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Report of the 5th proficiency test (PT) for pulsed field gel electrophoresis (PFGE) typing of Verocytotoxin-producing *E.coli* (VTEC) strains (PT-PFGE5) – 2016

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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 data and for the development of a database on molecular testing results on isolates of Salmonella, *L. monocytogenes* and VTEC from food, feed, animals and the related environment, in collaboration with the relevant European Union Reference Laboratories (EURLs). The data would be submitted to EFSA by the Member States while the EURLs would act as curators of the data and would contribute to the data analyses. In this context the Standard Operating Procedures for the production, interpretation and curation of the PFGE profiles were provided by the three EURLs and published by EFSA (http://www.efsa.europa.eu/sites/default/files/scientific_output/files/main_documents/704e.pdf ; /702e.pdf ; / 703e.pdf).

In December 2014, the EFSA published the "*Technical specifications for the pilot on the collection of data on molecular testing of food-borne pathogens from food, feed and animal samples*" (http://onlinelibrary.wiley.com/doi/10.2903/sp.efsa.2014.EN-712/epdf), where all the details on the metadata, on the data analysis and on the ownership of the data were provided.

In order to contribute to the EU preparedness to submit VTEC PFGE profiles to the upcoming EFSA database, the EURL-VTEC developed an external quality assessment (EQA) program to verify the capability of the NRLs to perform PFGE and the quality of the profiles produced.

This document represents the report of the fifth study organized by EURL-VTEC on PFGE

typing for the benefit of the network of NRLs (PT-PFGE5).

2. OBJECTIVES AND DESIGN OF THE STUDY

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 further 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 21 Laboratories joined the study. One of the MS was represented by a laboratory, which was not appointed as NRL for VTEC (Germany). Each participant received its own individual Laboratory code, which is reported in the result tables.

The Laboratories participating in the study were:

- Austria, Austrian Agency for Health and Food Safety (AGES)
- Belgium, Scientific Institute of Public Health
- Cyprus, Laboratory for the Control of Food of Animal Origin (LCFAO)
- Czech Republic, Veterinary Research Institute
- Finland, Finnish Food Safety Authority Evira, Kuopio
- France, *VetAgro Sup Campus Vétérinaire de* Lyon
- Germany, *Friedrich-Loeffler-Instituts* (FLI)
- Ireland, Veterinary Public Health Regulatory Laboratory, Department of Agriculture, Food and the Marine
- Italy, *Istituto Superiore di Sanità*
- Lithuania, National Food and Veterinary Risk Assessment Institute
- Luxemburg, *Ministère de l'Agriculture, de la Viticulture et de la Protection des consommateurs, Administration des services vétérinaires* (LMVE)
- Norway, Norwegian Veterinary Institute
- Poland, National Institute of Public Health, National Institute of Hygiene

- Romania, Institute for Hygiene and Veterinary Public Health
- 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, *Departamento de Bacteriología 2, LCV de Algete*
- Turkey, Public Health Institution of Turkey, Microbiology Reference Laboratories Dept, Nat. Ref. Lab. for Enteric Pathogens

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). 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 19 and 20 October. 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 24 October 2016 by testing two randomly selected sets of strains for the presence of known microbiologic characteristics. The test samples were stored at room temperature until November 2, when the samples were sent to the participating laboratories by courier.

The PFGE profiles of the test strains were pre-determined at the EURL-VTEC and were considered as reference profiles to evaluate the acceptability of those submitted by the NRLs (Figure 1).

