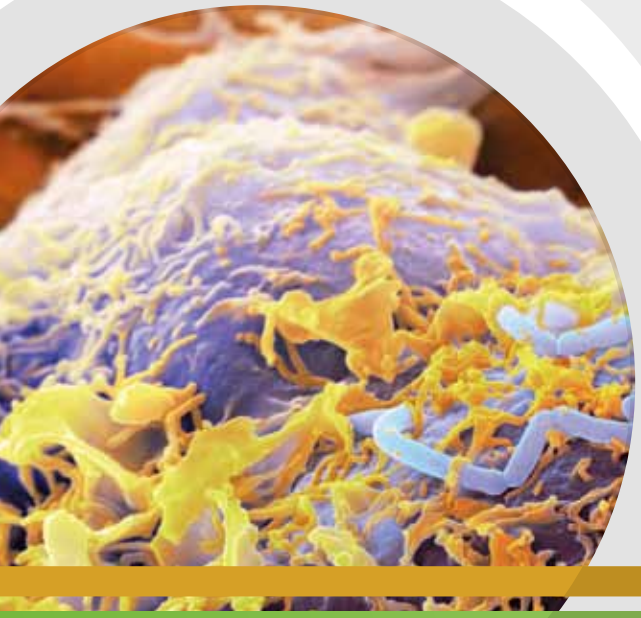

TECHNICAL REPORT

External quality assurance scheme for *Listeria monocytogenes* typing

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ECDC TECHNICAL REPORT

External quality assurance scheme for *Listeria monocytogenes* typing

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Contents

Abbreviations

BN	BioNumerics
ECDC	European Centre for Disease Prevention and Control
EEA	European Economic Area
EQA	External Quality Assurance
FWD	Food- and Waterborne Diseases and Zoonoses
PFGE	Pulsed Field Gel Electrophoresis
SSI	Statens Serum Institut
TESSY	The European Surveillance System

Executive summary

This report presents the results of the first round of the Listeria External Quality Assurance (EQA) scheme for the typing of *Listeria monocytogenes* (further EQA-1). The EQA covers the Pulsed Field Gel Electrophoresis (PFGE) method, conventional serological typing and PCR-based molecular typing. A total of 18 laboratories participated in the EQA-1 which took place between January and March 2013.

Listeriosis is a relatively rare but serious foodborne disease, with 1 476 confirmed human cases reported in EU in 2011 (0.32 cases per 100 000). Compared to other foodborne infections under EU surveillance, listeriosis caused the most severe human disease, with 93.6% of the cases hospitalised and 134 fatalities (case fatality rate 12.7%).

Since 2007, ECDC's Programme on Food- and Waterborne Diseases and Zoonoses (FWD) has been responsible for the EU-wide surveillance of listeriosis, including the facilitation of the detection and investigation of foodborne outbreaks. Surveillance data, including some basic typing parameters for the isolated pathogen, are reported by the Member States to the European Surveillance System (TESSy). In addition to the basic characterisation of the pathogens, there is added public health value to using more advanced and discriminatory typing techniques for surveillance of foodborne infections. In 2012, ECDC initiated a pilot project on enhanced surveillance through incorporation of molecular typing data ('molecular surveillance').

The objectives of this EQA are to assess the quality of PFGE and serotyping and the comparability of the collected test results among participating public health national reference laboratories in European Union (EU), European Economic Area (EEA) and EU candidate countries. Strains for the EQA were selected from strains which are currently relevant for public health in Europe. A set of ten strains was selected. The set included a broad range of the clinically relevant types for invasive listeriosis.

A total of 18 laboratories participated in at least one part of the EQA-1: 17 laboratories (94%) produced PFGE results, 16 laboratories (89%) participated in the serotyping exercise. Ten of these 16 laboratories performed conventional phenotypic serotyping, while seven performed molecular PCR-based serotyping.

The majority (59%) of the laboratories were able to produce a PFGE gel of sufficiently high quality to allow for comparison with profiles obtained by other laboratories. The profiles were then normalised and interpreted using the specialised software BioNumerics (BN). Thirteen laboratories completed the gel analysis and generally did so in accordance with the guidelines.

The average score for traditional serotyping among the participants was 94%. In the molecular (multiplex PCR based) serotyping one participant reported a faulty nomenclature and scored 0%. Among the remaining participants, the average score for correct results was 97%.

This EQA-1 scheme for the typing of *Listeria* was the first EQA for laboratories participating in the FWD-Net. Their level of performance in the EQA was encouraging, although the number of participants could have been higher. The molecular surveillance system that is about to be implemented as part of TESSy relies on the capacity of the European Food- and Waterborne Diseases and Zoonoses network (FWD-Net) laboratories to produce comparable typing results. At the moment the molecular typing method used for EU-wide surveillance is PFGE. Phenotypical serotyping is currently included in TESSy (PCR-based is in the process of being added) and used for surveillance purposes by several EU countries. In general, countries demonstrated a high proficiency level for serotyping. The results of the EQA-1 for PFGE typing of *Listeria* demonstrate that the majority of participating laboratories were able to produce good results, scoring 'Fair' and above in all parameters, which enables inter-laboratory comparisons. Less than half of the laboratories produced results that need to be improved for inter-laboratory exchange of data. However, the majority of the technical issues identified could have been overcome to achieve an acceptable quality by optimising procedures in laboratories, trouble-shooting assistance and training.

1. Introduction

1.1. Background

The European Centre for Disease Prevention and Control (ECDC) is a European Union (EU) agency with a mandate to operate the dedicated surveillance networks and to identify, assess and communicate current and emerging threats to human health from communicable diseases. Within its mission, ECDC shall 'foster the development of sufficient capacity within the Community for the diagnosis, detection, identification and characterisation of infectious agents which may threaten public health. The Centre shall maintain and extend such cooperation and support the implementation of quality assurance schemes' [1].

