EU Reference Laboratory for *E. coli*

Department of Veterinary Public Health and Food Safety
Unit of Foodborne Zoonoses

Istituto Superiore di Sanità

Report of the 3rd proficiency test (PT) for pulsed field gel electrophoresis (PFGE) typing of Verocytotoxin-producing *E.coli* (VTEC) strains (PT-PFGE3) – 2014

Edited by: *Alfredo Caprioli, Clarissa Ferreri, Antonella Maugliani, Valeria Michelacci, Stefano Morabito, Rosangela Tozzoli*

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.

The 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 first two PFGE typing inter-laboratory studies (PT-PFGE1 and PT-PFGE2) are available in the EU-RL website (http://www.iss.it/vtec), section Proficiency Tests. The results of the 3rd study (PT-PFGE3) 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 purposes of this PT were:

- To assess 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 EFSA database.
- To identify the aspects of the process of molecular data production and analysis that still need improvement.

The study was conducted according to the International Standard ISO/IEC 17043:2010 "Conformity assessment – General requirements for proficiency testing", by extending to PFGE the typing of the 7 strains of *E. coli* belonging to different groups of pathogenic *E. coli* sent to the NRLs in the framework of the 13rd inter-laboratory study on VTEC identification and typing (PT13, report available at http://www.iss.it/binary/vtec/cont/Report_PT13.pdf).

3. PARTICIPANTS

A total of 27 laboratories, including 25 NRLs representing 21 EU Member States and the NRLs of Norway and Switzerland, participated in 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)
- Belgium, Veterinary & Agrochemical Research Centre (VAR - CODA – CERVA)
- 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
- 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
- Romania, *Institutul de Igiena si Sanatate Publica Veterinara*
- 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 Food Agency (SLV)
- 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 7 *E. coli* strains (samples 1 to 7), selected among those present in the EU-RL VTEC reference collection. The preparation of the samples is described in the PT13 report, available at http://www.iss.it/binary/vtec/cont/Report_PT13.pdf. 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.

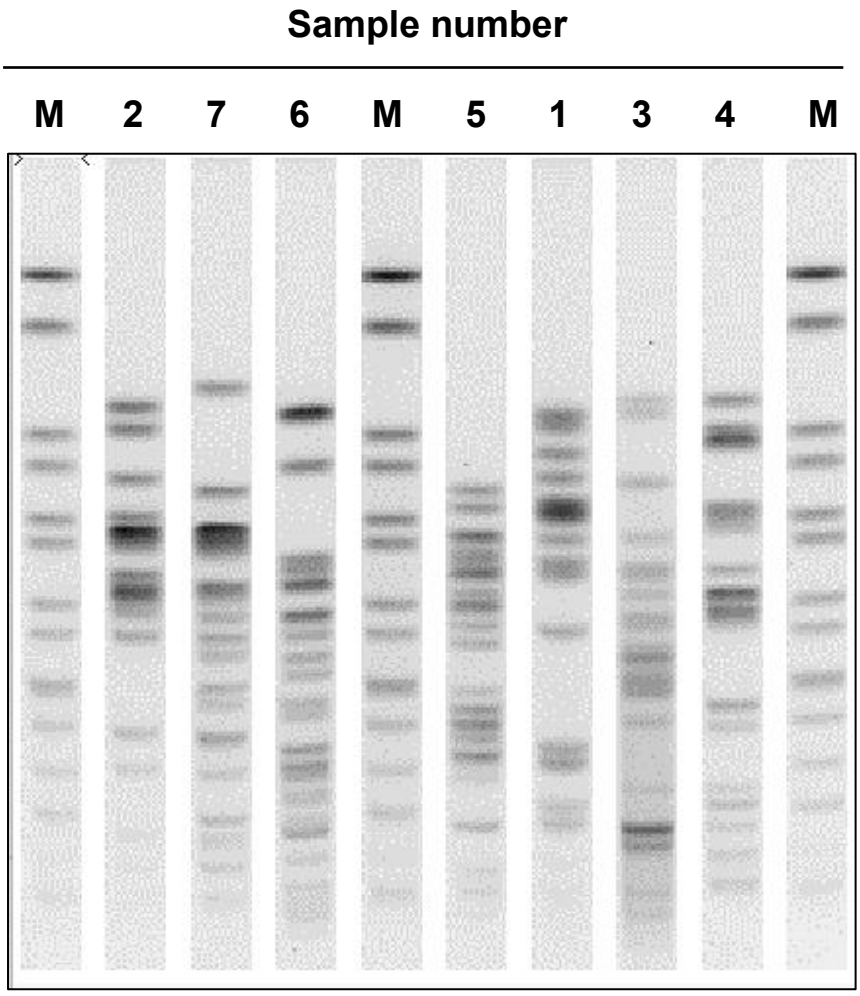


Figure 1: PFGE profiles of the *E. coli* strains included in the study. The samples correspond to those examined in the framework of PT13. M: *S. braenderup* H9812 molecular weight standard.