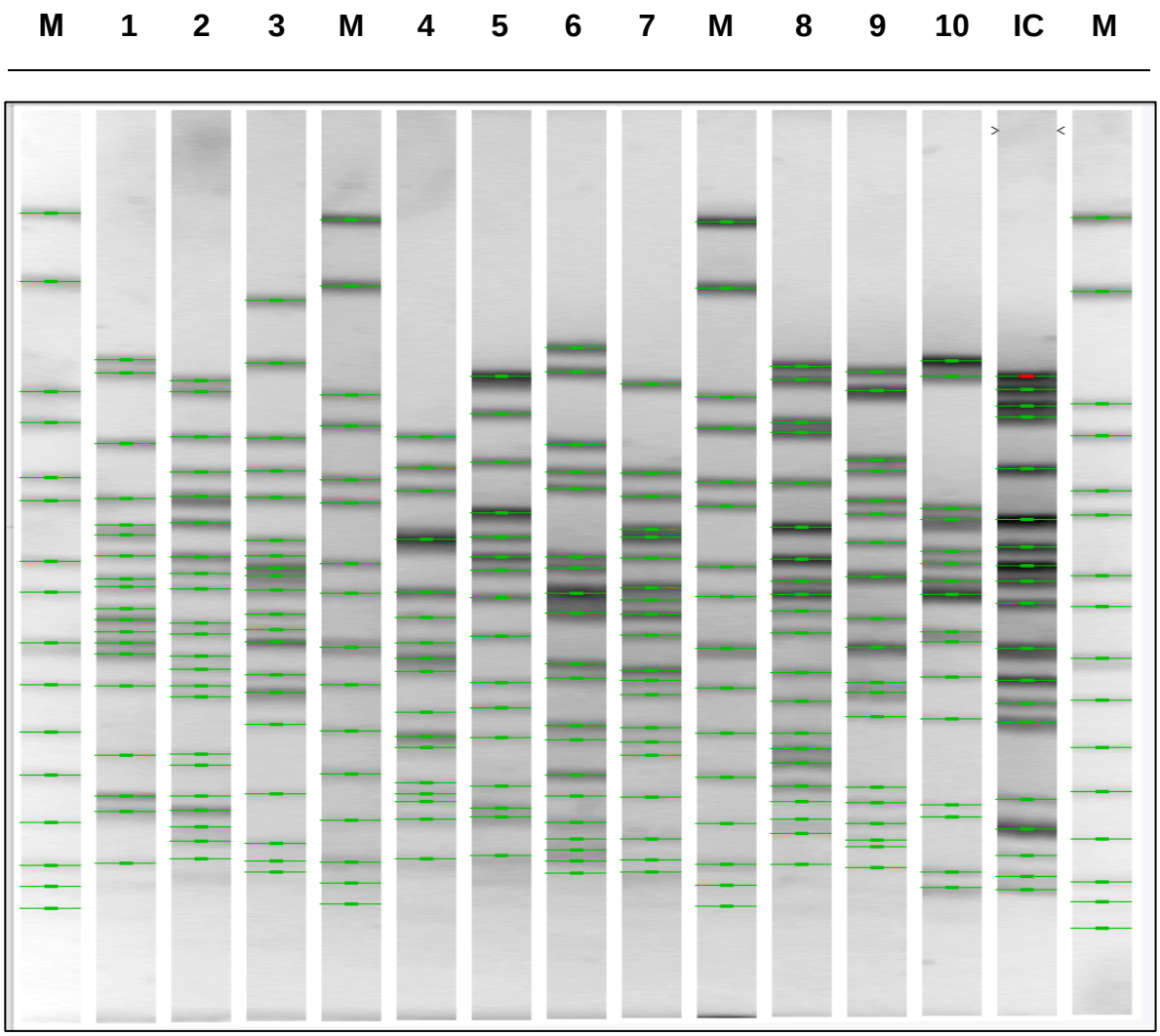

Figure 1: PFGE profiles of the *E. coli* strains included in the study. M: *S.* Braenderup H9812 molecular weight standard.

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 published by EFSA (http://www.efsa.europa.eu/en/supporting/doc/704e.pdf).

4.2.2. BioNumerics software analysis

The NRLs that accepted 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.

Briefly, the NRLs that did not perform 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 Tests Section of the EURL website.

The NRLs carrying out 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, including normalization and band assignment.

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

The gel images submitted 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 electrophoretic conditions.
- 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 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 EURL.

For the NRLs that made the *BioNumerics* analysis, the EURL used 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 EURL.

