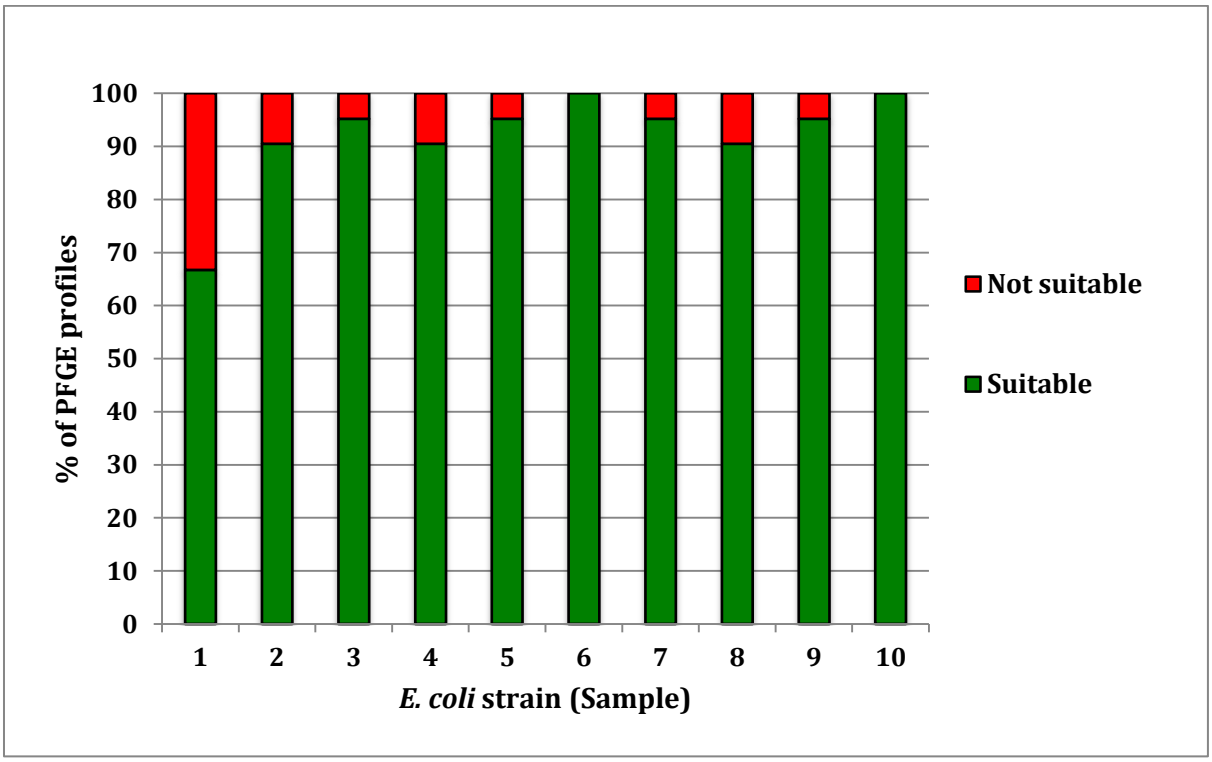


Figure 8. Rate of acceptable PFGE profiles submitted by the NRLs, by *E. coli* strain. The evaluation was carried out considering the profiles that could be submitted to cluster analysis.

5.2. Evaluation of the capacity of the NRLs to carry out the *BioNumerics* analysis

The capacity of the NRLs to carry out the *BioNumerics* analysis was evaluated on the basis of the modifications required by their XML files and the 16 NRLs that carried out the analysis were categorized according to the criteria described in section 4.7.2. The category assigned to each NRL is shown in Figure 9, while Figure 10 shows the number of NRLs grouped in each category.

Five NRLs (31 %), fell into categories A and B, showing a good capacity to analyze PFGE profiles with the *BioNumerics* software.