4.2. Laboratory methods

The NRLs were requested to use the laboratory procedure for PFGE of *E. coli* O157:H7, *E. coli* non-O157 (STEC), Salmonella serotypes, *Shigella sonnei* and *Shigella flexneri* in use in the PulseNet international network, available at URL: http://www.pulsenetinternational.org/assets/PulseNet/uploads/pfge/PNL05_Ec-Sal-ShigPFGEprotocol.pdf. In particular, the NRLs were requested to use the electrophoresis conditions specified for *E. coli* O157:H7 and *Shigella sonnei* strains (page 9 of the protocol), regardless the serotype of the *E. coli* strains to be tested.

4.3. Collection of the results

The NRLs were requested to submit by email the pictures of the PFGE gels as TIFF files, together with a scheme of sample loading in a separate word/excel file.

4.4. Analysis of the results: Visual assessment of the gel images

The gel images were visually inspected to evaluate their suitability for a further instrumental in-depth 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. In-depth 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 shown as 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).

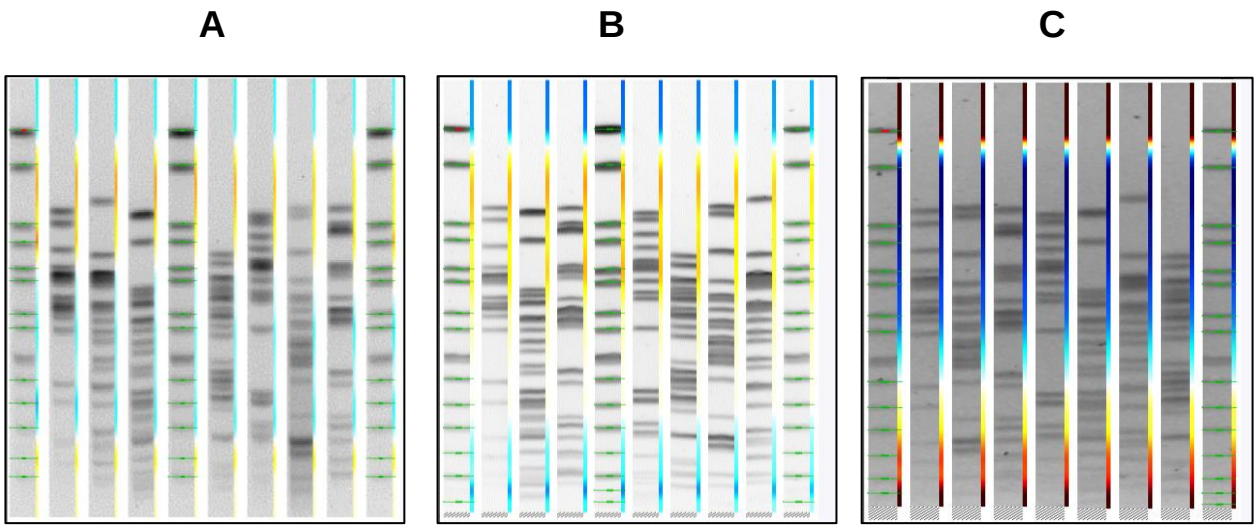


Figure 2. Examples of migration distortion analysis. Panel A: light colors, indicating an acceptable level of distortion. Panel B: darker colors, indicating a stronger distortion, which may be still compensated by the software. Panel C: black coloring, indicating distortions too strong to be compensated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

4.6. In-depth analysis of the PFGE profiles: cluster analysis

The submitted images that passed the migration distortion analysis step were further analyzed by band assignment and cluster analysis with the related reference PFGE profiles produced by the EU-RL (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”.

4.7. Evaluation of the NRL performance

The performance of each NRL in producing PFGE profiles suitable for inclusion in a database of molecular typing data was evaluated according to the following scheme:

Poor: when more than four of the seven profiles were not accepted.

Fair: when three to four of the seven profiles were not accepted.

Good: when one or two of the seven profiles were not accepted.

Excellent: when all the seven profiles were accepted.

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 1**.

5. RESULTS

The study was carried out by 27 NRLs. The 27 PFGE gel images submitted were first evaluated by visual assessment (paragraph 4.4). One of them was rejected due to the poor quality (Figure 3), while the other 26 underwent the distortion bar analysis (paragraph 4.5). The level of distortion was considered as acceptable for 24 images, and the resulting 168 profiles were further analyzed by cluster analysis with the related reference PFGE profiles (paragraph 4.6).