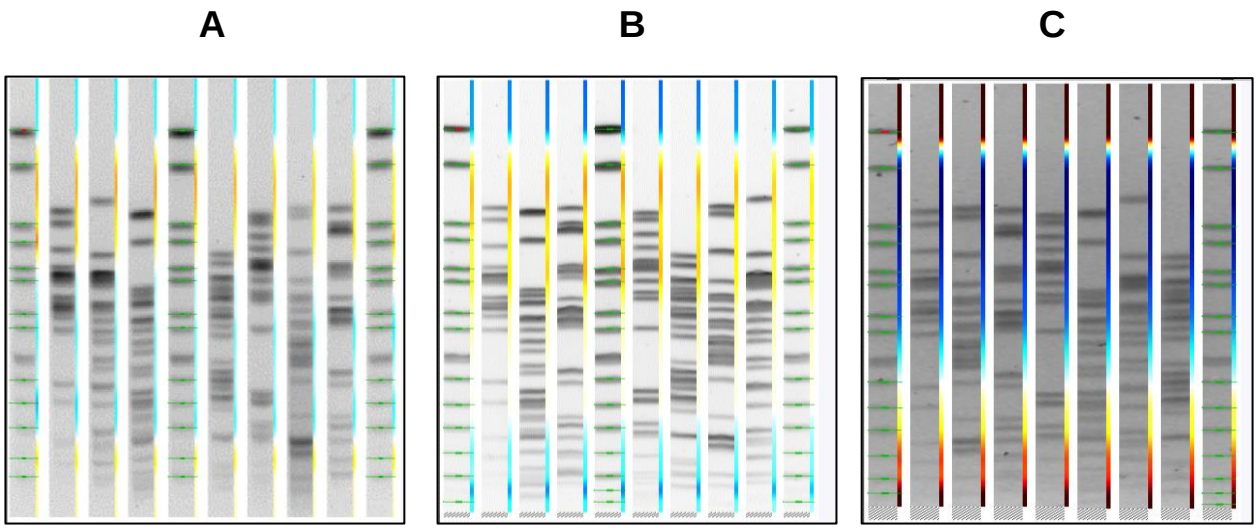

Figure 2. Examples of migration distortion analysis.

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 EURL 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 acceptable".

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 EURL.

For the NRLs that made the *BioNumerics* analysis, the band assignment made by the NRL was directly used for the cluster analysis. When the cluster analysis showed errors in the band assignment, the procedure was repeated by the EURL 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 an 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.

5. RESULTS

Nineteen Laboratories out of the 21 that joined the study submitted PFGE profiles, including 16 Laboratories representing 15 EU Member States and the NRLs of Turkey and Norway.

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

Thirteen NRLs submitted both the TIFF files and the XML export files, including normalization and band assignment, representing 68 % of the total (Figure 3).

5.1. Evaluation of the PFGE profiles submitted

The 19 PFGE gel images submitted were first evaluated by visual assessment, as described in paragraph 4.4: 187 out of the 190 profiles 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. The 157 profiles resulting from the accepted 16 images were further analyzed by cluster analysis with the related reference PFGE profiles produced by the EURL (paragraph 4.6). A hundred and fifty-six PFGE profiles of the total 190 submitted (82.1 %) returned a 97 % similarity and above with the related reference profiles and were considered suitable for inclusion in the database of molecular typing data (Figure 4).