External quality assurance (EQA) is an element of quality management systems and involves an external agency evaluating the performance of laboratories on material supplied specially for the purpose.

ECDC's disease-specific networks organise a series of EQAs for EU/EEA countries. The aim of the EQA is to identify areas for improvement in laboratory diagnostic capacity relevant to disease surveillance, as listed in Decision No 2119/98/EC [2], and to ensure comparability of results from laboratories in all EU/EEA countries. The main purposes of EQA schemes include:

- assessment of the general standard of performance ('state of the art')
- assessment of the effects of analytical procedures (method principle, instruments, reagents, calibration)
- evaluation of individual laboratory performance
- identification and justification of problem areas
- providing continuing education
- identification of needs for training activities.

In 2012, ECDC put out to tender a framework service contract on 'Microbiological characterisation services to support surveillance of *Salmonella*, STEC/VTEC and *Listeria* infections' for the period 2012–2016. The EQA framework contract was awarded to Foodborne Infections Unit at Statens Serum Institut in Denmark for the three lots covering *Salmonella*, Shiga toxin/verocytotoxin-producing *Escherichia coli* (STEC/VTEC) and *Listeria monocytogenes* respectively. The contract for lot 3 (*Listeria monocytogenes*) covers the organisation of an EQA exercise for PFGE, serotyping of *L. monocytogenes*, and molecular typing services. The present report presents the results of the first EQA-exercise under this contract (Listeria EQA-1).

1.2 Surveillance of listeriosis

Human listeriosis is a relatively rare but serious zoonotic disease, with high morbidity, hospitalisation and mortality in vulnerable populations. In 2011, 1 476 confirmed human cases were reported in the EU corresponding to a notification rate of 0.32 cases per 100 000 population [3]. Compared to other foodborne infections under EU surveillance, listeriosis caused the most severe human disease, with 93.6 % of the cases hospitalised and 134 fatalities (case fatality rate 12.7%).

Since 2007, ECDC's Programme on Food- and Waterborne Diseases and Zoonoses (FWD) has been responsible for the EU-wide surveillance of listeriosis, including facilitating the detection and investigation of foodborne outbreaks. One of the key objectives for the FWD programme is to improve and harmonise the surveillance system in the EU to increase scientific knowledge regarding aetiology, risk factors and burden of food- and waterborne diseases and zoonoses. The surveillance data, including some basic typing parameters for the isolated pathogen, are reported by the Member States to TESSy. In addition to the basic characterisation of the pathogens isolated from infections, there is a public health value to using more advanced and more discriminatory typing techniques in the surveillance of foodborne infections. Therefore, in 2012 ECDC initiated a pilot project on enhanced surveillance through the incorporation of molecular typing data ('molecular surveillance'). In the first pilot phase, three selected FWD-Net pathogens were included: *Salmonella*, *Listeria monocytogenes*, and Shiga toxin/verocytotoxin-producing *Escherichia coli* (STEC/VTEC). The overall aims of integrating molecular typing into EU level surveillance are:

- to foster rapid detection of dispersed international clusters/outbreaks
- to facilitate the detection and investigation of transmission chains and relatedness of strains across Member States and globally
- to detect emergence of new evolving pathogenic strains
- to support investigations to trace-back the source of an outbreak and identify new risk factors
- to aid the study of a particular pathogen's characteristics and behaviour in a community of hosts.

The molecular typing pilot project gives Member State users access to EU-wide molecular typing data for the pathogens included. The pilot also gives its users the opportunity to perform cluster searches and analyses of the data of the EU level data, to determine whether isolates characterised by molecular typing at the national level(s) are part of a multinational cluster that may require cross-border response collaboration.

Since 2009, ECDC's FWD Programme has supported EQA schemes for serotyping and antimicrobial resistance testing for *Salmonella* and VTEC. These EQA schemes have helped to strengthen laboratory capacity in EU/EEA countries in order to provide reliable and valid data for surveillance and research. As mentioned above, ECDC is now extending its centralised data collection capabilities to include detailed molecular typing data for surveillance of selected pathogens. The technical platform to support this will be molecular typing databases within TESSy. To ensure that the molecular typing data entered into the surveillance databases is of sufficiently high quality, expert support and EQA schemes are needed that cover these methods. Therefore, since 2012 ECDC's Food and Waterborne Disease Programme has been supporting EQA schemes focussing on expert assistance for molecular typing, namely Pulsed Field Gel Electrophoresis (PFGE) and Multiple-Locus Variable-number tandem repeat analysis (MLVA) of *Salmonella spp.,* PFGE of Shiga toxin/verocytoxin -producing *Escherichia coli* (STEC/VTEC) and *L. monocytogenes.* Quality assurance activities have also included virulence gene detection, phage typing and serotyping of the pathogens. The EQA schemes have targeted national reference laboratories that were already expected to be performing molecular surveillance at the national level.

1.3 Objectives of the EQA-1 scheme

1.3.1 Pulsed-Field Gel Electrophoresis (PFGE) typing

The objective of the EQA-1 was to assess the quality of the standard PFGE molecular typing and comparability of the collected test results among participating laboratories and countries. The exercise focused on the production of raw PFGE gels of high quality, normalisation of PFGE images and interpretation of the final results.

1.3.2 Serotyping

The EQA-1 scheme assessed the serotype determination by either conventional antigen-based typing of somatic 'O' antigens and flagellar 'H' antigens and/or PCR-based molecular serotyping.

2. Study design

2.1 Organisation

The Listeria EQA-1 was funded by ECDC and arranged by Statens Serum Institut (SSI) in accordance with the International Standard ISO/IEC 17043:2010 (4). The EQA-1 included PFGE and serotyping and was carried out between January and March 2013.

Invitations were emailed to ECDC contact points in the Food- and Waterborne Diseases Network (FWD-Net) (30 countries) by 15 October 2012. In addition, invitations were sent to EU acceding and candidate countries; Croatia, Montenegro, Serbia, the former Yugoslav Republic of Macedonia and Turkey, by the ECDC coordinator.