As a whole 134 PFGE profiles of the total 189 submitted (71 %) were accepted and considered suitable for inclusion in a database of molecular typing data (Figure 3).

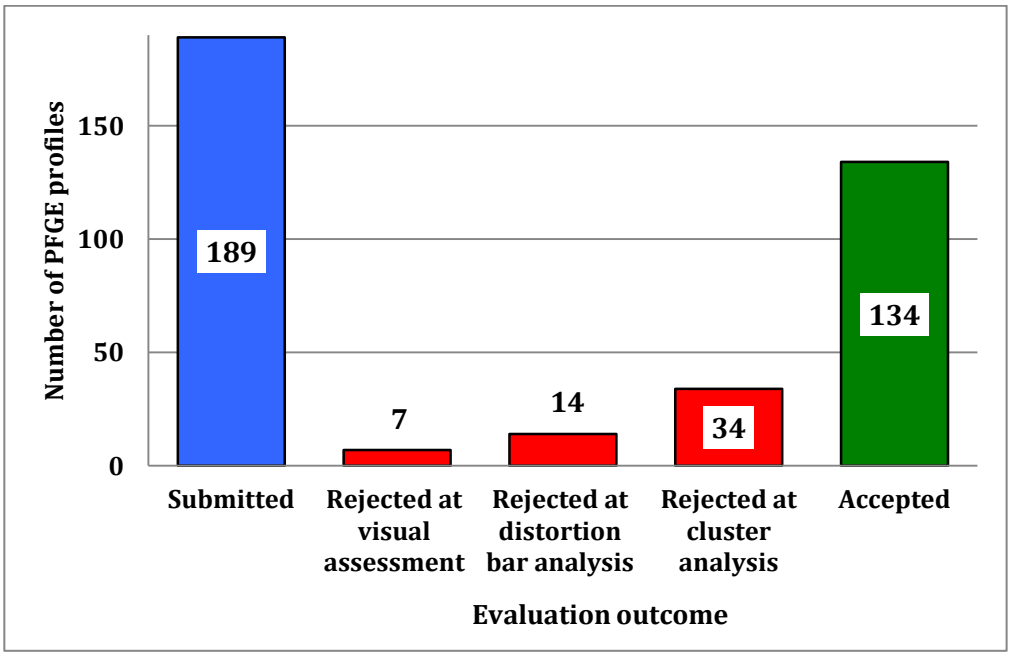


Figure 3. Three-step evaluation of the submitted PFGE profiles.

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

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 too poor 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
L206	97	100	100	100	100	100	100
L240	100	100	100	100	100	100	100
L258	100	100	100	100	100	100	100
L354	100	100	100	100	100	97.7	100
L367	NA	NA	NA	NA	NA	NA	NA
L402	100	100	100	100	100	100	100
L433	100	100	100	100	100	100	100
L534	NA	NA	NA	NA	NA	NA	NA
L544	100	100	94.1	94.4	97.3	92.7	97
L551	100	100	100	100	100	100	100
L553	100	100	100	100	100	100	100
L566	93.8	61.3	97.1	100	100	95.2	97
L633	93.8	100	94.1	97.1	97.3	92.7	97
L674	91.4	90.3	88.9	94.1	92.3	90	85.7
L679	100	100	100	97	100	100	100
L687	100	100	100	100	100	100	100
L691	100	100	97.1	97.1	97.3	95.2	97
L692	97	100	97.1	90.9	97.3	97.7	100
L699	97	100	94.1	90.9	91.4	87.2	93.8
L712	100	100	97.1	100	97.3	95.2	100
L737	97	100	97.1	94.1	97.3	92.7	100
L775	100	100	100	100	100	100	100
L776	93.8	100	94.1	94.4	97.3	95.2	97
L905	100	100	100	100	100	97.7	100
L949	100	100	100	100	100	100	100
L986	NA	NA	NA	NA	NA	NA	NA
L987	97	100	94.1	91.4	91.4	89.5	97

Twenty-three NRLs submitted suitable PFGE profiles, while four 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 poor quality of the gel images or excessive distortion (Figure 3). For L674, the cluster analysis was carried out but all the profiles returned similarity rates lower than 97 % (Table 1).

The number of accepted profiles provided by each NRL ranged from 7 to 2, with 13 NRLs having all their profiles accepted (Figure 4).