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

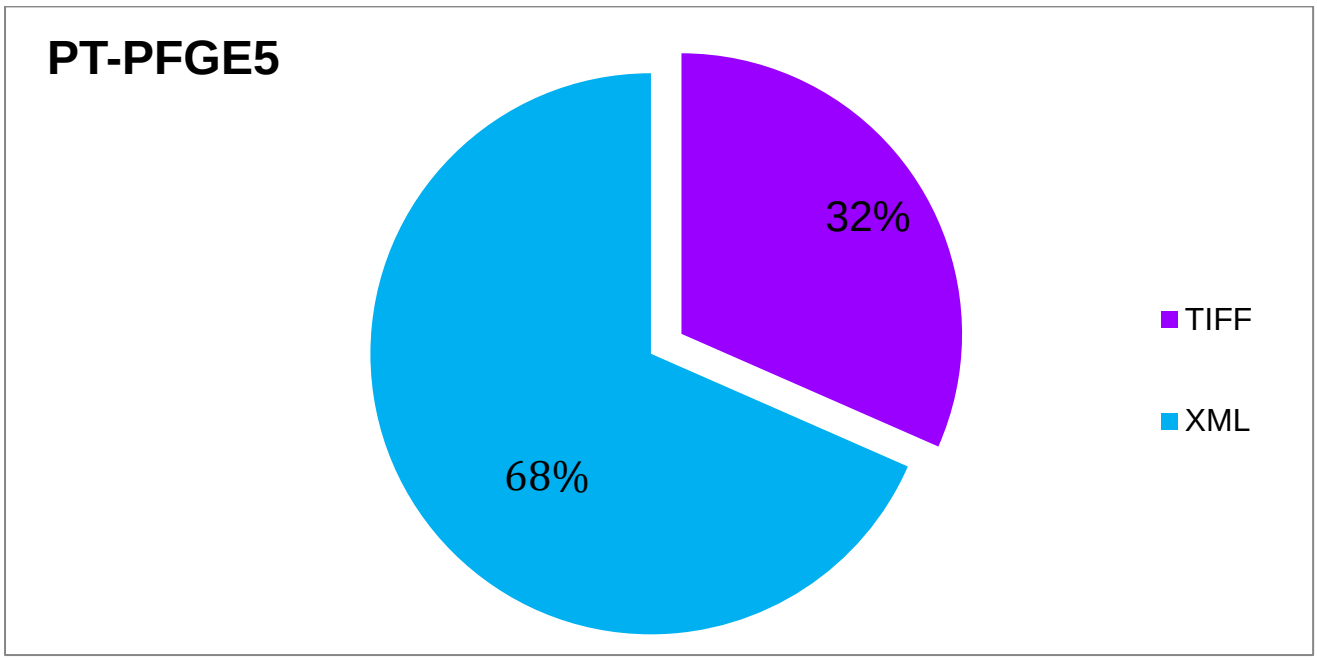

Figure 3: Percentage of the laboratories submitting the gel images either as TIFF or as XML files

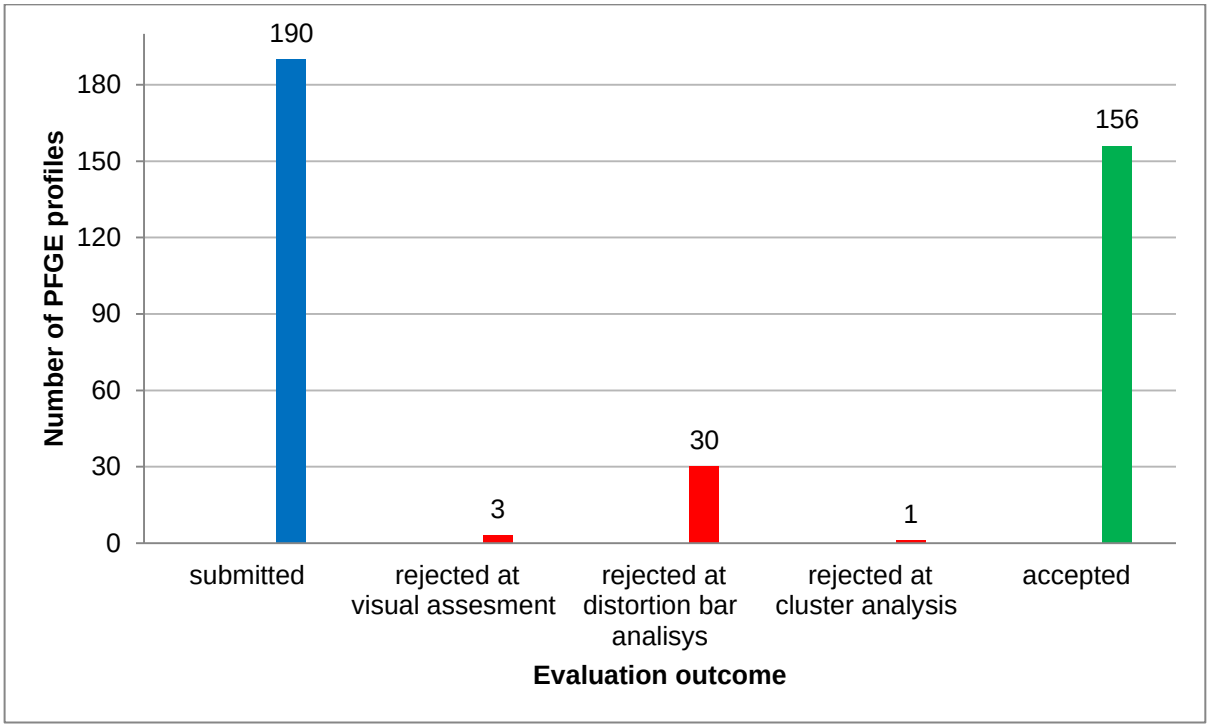

Figure 4. Three-step evaluation of the submitted PFGE profiles.