Twenty–two laboratories accepted the invitation to participate but two laboratories later communicated that they were unable to perform the tests and two laboratories never responded after receiving their dispatched strains. Therefore, a total of 18 laboratories are included in the result tables. The list of participants appears in Annex 1.

The EQA test-strains were sent to the laboratories at the end of January 2013. The participants were asked to submit their results by e-mail to list.eqa@ssi.dk by 25 March 2013.

2.2 Selection of strains

Strains were selected for the EQA-1 programme based on the following criteria:

- they should cover a broad range of the clinically relevant types for invasive listeriosis;
- they should remain stable during the preliminary testing period at the organising laboratory.

SSI tested 15 strains and 10 were selected. The 10 strains for the PFGE part were selected based on their PFGE profiles, containing both some 'easy' strains without difficult double bands and strains which were identical or very similar. A variety of different serotypes relevant for the epidemiological situation in Europe were selected from strains within serotypes 1/2a, 1/2b, 1/2c 4a/4c and 4b. The strain selection also included a recent outbreak strain. The characteristics of the ten *L. monocytogenes* test strains used in the EQA-1 are listed as 'original' together with the participants' results in the tables (Annex 2 and 6). In addition to the test strains, laboratories participating in the EQA-1 for PFGE could request the *Salmonella* Braenderup H9812 strain used as molecular size marker.

2.3 Carriage of strains

By the end of January all strains were blinded and packed and shipment was initiated on 21 January 2013. All of the participants received their dispatched strains within one to three days. The parcels were shipped from SSI labelled as UN 3373 Biological Substance. The participants were e-mailed their specific blinded number as an extra control. No participants reported damages of the shipment or errors in the specific strain number.

On 31 January, instructions on how to submit results were e-mailed to participants. This included Excel sheets for submission of PFGE setup and serotyping results. Also included were zip files for the BN database experiment settings (PFGE part) and guidelines on how to export XML files from BN (Annex 7 and 8).

2.4 Testing

In the PFGE part, 10 *L. monocytogenes* strains were tested and participants could choose to take part in the laboratory part only (submit the PFGE gel) or to also complete an additional analysis of the gel (submit normalised profiles with assigned bands). For the laboratory procedures, the participants were instructed to use the laboratory protocol *Standard PulseNet Listeria PFGE -One-Day (24-28 h) Standardized Laboratory Protocol for Molecular Subtyping of Listeria monocytogenes by Pulsed Field Gel Electrophoresis (PFGE)* (4)

For the gel analysis, laboratories were instructed to create a local database and analyse the PFGE gel in BN, including normalisation and band assignment. Submission of results included emailing PFGE images, either as a TIFF file alone or as XML export files of the BN analysis.

In the serotyping part the same 10 *L. monocytogenes* strains were tested to assess the participants' ability to obtain the correct serotype. The participants could choose to use either conventional serological methods or multiplex PCR, according to the protocol suggested by Doumith et al. 2005 (6). The serotypes were submitted by email using the Excel sheet provided.

2.5 Data analysis

When the results from the laboratories were received at SSI, the PFGE and serotyping results were added to the dedicated *Listeria* EQA BN database at SSI. For PFGE, the gel quality was evaluated according to the ECDC Food and Waterborne Disease MolSurv Pilot - SOPs 1.0 - Annex 5 - PulseNet US protocol PFGE Image Quality Assessment (TIFF Quality Grading Guidelines - Annex 3) by scoring the gel with respect to seven parameters (scores in the range 1–4, 4 being the top score). The BN analysis was evaluated according to the BioNumerics Gel Analysis Quality Guidelines (Annex 4) with respect to five parameters (scores in the range 1–4, 4 being the top score). The serotyping results were evaluated on the basis of correct results and a total score was obtained.

3. Results

3.1 Participation

The laboratories could choose to participate in the full scheme or only one of the methods. Of the 18 participants, 17 laboratories (94%) participated in the PFGE part and 16 (89%) in serotyping of *Listeria*. Conventional serotyping results were provided by 10 laboratories (56%) and results of the PCR-based method were provided by seven (39%) laboratories (one laboratory performed both methods). Both PFGE and serotyping were completed for 83% of the laboratories (Table 1).

Table 1. Number of FWD-Net laboratories submitting results for each method

Methods	PFGE	Serotyping		PFGE and serotyping
		Traditional	Molecular	
Number of participants	17	10	7	15
% of participants	94	56	39	83

Eighteen laboratories participated in at least one of the methods.

3.2 Pulsed Field Gel Electrophoresis (PFGE)

Seventeen laboratories participated in the *Listeria* PFGE, submitting raw gel images (TIFF files). Thirteen of these laboratories had also analysed the gel using BioNumerics (BN) and submitted the results in the form of an XML file.

3.2.1 Gel quality

All laboratories were able to produce profiles that were recognisable for the relevant EQA strain. The gels, and therefore the profiles for individual strains, varied considerably in quality (Table 2). All gels were graded according to the TIFF Quality Grading Guidelines, with seven parameters being evaluated (Annex 3). In general, an acceptable quality should be achieved for each parameter since a low-quality score in just one category can have a high impact on the ability to analyse the TIFF file further and compare it with other profiles.

For three parameters – 'Cell suspension', 'Lanes' and 'Restriction' – a high average score of 3.5 (between 'good' and 'excellent') was obtained (Table 2). Two parameters – 'DNA degradation' and 'Gel background' – had an average score of 3.2 (which was also between 'good' and 'excellent'). The two remaining parameters – 'Bands' and 'Image acquisition and running conditions' – had an average score below 3 (2.5 and 2.1 respectively, which is between 'fair' and 'good').