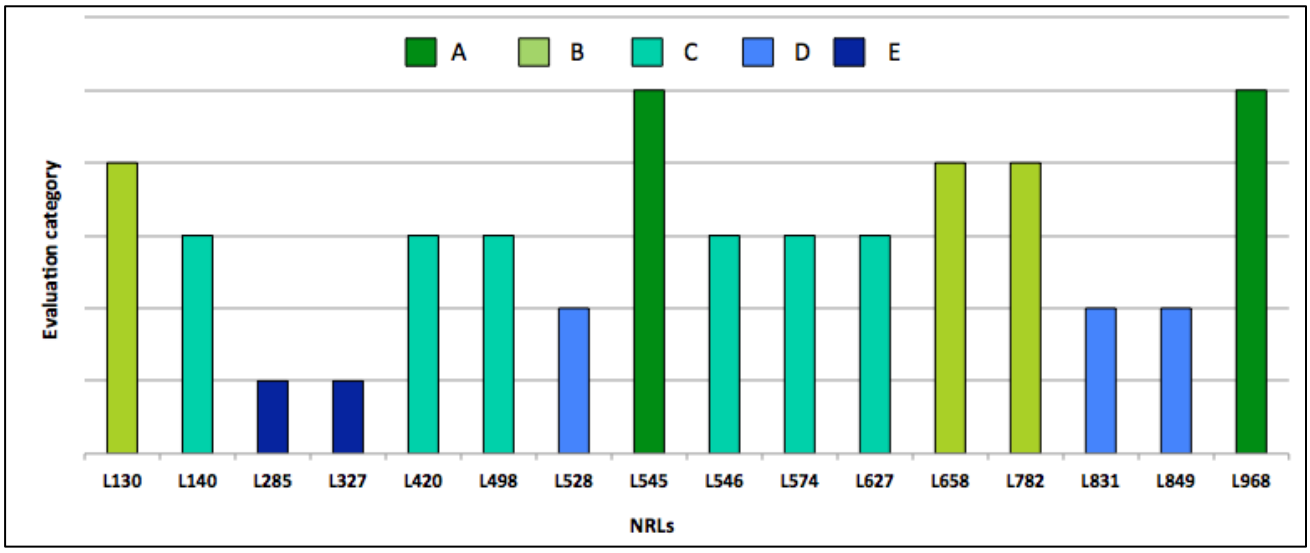


Figure 9. Evaluation of the capacity of the NRLs to carry out the *BioNumerics* analysis. Categories are defined in section 4.7.2.

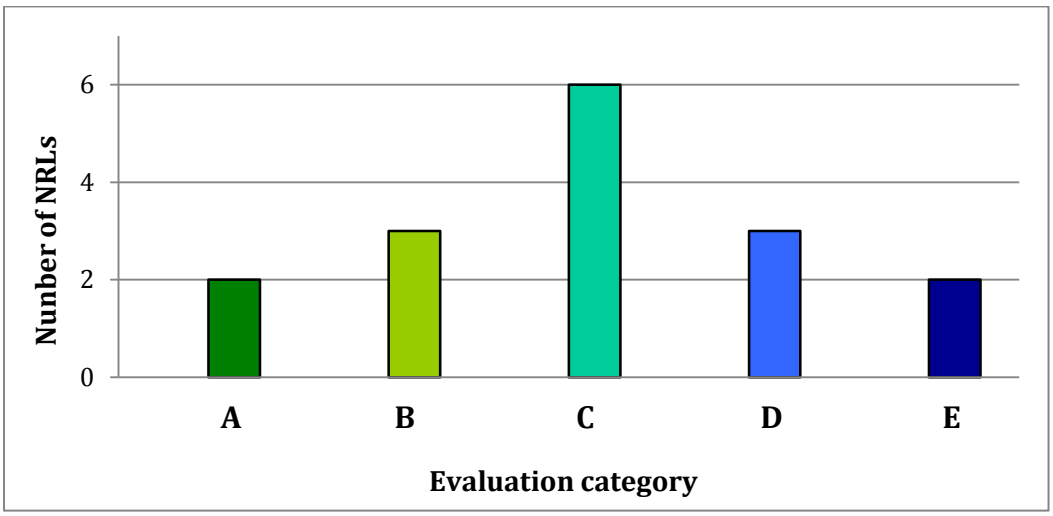


Figure 10. Evaluation of the capacity of the NRLs to carry out the *BioNumerics* analysis. The NRLs that submitted XML files are grouped by category. Categories are defined in section 4.7.2.