NRL	Similarity (%) of the PFGE profile submitted by the NRL with the related reference profile for: with the related reference profile for:									
	STRAIN 1	STRAIN 2	STRAIN 3	STRAIN 4	STRAIN 5	STRAIN 6	STRAIN 7	STRAIN 8	STRAIN 9	STRAIN 10
L170	97,0	100	97,4	100	97,4	97,3	100	97,4	100	100
L181	100	100	100	100	100	100	100	100	100	100
L280	97,0	100	97,4	97,4	100	97,3	97,4	97,3	100	100
L405	97,0	100	100	97,4	97,0	100	97,4	97,4	97,6	100
L417	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.
L435	97,0	100	97,4	97,4	100	100	100	97,3	100	100
L492	97,0	97,0	97,4	97,6	100	100	97,4	97,3	100	100
L597	97,0	96,8	100	100	100	97,3	97,4	97,3	97,6	97,7
L647	100	100	100	100	100	97,3	100	97,3	97,6	100
L653	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.
L721	97,0	100	97,4	97,4	100	100	97,4	97,3	97,6	100
L731	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.
L782	100	100	100	100	100	100	100	100	100	100
L789	100	96,8	97,4	97,4	100	97,3	97,4	97,3	100	100
L817	97,0	89,7	97,4	N.A.	97,0	N.A.	97,6	97,3	100	N.A.
L844	100	100	100	97,4	97,0	100	97,4	97,4	100	97,8
L907	97,0	100	97,4	97,4	97,0	97,3	97,4	100	100	100
L973	97,0	100	100	97,6	100	100	97,4	100	97,7	97,8
L975	100	100	100	100	100	100	100	100	100	100

Table 1. Cluster analysis of the PFGE profiles submitted by the NRLs with the related reference PFGE profiles. 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).

Sixteen NRLs submitted suitable PFGE profiles, one of them (L817) sent images from which one of the profiles was not acceptable based on the cluster analysis and three profiles were not analyzable based on the visual inspection of the gel image. For three laboratories (L417, L653 and L731) the profiles could not be evaluated by cluster analysis due to the excessive distortion at normalization step (Figure 4). For all the others laboratories, the cluster analysis returned similarity rates equal or higher than 97 % for all the profiles (Table 1).

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.

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 three NRLs (15.8 %), “fair” for one NRL (5.3 %) and “excellent” for 15 NRLs (78.9 %).

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”, while the orange bar indicates a “fair” performance. The green bars correspond to “excellent”.

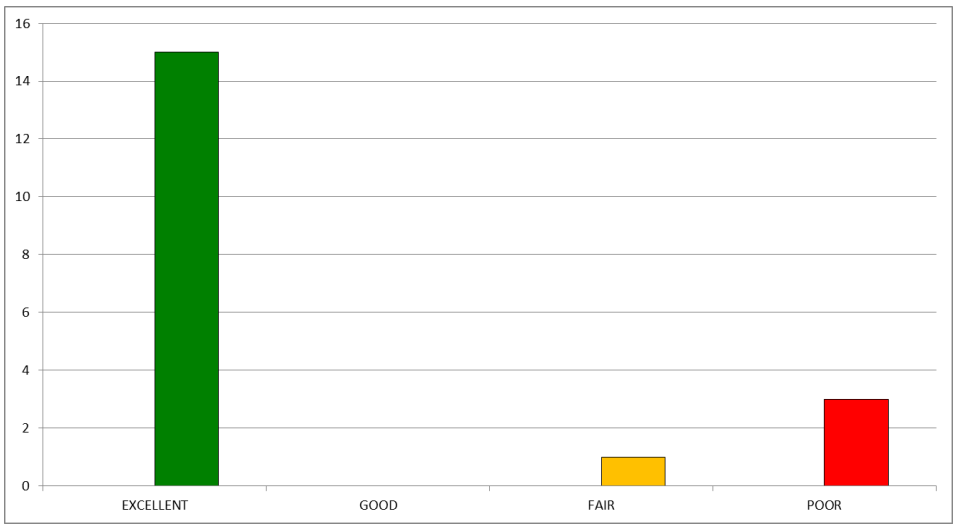

Figure 6. Evaluation of the NRL performance in producing PFGE profiles. The red bars indicate the NRLs whose performance was considered as "poor", while the orange bar indicates a "fair" performance.