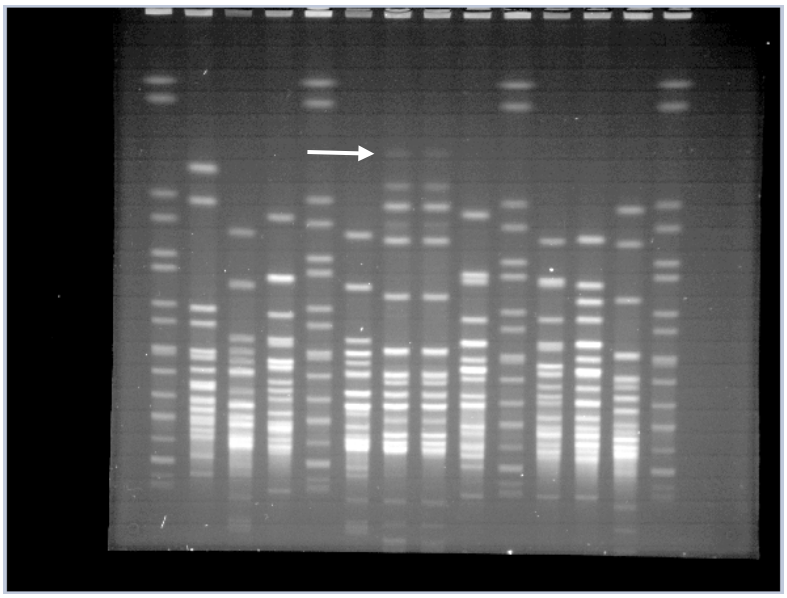
Table 2. Results of PFGE gel quality for 17 participating laboratories

The table shows the average score and the percentage of laboratories obtaining scores 1–4 for the seven TIFF Quality Grading Guideline parameters.

Parameters	1. Poor	2. Fair	3. Good	4. Excellent	Average
Image and running conditions	12%	65%	24%	0%	2.1
Cell suspension	0%	6%	12%	82%	3.8
Bands	24%	35%	6%	35%	2.5
Lanes	0%	6%	12%	82%	3.8
Restriction	0%	12%	24%	65%	3.5
Gel background	0%	6%	65%	29%	3.2
DNA degradation	18%	6%	18%	59%	3.2

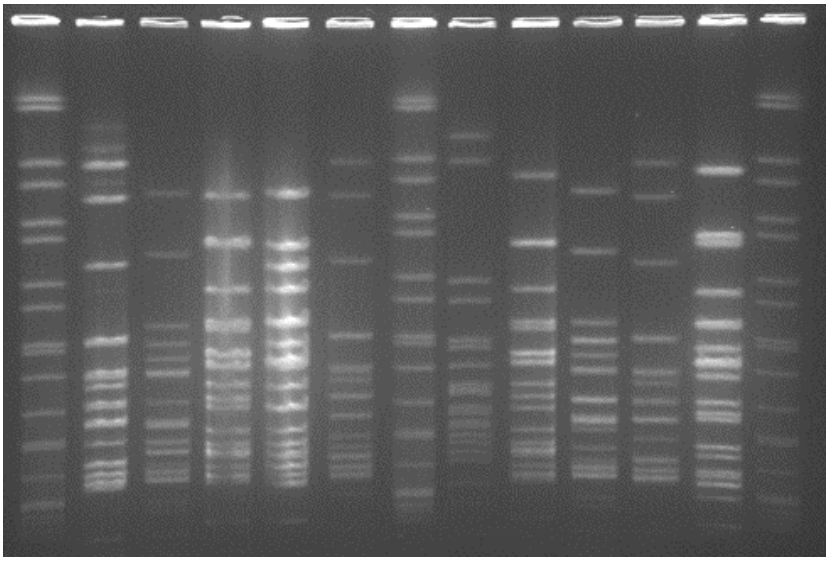
An analysis of the parameter 'Image acquisition and running conditions' shows that scores vary considerably among the participating laboratories (Table 2). Only 24% of participants were graded as 'good' (3), none as 'excellent' (4) and 67% obtained a critical low score ('poor' or 'fair' – 1 and 2). In the parameter 'Bands', 35% of laboratories were graded with a top score (4), while 24% of participants were graded 1, making a proper analysis of the gel impossible in BN.

Figure 1. A gel with a low score in 'Running conditions' and 'DNA degradation'



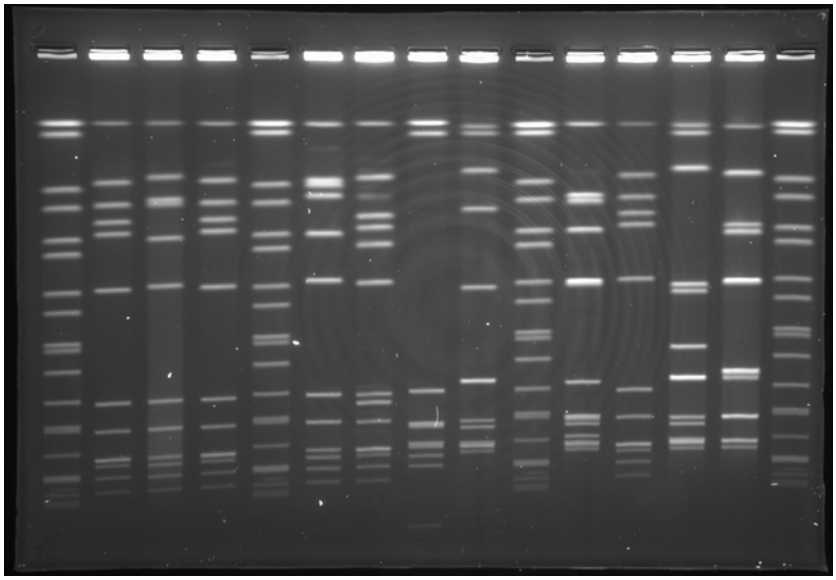
In Figure 1, the gel is scored as 'poor' (1) in the parameter 'Image acquisition and running conditions'. The low score was caused by the running conditions being completely incorrect when compared to the PulseNet International protocol. This meant that the strains were impossible to compare and the gel was impossible to analyse (see also Figure 5 in next section on BN analysis). In Figure 1 the clear presence of shadow bands in lanes 7 and 8 (marked with an arrow) is also noteworthy.