6. CONCLUDING REMARKS

PFGE typing of VTEC and other pathogens has greatly contributed to the surveillance of foodborne infections. However, PFGE typing is a complex laboratory procedure, potentially affected by several troubles, and the establishment of a network of laboratories producing PFGE profiles to be uploaded in a common database requires an EQA program to continuously monitor the capability of the laboratories to produce high quality PFGE profiles. Therefore, the EU-RL VTEC has set up a program of EQA for the *E. coli* NRLs of EU and EEA Member States, in the framework of the upcoming collection of PFGE typing data on food and animal isolates of VTEC, coordinated by EFSA.

The set of *E. coli* strains sent for PT-PFGE4 was the same used in the 6th external quality assessment round for typing of VTEC referring to the Program for Food- and Waterborne Diseases and Zoonoses (FWD) surveillance of the ECDC. The use of the same test strains in both the EQA programs will facilitate the harmonization of the procedures used within the two NRL networks and the forthcoming comparison of PFGE profiles of VTEC strains isolated from human and non-human sources.

A total of 28 laboratories, including 24 NRLs representing 22 EU Member States and the NRLs of Norway, Switzerland, Macedonia and Russia, joined this 4th PT.

The results were submitted by 26 laboratories and their evaluation was carried out keeping in mind the objective of the EQA program: to verify the capability of the NRLs to provide PFGE profiles to be included in the upcoming EFSA database of molecular typing data. Therefore, the performance of each NRL was evaluated according to the number of PFGE profiles provided that were considered as suitable for the inclusion in the database and for the comparison with the PFGE profiles of VTEC strains from human infections, according to the standard operating procedures for PFGE profiles interpretation and curation recently published by EFSA (http://www.efsa.europa.eu/en/supporting/doc/704e.pdf).

A step-wise evaluation process was applied:

1. At the first evaluation by visual assessment, all the PFGE images submitted were considered as suitable for further analysis.
2. Three images did not pass the second step of the migration distortion analysis.
3. The images provided by 23 NRLs were subjected to cluster analysis with the related reference PFGE profiles.
4. Of the total 260 profiles submitted, 193 (74.2 %) provided by 21 of the 26 NRLs (81 %) were considered suitable for inclusion in a database of molecular typing data.

As a whole, 14 NRLs (53.8 %) submitted profiles that were all accepted and their performance was considered as “excellent”, while, on the contrary, 6 NRLs (23.1 %) had more than 60 % of their profiles that were considered as not suitable and their performance was considered as “poor”.

The problems more frequently encountered by the NRLs in producing good quality PFGE profiles were those related with the gel background and the presence of bands that appeared fuzzy and/or compressed in the central part of the lanes. Specific suggestions and advice to overcome such problems were provided to each NRL in the individual report.

The rate of accepted profiles also depended on the *E. coli* strain tested, ranging from 100 % for strains 6 and 10 to 67 % for strain 1. This highlighted that the electrophoresis run conditions used, those developed for VTEC O157, might not be optimal for some VTEC strains belonging to other serogroups.

PT-PFGE4 also provided the opportunity to carry out a first evaluation of the capacity of the NRLs to carry out the normalization and band assignment of their PFGE profiles using the *BioNumerics* software. Sixteen NRLs made such analysis on a voluntary basis and submitted the resulting XML files, which were used by the EU-RL to carry out the profile evaluation and, when needed, were modified for the purpose. An evaluation of the capacity of the NRLs to carry out the *BioNumerics* analysis was based on the extent of modifications required by their XML files for a correct analysis. Such an evaluation showed that at least five NRLs have at the moment a satisfactory capacity to analyze PFGE profiles by using the *BioNumerics* software.