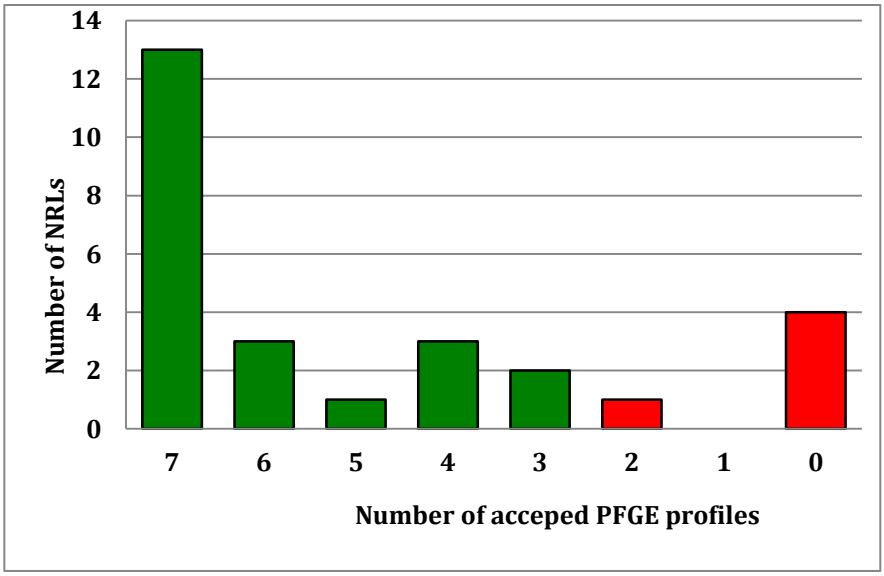


Figure 4. Number of suitable PFGE profiles, by number of NRLs. The red bars indicate the NRLs whose performance was considered as “poor”.

The performance of each NRL was evaluated according to the criteria described in section 4.7, on the basis of the number of PFGE profiles considered as suitable for inclusion in a database of molecular typing data. 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 5 NRLs (18 %), as “fair” for 5 NRLs (18 %), as “good” for 4 NRLs (15 %) and as “excellent” for 13 NRLs (48 %).