Figure 1. A gel with low score in 'Bands' and 'Running conditions'



The gel shown in Figure 2 is scored 'poor' (1) in the parameter 'Bands'. The low score is due to the fuzziness of many of the bands and band distortion in several lanes, making the gel analysis difficult.

Figure 3. A gel with high score in all seven parameters



Finally, a gel with a high score in all seven parameters is shown in Figure 3. The image has been captured correctly, there is an even distribution of DNA, the bands are clear and there is no background distortion, and only minor debris and shadow bands.

3.2.2 Gel analysis by the use of BioNumerics

Thirteen laboratories had analysed the gel and were able to produce XML files according to the protocol attached to the invitation letter (Annexes 7 and 8). The BN analysis was graded according to the BioNumerics Gel Quality Grading Guidelines developed at SSI, which involves the grading of five parameters (Annex 4).

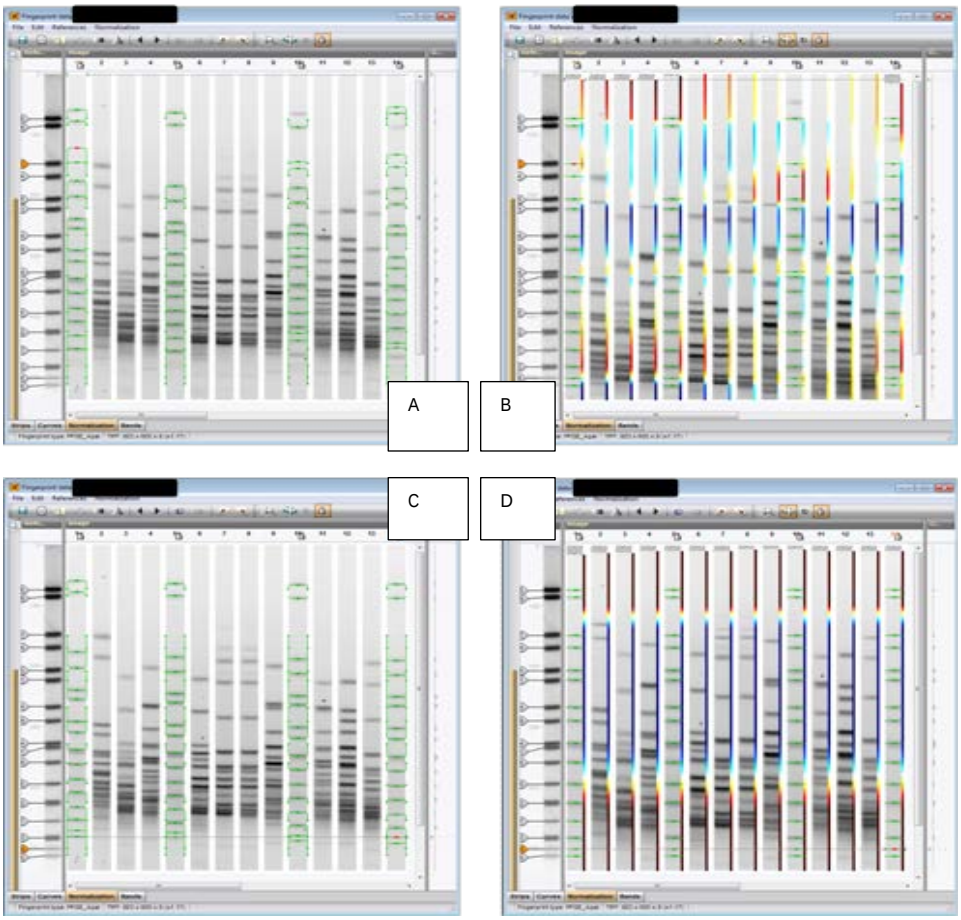
Table 3. Results of the BN analysis obtained by 13 laboratories

The table shows the five gel analysis parameters for the BioNumerics Quality Guidelines, the percentage of laboratories scoring 1–4 and the average score for all laboratories.

Parameters	1. Poor	2. Fair	3. Good	4. Excellent	Average
Position of the gel	15%	0%	46%	38%	3.1
Strips	0%	23%	0%	77%	3.5
Curves	0%	0%	46%	54%	3.5
Normalisation	15%	0%	38%	46%	3.2
Band assignment	0%	0%	38%	54%	3.3

Two parameters – ‘Strips’ and ‘Curves’ – had a high average score, above 3.5. Two parameters – ‘Position of the gel’, ‘Normalisation’ and ‘Band assignment’, were graded slightly lower with an average of 3.1, 3.2 and 3.3, respectively. Fifteen per cent of the laboratories were unable to correctly position the gel in the frame and perform normalisation.

Figure 2. Normalisation and band assignment using BN

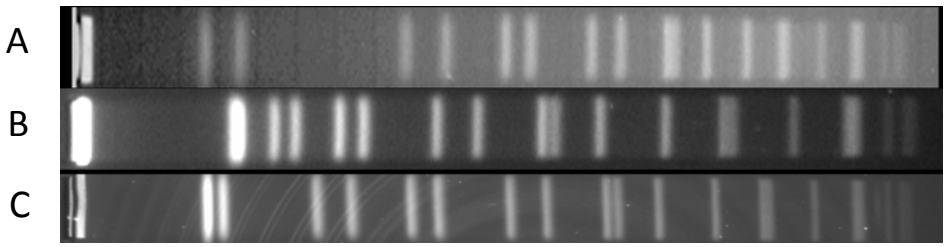


One of the very critical steps is normalisation of the gel, which depends on the running conditions and the correct assignment of the bands in the reference lanes. Figure 4 shows an example of the problems that can arise when normalising a gel. Panel A shows the reference lane band assignment, as performed by the participant. Many of the bands are in the wrong positions and Panel B shows how difficult it is to normalise this gel. In Panel C, the reference band assignment has been corrected and the resulting normalisation is shown in Panel D. The dark colours of distortion bars after normalisation indicate that the electrophoresis was not carried out according to protocol.