In conclusion, most of the PFGE profiles provided by the NRLs participating in this 4thPT on PFGE typing of *E. coli* showed a quality that was suitable for inclusion in the upcoming EFSA database of molecular typing data on VTEC strains isolated from food and animals.

Annex 1: Laboratory Procedure

EU Reference Laboratory for *E.coli*

Department of Veterinary Public Health and Food Safety

Unit of Foodborne Zoonoses

Istituto Superiore di Sanità

4th inter-laboratory study on Pulsed Field Gel Electrophoresis typing of *E. coli strains* (PT-PFGE4)

Laboratory Guideline

The 4th round of proficiency testing on PFGE typing (**PT-PFGE4**) will be carried out in view of the upcoming collection of molecular typing data on VTEC of food and animal origin, coordinated by EFSA.

The **objectives** are:

- A further evaluation of the capability of the NRLs to produce PFGE profiles suitable for the inclusion in the upcoming EFSA database of molecular typing data.
- The assessment of the capacity of NRLs to analyze PFGE gels and to assign the profile's bands using the BioNumerics software.

1. Test materials

The study will be carried out on a set of 11 *E. coli* strains, including 10 test strains and a VTEC O157 strain to be used as internal control (IC) in each gel, in addition to the *S. braenderup* H9812 standard.

The test strains are the same recently sent for PFGE typing to the network of public health NRLs for VTEC referring to the Program for Food- and Waterborne Diseases and Zoonoses (FWD) surveillance of the European Center for Disease Prevention and Control (ECDC), in the framework of their ongoing 6th external quality assessment (EQA-6) scheme for VTEC typing.

The test strains will be sent as bacterial cultures seeded into soft (0.3 %) nutrient agar in glass vials, labeled with numbers from 1 to 10. The parcel will also include the reference VTEC O157 strain ED398, to be used as additional IC in each gel in addition to the *S. braenderup* H9812 standard (see point 2). The *S. braenderup* H9812 will also be included.

The strains are provided as freshly seeded stab agar. They can be stored at room temperature and are stable for up to three weeks since the date of receipt. Therefore, the strains must be passaged before that date and the sub-cultures should be stored frozen after the addition of crio-preservatives.

These *E. coli* strains may **produce Verocytotoxin** and, for **safety** reasons, should be handled accordingly.

2. PFGE conditions.

PFGE will be carried out using the standard operating procedures for the production of PFGE profiles of VTEC recently published by EFSA (http://www.efsa.europa.eu/en/supporting/doc/704e.pdf).

According to this EFSA SOP, the additional IC must be included in each gel in the lane preceding that of the last *S. braenderup* H9812 standard placed on the right side of the gel.

The sample loading scheme to be used for this PT is shown in the Table below.

Number	**Wells**														
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**
Strain	*S.braend. H9812*	1	2	3	*S.braend.* H9812	4	5	6	7	*S.braend*. H9812	8	9	10	ED398	*S.braend.* H9812

3. Submission of the results

The NRLs are requested to submit the PFGE profiles both as TIFF files and XML export files. The files must be uploaded in the restricted area of the Proficiency Test section of the EU-RL web page (https://www.iss.it/site/pt_CRLEC/login.aspx). **The log-in instructions will be sent later on.**

The TIFF file will be named as: **Username_Lcode_PT-PFGE4 (e.g. EC_Italy_ISS_Lxxx_PT-PFGE4).**

The NRLs that don't have the BioNumerics software will upload the TIFF image and will provide details on the equipment and laboratory reagents used to produce the PFGE profiles by filling in the xls file provided together with this Procedure, named *PFGE_details*.xls. The xls file will be renamed by each NRL as Username_Lcode_PT-PFGE4 (e.g. EC_Italy_ISS_Lxxx_PT-PFGE4) and uploaded together with the TIFF file.