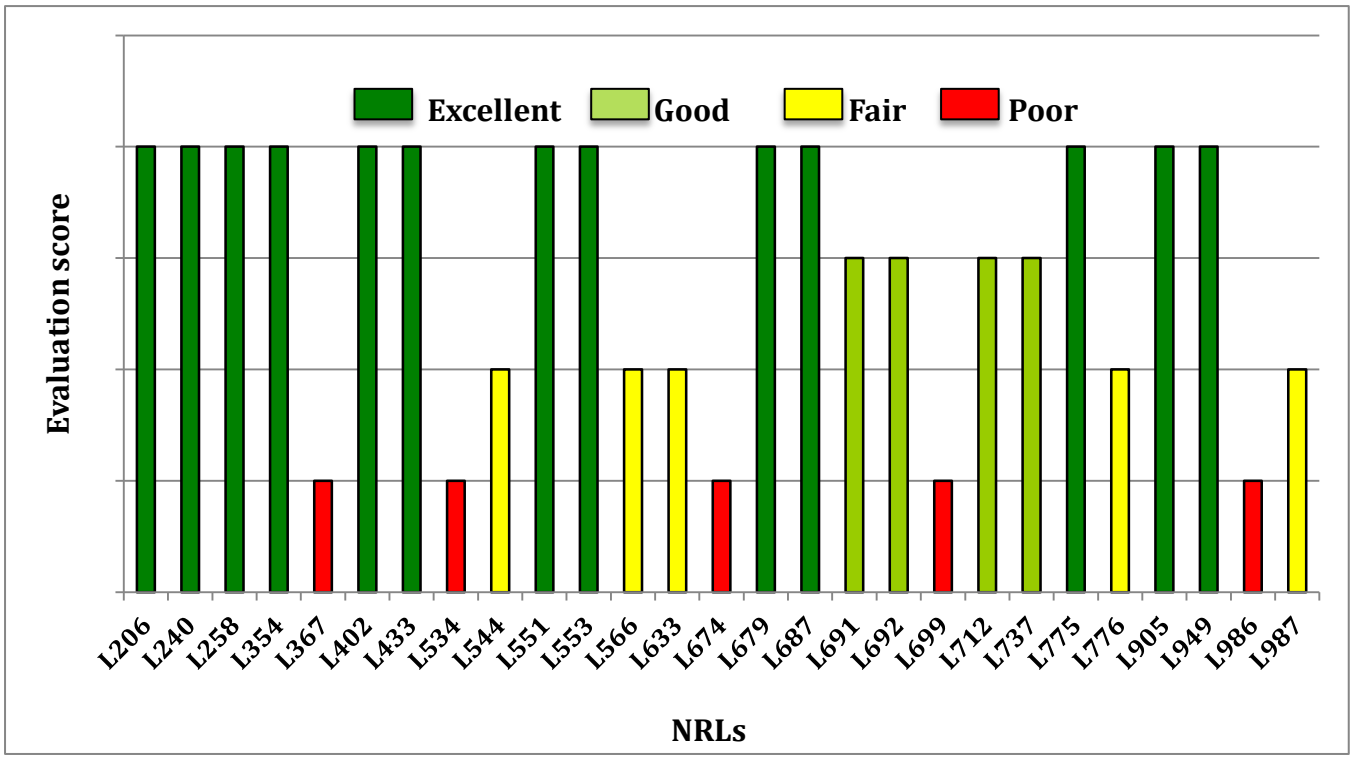


Figure 5. Evaluation of the performance of each NRL in producing PFGE images. The red bars indicate the NRLs whose performance was considered as "poor".

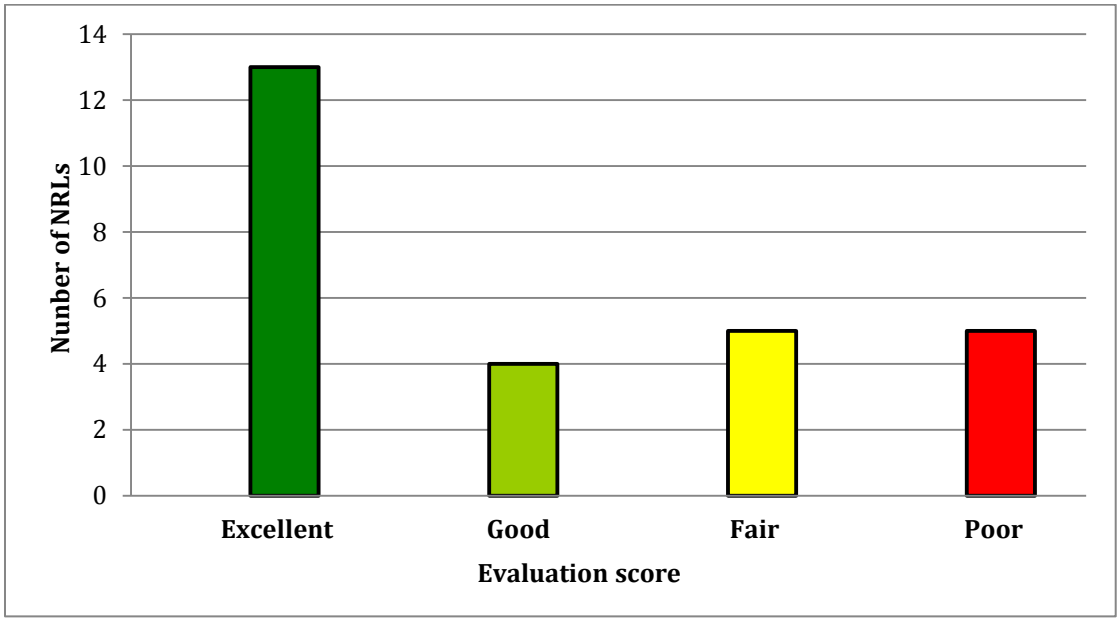


Figure 6. Evaluation of the NRL performance in producing PFGE images. The red bars indicate the NRLs whose performance was considered as "poor".