Panel A shows the band assignment in the reference lanes, as performed by the participant; bands were not assigned according to the global reference. Panel B shows what happens when normalising according to the band assignment from Panel A. Darker colours indicate more severe adjustments to the standard. Red and blue hues indicate adjustments in different directions. Panel C is similar to Panel A but with corrected band assignment. Panel D shows what happens when normalising according to the new band assignment from Panel C. Another example of the participant's difficulties with the correct running conditions is the comparison of the three reference lanes in Figure 5.

Figure 3. Comparison of three reference systems under different running conditions

Lane C shows *S. Braenderup* H9812 run with the correct protocol. Lanes A and B show examples of *S. Braenderup* not run according to protocol.



Lane B has too little separation in the heavy bands and too much in the light compared to the correct separation shown in Lane C. Lane A is the opposite: too much separation in the heavy bands and too little in the light ones.

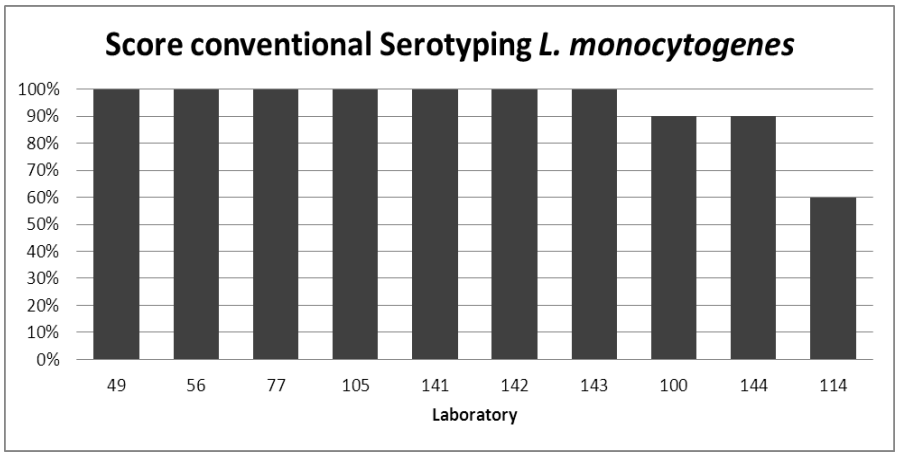
These errors are beyond what the normalisation process can compensate for and the samples run on these gels cannot be used for further comparative analysis.

3.3 Serotyping

3.3.1 Conventional serotyping

Ten laboratories performed the conventional serotyping of *L. monocytogenes* (Figure 6). Seven of these were able to correctly serotype all ten EQA test strains. Two participants mistyped a single isolate, while one participant mistyped four. The laboratory with four errors seems to have had a general problem with the H-types in O-group 1/2.

Figure 6. Results of conventional serotyping of *L. monocytogenes*

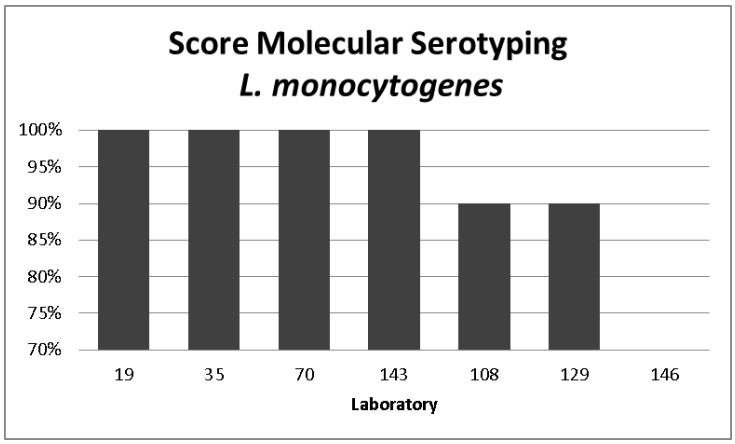


The 10 participating laboratories are represented by arbitrary numbers. Bars represent the percentage of correctly assigned serotypes.

3.3.2 Molecular serotyping

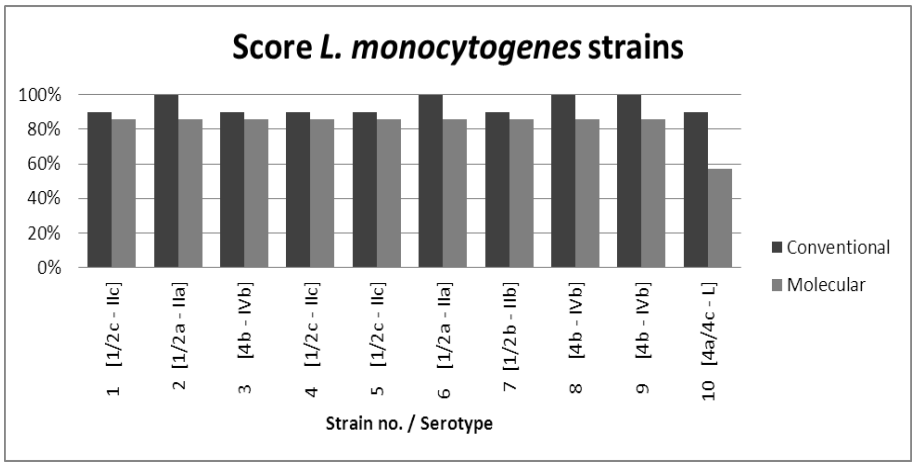
Seven laboratories performed the molecular serotyping of *L. monocytogenes* (Figure 7). Four of these were able to correctly serotype all 10 EQA test strains. Two laboratories had 90% correct and one laboratory, which scored 0%, had used a nomenclature that was not in accordance with Doumith et al. (6).