The NRLs that have the BioNumerics software will analyze the TIFF image by themselves. Then they will upload both the TIFF file and the XML file created by the BioNumerics analysis. For this purpose, an "*ad hoc*" database must be created in BioNumerics. This

database will be named **E coli_EQA_EURLtest_Username** (e.g. E coli_EQA_EURLtest_ EC_Italy_ISS). An XML file provided by the EU-RL together with this procedure will be used to properly set up the database.

After the analysis, the generated XML export file will be uploaded in the restricted area as a zip file, together with the TIFF file. This process is described in the following paragraphs, where two procedures are reported: one (**3.1**) describing the use of BioNumerics versions up to 6.6, and one (**3.2**, **page 11**) the use BioNumerics versions 7 and more.

NOTE: ONLY ONE FILE FOR EACH TYPE CAN BE UPLOADED. THE SYSTEM WILL AUTOMATICALLY REPLACE THE EXISTING FILES WITH THE NEWLY UPLOADED FILES WITH THE SAME EXTENSION.

3.1. Procedure for importing XML files using BioNumerics version up to 6.6

1. Set up a new empty database in your BioNumerics and name it as **E coli_EQA_EURLtest_Username** (e.g. E coli_EQA_EURLtest_ EC_Italy_ISS). Do not use any of your existing databases.
2. Download the XML file (corresponding to the BioNumerics version 6, named as "xml_BN6") prepared by the EU-RL. Unzip it and save the XML files in your computer and take note of the location.
3. Import these XML files into the E coli_EQA_EURLtest database. This action will import all the setups needed for a proper setting of the database.
   All the steps of the procedure are described in detail below.

- In your empty database, add the XML Tools plugin, by selecting the plugin in the list (from the file menu) and press "*Install*".
- Select the "*Import entries from XML*" from the file menu.
- Locate your newly unzipped files. Select all of them and click "*Open*".
- Mark the box "*Overwrite experiment settings*" And click *OK*.
- Restart the database.
- Your database should have this aspect.

In the *database entries* window (on the left) you can find all the fields that **must be filled** for each sample (see as example the above figure – NOTE the Strain_Name field). In the *experiment type* windows (on the right) you can also find the PFGE_XbaI experiment.

The database is now ready for the analysis of the PFGE gel image.

Remember to rename your gel as: **Username_Lcode_PT-PFGE4** (e.g. EC_Italy_ISS_Lxxx_PT-PFGE4).

After the analysis, the XML file will be created following the procedures described in the next paragraph.

3.1.1. Exporting XML files from BioNumerics version up to 6.6

1. Analyze your gel by using the *S. braenderup* H9812 setting imported (remember to add all the lanes to the database).
2. Fill in all the fields requested.
3. Select the entries that must be exported as XML file in the main window.
4. Click on export selection as XML from the file menu.
5. Export the TIFF file.
6. Make a compressed folder of all these files, name it as: Username_Lcode_PT-PFGE4 (e.g. EC_Italy_ISS_Lxxx_PT-PFGE4) and upload it in the restricted area of the Proficiency Test section of the EU-RL web site; also the TIFF file of the gel shall be uploaded, in a second upload.

All the steps of the procedure are described in detail below.

- Add all lanes to the data base.
- Go to the main window and select all the lanes that must be exported, select "*export selection as XML*" from the file menu.
- Select "*export all fingerprint types*", make sure that all the experiments and all the fields are marked, then click *OK.*
- Export the TIFF files by selecting "*export tiff files for selected entries*" from the file menu.
- Select the experiment to be exported (*PFGE_XbaI*)
- Remember to take note of the location of these files: they should be located in the *export* folder.
- Select all the files and compress them as zip file.
- Now you can upload all the compressed files in the restricted area in the Proficiency Test Section of EU-RL web page.

3.2. Procedure for importing XML files using BioNumerics version 7

1. Set up a <u>new empty database</u> in your BioNumerics and name it as **E coli_EQA_EURLtest_Username** (e.g. E coli_EQA_EURLtest_ EC_Italy_ISS). Do not use any of your existing databases.

2. Download the XML file (corresponding to the BioNumerics version 7, named as "xml_BN7") prepared by the EU-RL. Unzip it, save the XML files in your computer, and take note of the location.
3. Import these XML files into the E coli_EQA_EURLtest database. This action will import all the setups needed for a proper setting of the database.