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

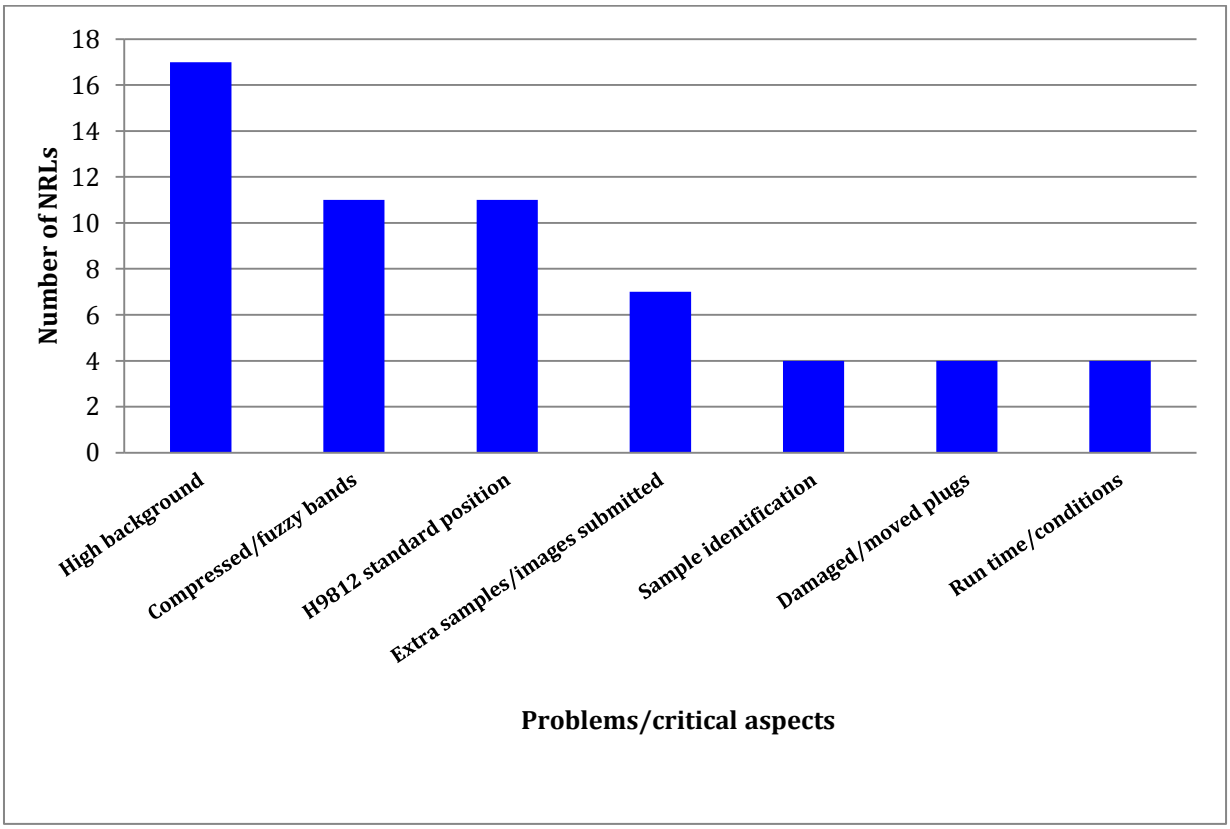


Figure 7. Technical problems most frequently encountered by the NRLs in PFGE profile production.

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. Several NRLs placed the *S. braenderup* H9812 standard in incorrect positions, which could hamper a proper comparison of PFGE profiles from different gels, or made errors in the correct matching of the strains with the sample codes assigned to the each NRL. Each NRL received proper advice to overcome its specific problems in the individual report (**Annex 1**).

The rate of accepted profiles varied with the *E. coli* strain tested, and ranged from 92 % of strains 2 and 7 to 58 % of strain 6.

Figure 8 shows the rate of accepted profiles by single strains, carried out considering the profiles submitted to cluster analysis.