The problems most commonly encountered by the NRLs in producing PFGE profiles are shown in Figure 7. The most frequent problems were related with the band assignment performed by the NRLs submitting the xml file and the samples identification. Each NRL received proper advice to overcome specific problems in the individual report.

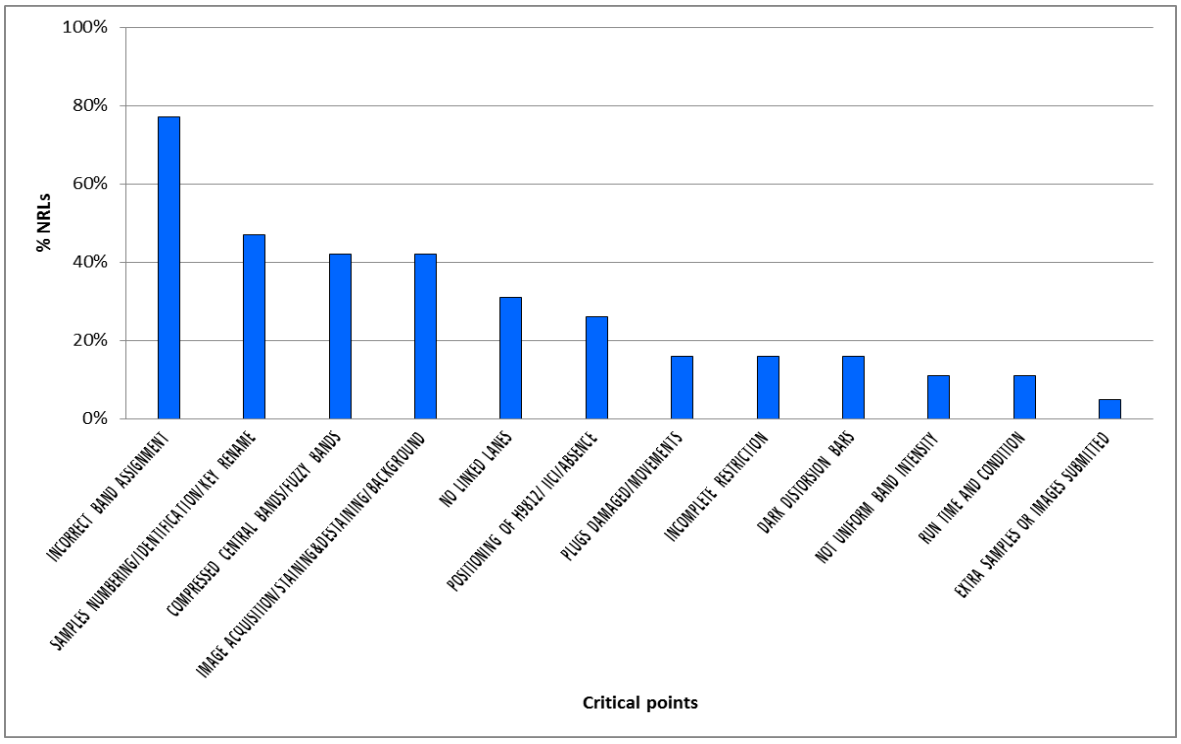

Figure 7. Technical problems most frequently encountered by the NRLs in PFGE profile production.

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

The capability of the NRLs to carry out the *BioNumerics* analysis was evaluated on the basis of the modifications required by their XML files and the 13 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 8, while Figure 9 shows the number of NRLs grouped in each category. Six NRLs (46.2 %) were categorized as “category C”, meaning that major modifications of the band assignment or complete re-assignment were done, and indicating improper analysis of PFGE profiles with BioNumerics Software was carried out. A total of six NRLs, fells into categories A, B and D, representing the 15.4 % each, while just one laboratory obtain the category “E” (XML file not usable for cluster analysis).