All the steps of the procedure are described in detail below.

- In the new data base (E coli_EQA_EURLtest_Username) select "*import data into the data base*" from the file menu.

Exporting_XML PROCEDURE -1a (BN ver.6)

Select all isolates that you would like to export

- Select the type of data that must be imported (*Data exchange* and then *Import data exchange data*), and click *Import*.
- Browse the XML files (or copy and paste the path describing the file location), flag the options described in the next image, then click *OK*.
- The XML file and the database structure are now in your database. Click *YES* for restarting the program.
- Now your data base should have this aspect.

In the *database entries* window (on the left) you can find all the fields that **must be filled** for each sample (see as example the above figure – NOTE the Strain_Name field). In the *experiment type* windows (on the right) you can also find the PFGE_XbaI experiment.

The database is now ready for the analysis of the PFGE gel image.

Remember to rename your gel as: Username_Lcode_PT-PFGE4 (e.g. EC_Italy_ISS_Lxxx_PT-PFGE4).

After the analysis, the XML file will be created following the procedures described in the next paragraph.

3.2.1. Exporting XML files from BioNumerics version 7

1. Analyze your gel by using the *S. braenderup* H9812 setting imported (remember to add all the lanes to data base).
2. Fill in all the fields requested.
3. Select the entries that must be exported as XML files in the main window.
4. Click on *export selection* as XML from the file menu.

5. Export the TIFF file.
6. Make a compressed folder of all these files, name it as: Username_Lcode_PT-PFGE4 (e.g. EC_Italy_ISS_Lxxx_PT-PFGE4) and upload it in the restricted area of the Proficiency Test section of the EU-RL web site; also the TIFF file of the gel shall be uploaded, in a second upload.

All the steps of the procedure are described in detail below.

- Add all the lanes to data base.
- Go to the main window and select all the lanes that must be exported; select "*export*" from the file menu.
- Select *Data exchange* in the data exchange folder. then click on *export.*
- The export data exchange window will appear. Do not change the default settings. Just flag the option "*export fingerprint files*", then click *OK.*
- The XML export process is done and this window will appear on your screen. Click *OK.*
- This second window will appear. REMEMBER TO TAKE NOTE OF THE LOCATION OF YOUR XML FILES (they should be in the export folder of your saved database).
- The files to be sent should appear as below.

Now you can upload all the compressed files in the restricted area in the Proficiency Test section of EU-RL-VTEC web site.

Annex 2: Example of Individual evaluation report

EU Reference Laboratory for *E. coli*

Department of Veterinary Public Health and Food Safety

Unit of Foodborne Zoonoses

Istituto Superiore di Sanità

4th inter-laboratory study on Pulsed Field Gel Electrophoresis (PT-PFGE4)

April - June 2015

Laboratory code: LXXX

Rome, 7 December 2015

Dear Colleague,

Please find enclosed the assessment of the PFGE results submitted by your laboratory, based on the evaluation grid described in the invitation.

Thank you very much for your participation in PT-PFGE4.

Alfredo Caprioli

Director, EU-RL for *Escherichia coli*

PFGE ASSESSMENT AND COMMENTS

Visual Assessment

Gel quality:

The gel has a high background. An increase in the de-staining time might be of help in solving the problem. The image acquisition process could be improved by adjusting the exposure. Some fuzzy bands are present.

Profiles' suitability for comparison:

The lanes are straight and defined, without incomplete restriction.

In-depth analysis

The assessment of the quality of the PFGE profiles with respect to their usability for comparison was conducted according to the guidelines described in the Standard Operating Procedures published by EFSA (http://www.efsa.europa.eu/en/supporting/doc/704e.pdf). The submitted PFGE profiles were analyzed with the BioNumerics software and compared with the reference profiles produced by the EU-RL. The submitted profiles were considered as suitable for the following cluster analysis when the normalization distortion bars displayed clear colors (yellow to light blue). Otherwise they were rejected at this stage.