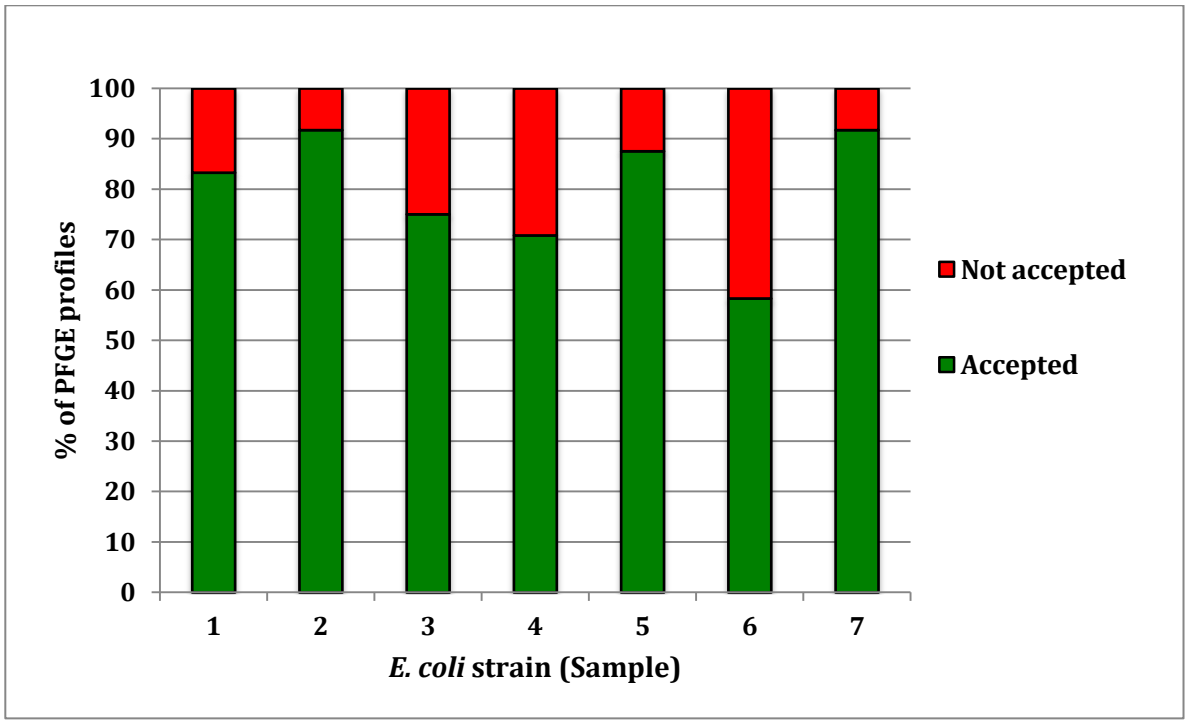


Figure 8. Rate of acceptable PFGE profiles submitted by the NRLs, by *E. coli* strain. The evaluation was carried out considering the profiles that could be submitted to cluster analysis.

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 verify the capability of the laboratories to perform PFGE and the quality of the profiles produced. Therefore, the EU-RL VTEC organized and EQA for the *E. coli* NRLs of EU and EEA Member States, which will take part in the upcoming collection of PFGE typing data on food and animal isolates of VTEC, coordinated by EFSA.

Twenty-seven NRLs, representing 21 EU Member States, Norway and Switzerland participated in this 3rd PT. This represented an increase with respect to the participation in the first two PT rounds on PFGE (reports available in the EU-RL web site, Section Proficiency Tests), which involved 19 and 23 NRLs, respectively.

In this PT, the evaluation of the results 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 a database, 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 three-step evaluation process was applied. At the first evaluation by visual assessment, one gel image was rejected, and other two did not pass the second step of the migration distortion analysis. The images provided by 24 NRLs were subjected to cluster analysis with the related reference PFGE profiles. At the end of the evaluation process, 134 PFGE profiles (71 % of those submitted) provided by 23 of the 27 NRLs (85 %) were accepted and considered suitable for inclusion in a database of molecular typing data. As a whole, five NRLs submitted more than four profiles that were not accepted, and their performance was considered as “poor”.

As for the specific technical problems, those more frequently encountered were related with the gel background and the presence of bands that appeared fuzzy and/or compressed in the central part of the lanes. Several NRLs placed the *S. braenderup* H9812 standard in incorrect positions, which could hamper a proper comparison of PFGE profiles from different gels. Specific suggestions and advice to overcome such problems were given to each NRL in its own individual report.

The rate of accepted profiles also depended on the *E. coli* strain tested, ranging from 92 % of strains 2 and 7 to 58 % only of strain 6. This was mainly due to the profile of strain 6 that, in the given PFGE conditions, had a high number of DNA bands, particularly in the central and low parts of the gel.

In conclusion, most of the NRLs participating in this 3rd PT on PFGE typing of *E. coli* were able to provide profiles suitable for inclusion in the upcoming EFSA database of molecular typing data on VTEC strains isolated from food and animals.

Annex 1: 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

3rd inter-laboratory study on Pulsed Field Gel Electrophoresis (PT-PFGE3)
conducted in the framework of the 13th inter-laboratory study (PT13)
on Verocytotoxin-producing *E. coli* (VTEC) identification and typing
June - September 2014

Laboratory code: xxx

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-PFGE3.

Alfredo Caprioli
Director, EU-RL for *Escherichia coli*

PFGE assessment and comments

Visual Assessment:

Gel quality:

In the submitted image, the lanes containing the *S. braenderup* H9812 standard are not properly placed. The standard must be loaded in lanes 1, 5, 10 (10-well gel) or in wells 1, 5, 10, 15 (15-well gel), as specified in section 5.7 of the Standard Operating Procedure for PFGE published by EFSA (http://www.efsa.europa.eu/en/supporting/doc/704e.pdf). This is of the utmost importance for gel analysis and normalization and allows comparing lanes from different runs and different laboratories. The gel has a high background. An increase in the de-staining time might be of help in obtaining clearer images.

Profiles' suitability for comparison:

The lanes are straight and defined, without incomplete restriction. Some bands are fuzzy and compressed in the central part of the lanes, but appear to be still analysable.

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. The submitted PFGE profiles were analyzed with the BioNumerics software and compared with the reference profiles produced by the EU-RL.

The submitted profiles are suitable for the following cluster analysis when the normalization distortion bars display clear colors (yellow to light blue). Otherwise they might be rejected at this stage.

In the submitted image, the distortion bars of the normalised gel all showed clear colours (figure 1). Therefore, the profiles were accepted and introduced into the cluster analysis step.

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.

One profile (sample xxxx) did not match the requirements: two bands in the lower part could not be assigned due to a fuzzy area. The resulting profile showed 95.2 % similarity with the reference profile and was therefore rejected (Figure 2).