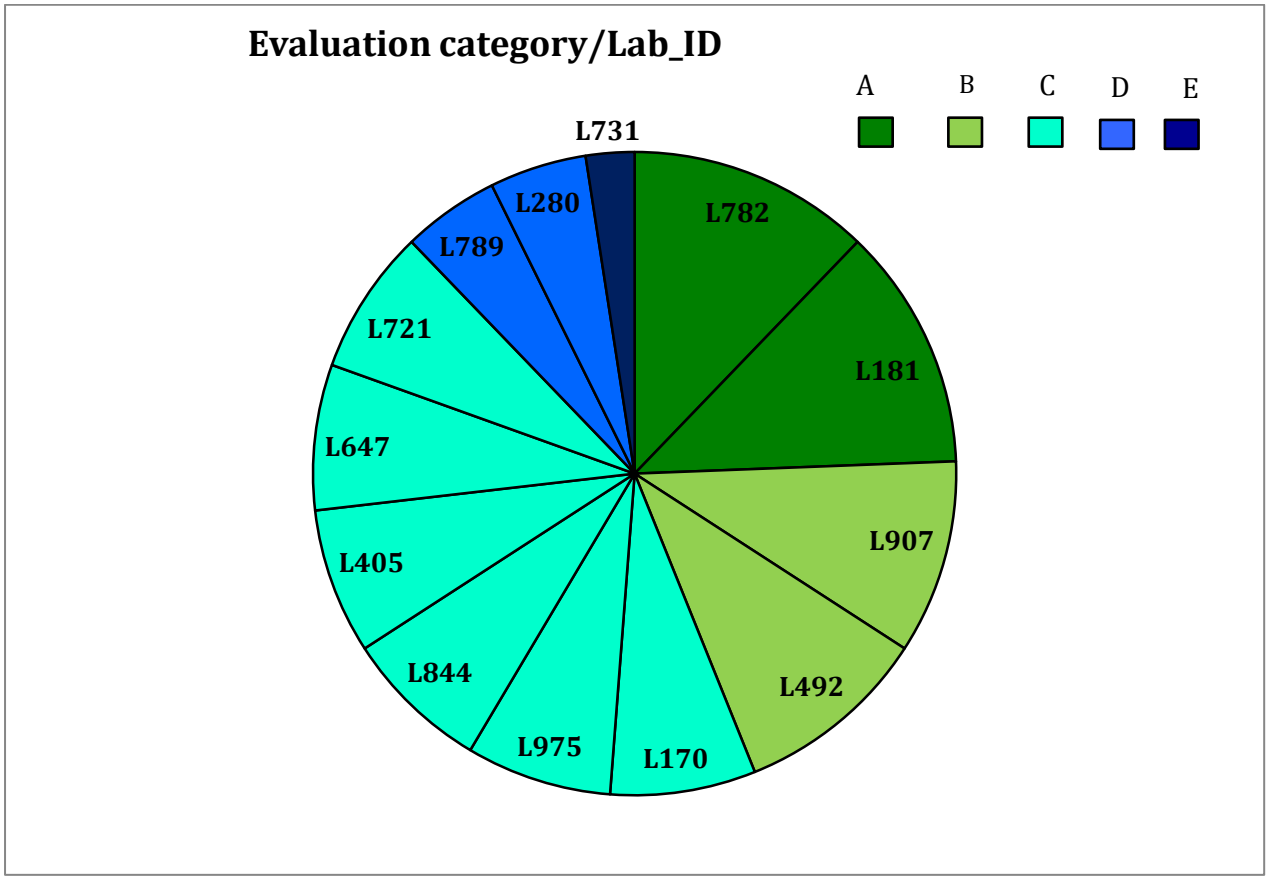

Figure 8. Evaluation of the capacity of the NRLs to carry out the *BioNumerics* analysis. Categories are defined in section 4.7.2.