Figure 4. Results of molecular serotyping for L. monocytogenes



The seven participating laboratories are represented by arbitrary numbers. Bars represent the percentage of correctly assigned serotypes.

Figure 5. EQA strains and average percentage score for each of the ten strains



An analysis of each individual strain in Figure 8 shows that none of the strains were serotyped 100% correctly using molecular serotyping. However, four strains were assigned correctly by all participants using a conventional serotyping method (serotypes (1/2a and 4b). The molecular serotyping of strain 10 was the one that caused the most problems.

Overall, participants performing conventional serotyping identified 94% of the isolates correctly, while participants using molecular serotyping identified 83% correctly.

4. Conclusions

A total of 18 laboratories participated in the EQA-1 scheme. Of these 17 (94%) produced PFGE results and 16 (89%) performed serotyping. Ten laboratories serotyped using the conventional method (56%) and seven laboratories (39%) used molecular serotyping. Fifteen laboratories (83%) completed both parts of the EQA.

PFGE is the gold standard for high-discriminatory typing of *Listeria* and the method is commonly performed with two enzymes (*Apa*I and *Asc*I) for extra discriminatory power. The majority (59%) of the laboratories were able to produce PFGE gels of sufficiently high quality to allow for the profiles to be comparable to those obtained by other laboratories. This comparability primarily relies on the use of correct running conditions, good quality image acquisition and distinct bands. The profiles are subsequently normalised and interpreted using the specialised software BioNumerics. Thirteen of the laboratories (76%) carried out this analysis of their gel and 85% performed well in accordance with the guidelines.

Serotyping of *L. monocytogenes* was included in the EQA-1, both as a phenotypic and a multiplex PCR-based method. The serotyping schemes have been used for surveillance in some parts of Europe for decades. The level obtained for the conventional serotyping was high, with the ten participating laboratories achieving an average score of 94%. Seven out of ten laboratories had 100% correct results. In the molecular (multiplex PCR-based) serotyping one participant reported using a faulty nomenclature and therefore scored 0%. Among the others, the average score for correct results was 97%. Test strains were chosen to cover most of the serotypes present in human isolates. The conventional phenotypic serotyping is much more laborious, requires experienced personnel and takes longer than the PCR-based typing. The PCR-based method can discriminate between five groups while the phenotypic method discriminates between 14 serotypes. It should be noted that either method can be used to identify the vast majority of human strains of listeriosis.

This EQA-1 scheme for typing *L. monocytogenes* is the first EQA organised for the laboratories participating in the FWD-Net on *Listeria* typing methods. The performance of the laboratories in the EQA was encouraging, although the number of participants could have been higher. The molecular surveillance system that is about to be implemented as part of TESSy relies on the capacity of the FWD-Net laboratories to produce comparable typing results. At the moment, the molecular typing method used for EU-wide surveillance is PFGE. Furthermore, phenotypical serotyping is currently included (PCR-based is currently being added) in TESSy and used for surveillance purposes by several EU countries. In general, the quality of serotyping was high. This first EQA for PFGE typing of *Listeria* demonstrates that the majority of participating laboratories were able to produce good results. Less than half of the laboratories produced results that would need to be improved for the inter-laboratory exchange of data. However, for the majority of the technical issues identified, an acceptable quality could be achieved through optimisation of procedures in laboratories, trouble-shooting assistance and training.

5. Discussion

5.1 Pulsed Field Gel Electrophoresis (PFGE)

Seventeen laboratories participated in the PFGE part of the EQA-1. All laboratories were able to produce a PFGE gel and generate an image of the gel (TIFF file). We graded the gel quality according to the TIFF Quality Grading Guidelines which involved evaluation of a gel using seven parameters. Scores were given between 1 and 4 (poor, fair, good and excellent). The majority of the laboratories were able to produce gels of acceptable quality. Evaluation without any parameters was scored as 'poor' (1).

All participants scored 'fair' and above for four of the seven parameters: 'Cell suspension', 'Lanes', 'Restriction' and 'Gel background' (Table 2). The parameters 'Cell suspension', 'Lanes' and 'Gel background' only had one participant each that scored 2. For the 'Restriction' parameter two laboratories scored 2. Consequently there is no need to focus on gel quality issues.

For the parameter 'DNA degradation', three of the participants' gels had so much smearing that it was impossible to analyse them (score 1 – 'poor'). For a highly sensitive method such as PFGE it is very important to follow the protocol. In order to improve DNA degradation, significant efforts can be made by carefully following the instructions regarding the plug preparation, which includes the lysis step, recommended time of restriction for the relevant enzyme, and washing plugs six times, as recommended.

In the category 'Bands', four laboratories were given the lowest score (1 – 'poor') and another five were given the second lowest (2 – 'fair'). Most of these low grades were due to thick or fuzzy bands. In a few cases the bands were too light at the bottom. Both thick and fuzzy bands make close bands much harder to detect properly. The problem is mostly linked to the imaging of the gel where, generally, major improvements can be made regarding exposure time and focus. Many laboratories seemed to enhance the contrast at image acquisition in order to enhance weak bands. Unfortunately, this results in thicker bands and makes it hard to distinguish double bands. This and the overloading of plugs with DNA are the main reasons for a low score in the category 'Bands'.