Figure 1. NORMALISATION - MIGRATION DISTORTION ANALYSIS: Gels are analysed with the BioNumerics software for the distortion introduced while stretching the lanes for the alignment with the reference standard.

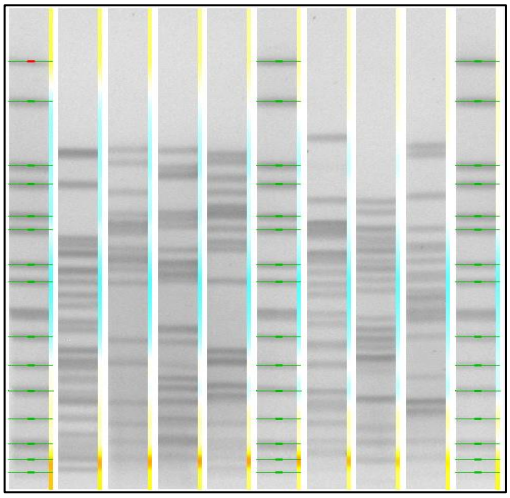
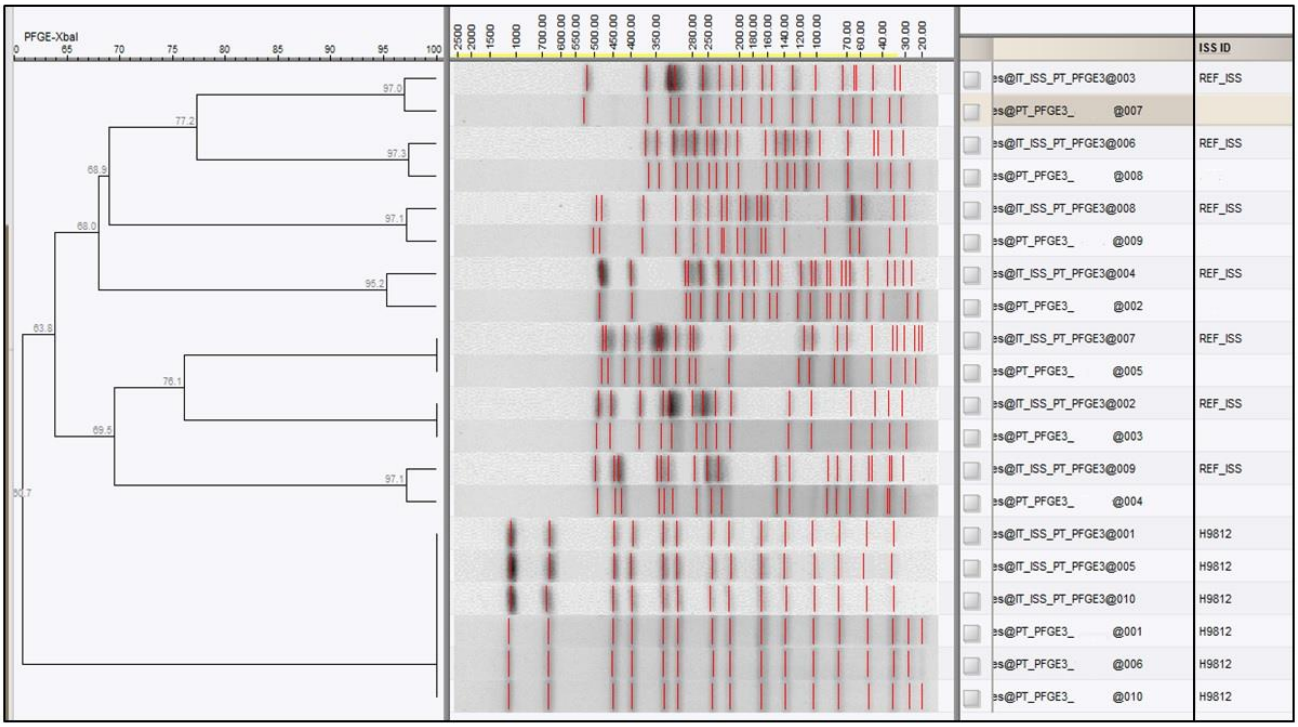


Figure 2. BAND ASSIGNMENT AND COMPARISON: Clustering of the submitted PFGE profiles with the corresponding reference PFGE profiles produced by the EU-RL. The band assignment is shown as red lines overlaying the bands on the lanes.



The overall grading of the gel images is done according to the following scheme:

Poor: More than four lanes are not accepted.

Fair: Three to four lanes are not accepted.

Good: One or two lanes are not accepted.

Excellent: All lanes are accepted.

Overall evaluation: The quality of the gel image provided is considered as **Good.**