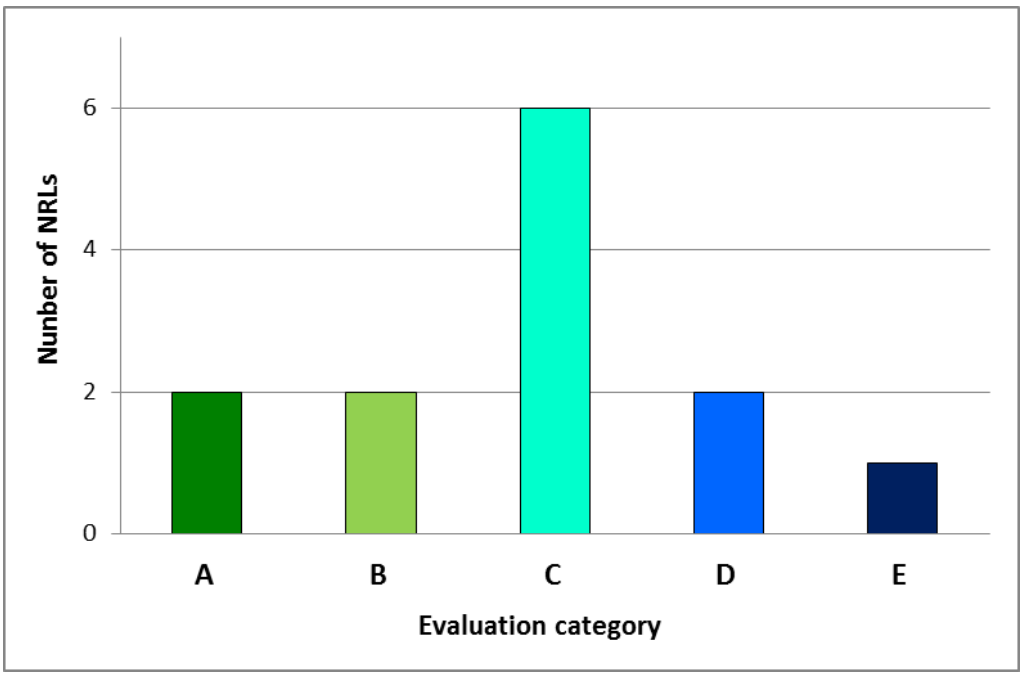

Figure 9. Evaluation of the capacity of the NRLs to carry out the *BioNumerics* analysis. The NRLs that submitted XML files are grouped by category. Categories are defined in section 4.7.2.

6. CONCLUDING REMARKS

PFGE typing 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.

The set of *E. coli* strains sent for PT-PFGE5 was the same used in the PT18, the inter-laboratory study organized by EURL-VTEC for serotyping and genotyping *E. coli* strains.

A total of 19 laboratories, out of 21 accepting to participate, including 16 NRLs representing 15 EU Member States plus a German laboratory, different from the NRL, and the NRLs from Norway and Turkey, submitted the profiles for this 5th PT on PFGE.

The performance of each NRL was evaluated according to the number of PFGE profiles that were considered as suitable for the inclusion in the non-human PFGE profiles database, according to the standard operating procedures for PFGE profiles interpretation and curation 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 accepted and introduced into the further analyses with the exception of three lanes from one gel image.
2. Three gel images did not pass the second step of the migration distortion analysis.
3. The images provided by 16 NRLs were subjected to cluster analysis with the related reference PFGE profiles.
4. Of the total 190 profiles submitted, 156 provided were considered suitable for inclusion in a database of molecular typing data.

As a whole, 15 NRLs (78.9 %) submitted profiles that were all accepted and their performance was considered as "excellent", while, one NRL (5.3 %) had more than 30 % of their profiles that were considered as not suitable and its performance was considered as "fair".

A total of 13 gels were submitted as XML file (68.4 %) and 6 as TIFF images (31.6 %); at the distortion bars evaluation step, 2 TIFF images and one XML file were considered as "poor".

All but one of the profiles of the *E. coli* strains, returned percentage higher than 97 % at the cluster analysis. However, for a total of 52 profiles (40 %) the band assignment was repeated by the EURL. The 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 evaluation showed that 4 laboratories (30.8 %) have at the moment a satisfactory capacity to analyze PFGE profiles by using the *BioNumerics* software, while the others need to improve their skills.

In conclusion in this 5th PT on PFGE typing of *E. coli*, 78.9 % of the laboratories that contributed the results obtained an excellent rate and all their PFGE profiles showed a quality suitable for inclusion in the upcoming EFSA database of molecular typing data on VTEC strains isolated from food and animals