For the submitted xml files, the distortion bars of the normalised gel all showed clear colours, indicating a satisfactory normalisation (Figure 1).

The acceptability of the submitted profiles for comparison purposes was evaluated by clustering with the related reference PFGE profiles produced by the EU-RL. The profiles were considered acceptable when the analysis returned 97 % similarity and above.

Using the submitted xml files, the cluster analysis returned similarity values below the 97 % cut-off value for 3 out of the 10 submitted profiles (Figure 2): lane 8/strain 6 (92.7 %), lane 9/strain 7 (95.5 %), and lane 2/strain 1 (92.3 %). For these 3 profiles, the band assignment

was repeated by the EU-RL and the differences between the two band assignment results are reported below.

- Strain 6_782: -30 -150 -450
- Strain 7_782: -430
- Strain 1_782: -30

All the bands below the 33.3kb were deleted in all the profiles.

The bands removed in the EU-RL band assignment are indicated with "–" and the molecular weight of the band (i.e. "-30" means that a band at 30 Kb was removed).

The bands added in the EU-RL band assignment are indicated with "+" and the molecular weight of the band (i.e. "+60" means that a band at 60 Kb was added).

The cluster analysis performed after the bands re-assignment (Figure 3) showed a similarity above 97 % for the profiles of strains 6 and 7, but not for the profile of strain 1 (94.7 %).

Figure 1. Normalisation - Migration Distortion Analysis. The gel analysed with the BioNumerics software by the participant was suitable for the following cluster analysis.

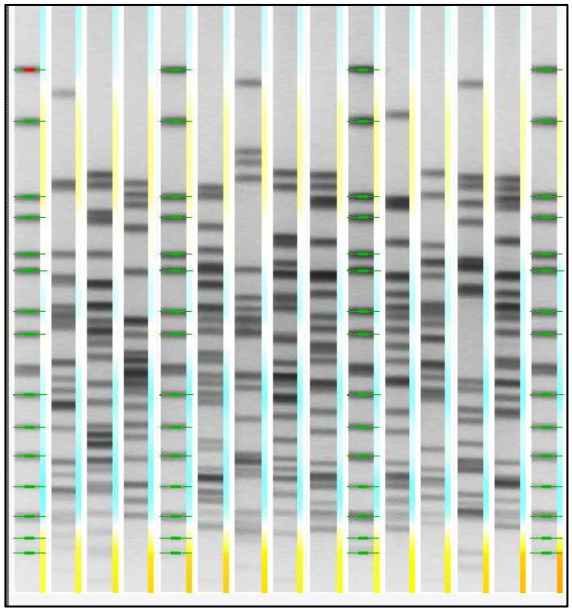


Figure 2. Cluster analysis of the submitted PFGE profiles (with band assignment performed by the NRL) with the corresponding reference PFGE profiles. The band assignment is shown as red lines overlaying the bands on the lanes.

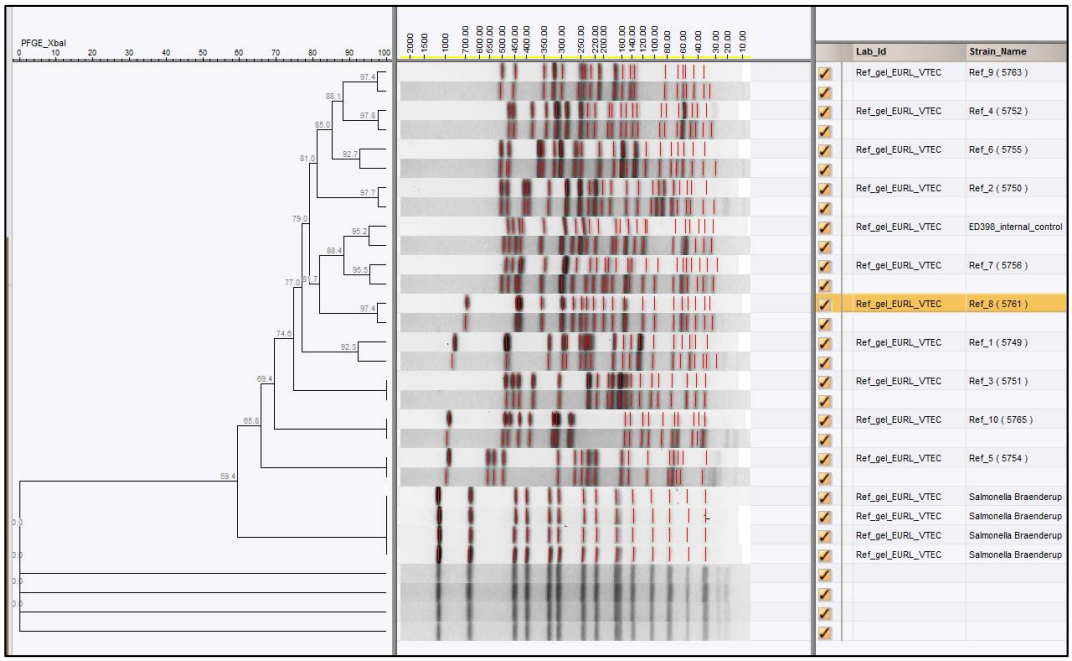
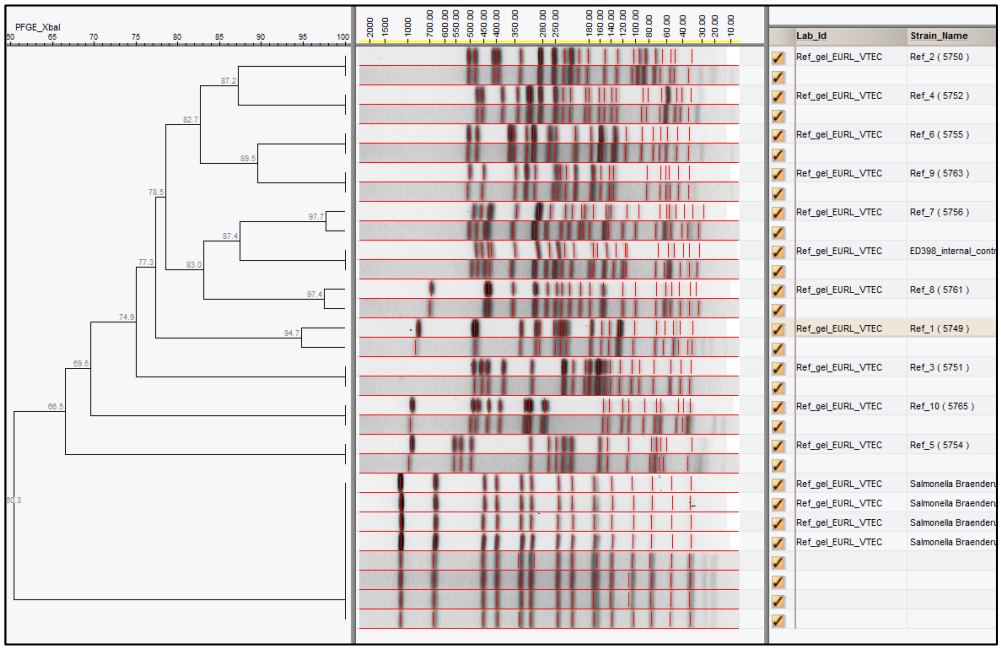


Figure 3. Cluster analysis of the submitted PFGE profiles (with band assignment performed by the EU-RL) with the corresponding reference PFGE profiles produced by the EU-RL. The band assignment is shown as red lines overlaying the bands on the lanes.



The overall evaluation of the set of PFGE profiles provided was done according to the following scheme:

Excellent: No rejected profiles

Good: < 30 % of rejected profiles

Fair: between 30 % and 60 % of rejected profiles

Poor: > 60 % of rejected profiles

Overall evaluation:

The quality of the set of PFGE profiles provided was considered as **Good.**