Some laboratories had problems with the critical category 'Image acquisition and running conditions'. This is critical because incorrect running conditions will make the gel impossible to compare with other gels run under correct conditions. It is very important to apply the running conditions described for the relevant organism as these vary significantly among species. It is also important to have equipment that runs properly as well as making sure that the running temperatures are as described in the protocol. There were a number of other common deviations from the protocol for 'Image acquisition' such as the many laboratories that forgot to fill the whole image with the gel, include wells and leave 1–1.5 cm below the smallest band on the gel. Although less critical than not using the correct running conditions, this can still have a major impact on the ability to assign bands correctly.

Only 76% of the laboratories that performed PFGE did the subsequent gel analysis (i.e. the normalisation and band assignment that provides the actual PFGE profiles for comparison). This analysis has to be done using specialised software, BioNumerics, and some laboratories may not have access to this or may only have limited experience in using BN databases for PFGE analysis. However, to be able to perform national surveillance and submit profiles to the EU-wide Molecular Surveillance System within TESSy, it is important to have the capacity to analyse and interpret PFGE gels. Most of the 11 laboratories (85%) that submitted gel analysis data had performed well in accordance with the guidelines. Only two laboratories had a 'poor' score in the 'Image acquisition and running conditions' category.

5.2 Serotyping

Sixteen laboratories participated in the EQA-1 serotyping exercise. Ten laboratories submitted results from conventional phenotypic serotyping and seven laboratories submitted multiplex PCR-based results. In general the results were very good, with 94% correct from conventional and 83% correct from multiplex PCR-based serotyping.

5.2.1 Conventional serotyping

For the conventional serotyping, 70% of the laboratories were in full agreement with the reference data set, as typed by the EQA organisers. Two participants each had a deviation in a single O-group 4 isolate; one 4b mistyped as 4e and one 4a/4c isolate that lacked the H-type assignment. Strain 10 was originally characterised as serotype 4a, but in the re-check the somatic O-type antigen IX was not clear and the strain was typed to 4c. Therefore both of these answers were accepted in the EQA.

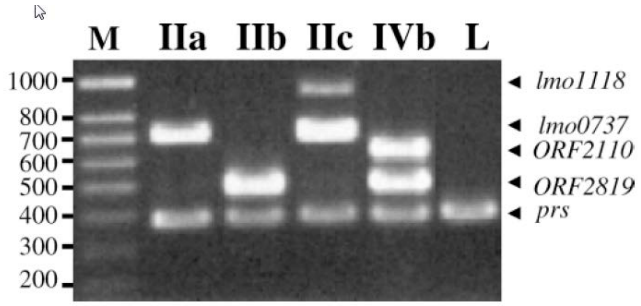
The third participant with deviations had more general problems with the H-antigens in O-group 1/2, mistyping 1/2c as both 1/2a and 1/2b and a 1/2b as 1/2a. This indicates that the participant has problems with both the 'A' and the 'C' H-antigens.

5.2.2 Molecular serotyping

In the PCR multiplex serotyping, 57% of the laboratories were in full agreement with the reference data as typed by the EQA organisers. Most of the errors were due to issues with nomenclature. One participant filled out the form with an incorrect nomenclature – using conventional serological symbols – and therefore failed in all samples. The remaining two participants with errors both had problems with isolate 10 (PCR profile 'L', conventional type '4a/4c'). Furthermore, one laboratory wrote IVa, which is not part of the specified nomenclature according to Doumith et al. (6), and one left the field empty. It is not surprising that this strain caused some problems, since among the serotypes in the EQA, this is the rarest form found in humans. In addition, the multiplex PCR reaction should be negative in all amplifications, with exception of the positive control (Figure 9) and was the probable cause of the problems.

Figure 6. Possible PCR multiplex results - the problematic type 'L'

The isolate 10 – conventional type 4a/4c is located to the right (6).



6. Recommendations

6.1 Laboratories

By evaluating the results obtained from the FWD-Net laboratories in this EQA, it has been possible to identify a number of technical issues that have an impact on the quality of typing results. For each method, performance improvements can be expected by introducing a range of measures.

The quality of PFGE profiles is highly dependent on well-controlled laboratory procedures. Therefore, laboratories should optimise their performance by strictly adhering to the detailed protocols. It might be tempting to take a few shortcuts in some steps, but high quality is dependent on small details such as adhering to the prescribed temperatures, times, number of repeated washing steps, etc. Deviations from the protocol should be avoided unless thoroughly evaluated in each laboratory. Certain elements have to be exactly as described in the protocol, especially the electrophoresis conditions, including temperature and switch times. It should be noted that although many steps are similar for different organisms, there are important differences specific to each species. Several laboratories probably produced a high quality gel, but failed to document this due to sub-optimal staining, de-staining and image capture. It is therefore highly recommended that laboratories take the time to get familiar with the image acquisition and electrophoresis equipment and ensure that this is maintained.

Most laboratories participated in the serotyping exercise which was relatively evenly divided between conventional phenotypic and PCR multiplex-based methods. The results indicate that one of the unexpected problems was nomenclature problems with the PCR-based serotyping. ECDC will standardise the TESSy system using the original Doumith (6) nomenclature for PCR multiplex serotyping, which was the indicated nomenclature for this EQA as well.

A number of other errors were made which could easily have been avoided, such as reading the instructions on how to create and send TIFF and XML files of the PFGE results; following indicated nomenclatures and proofreading the results before submission. For this first EQA on molecular typing, some errors in procedure were accepted and extra results/information/corrections forwarded after the deadline were accepted in some instances. However, this will not be the case in future EQA rounds.

6.2 ECDC and FWD-Net

A total of 18 laboratories participated in the EQA-1 scheme, which is only half of those invited. Future EQAs should aim to have a higher number of participating laboratories. It is encouraging, however, that among the 18 laboratories, both PFGE and serotyping had a high participation rate.

About 40% of the laboratories did not produce PFGE profiles of sufficiently high quality for inter-laboratory comparison and only 13 of the laboratories were able to both produce the raw gel image and perform the data analysis. This indicates that there is a need for capacity-building in the laboratory procedure as well as in the gel analysis and interpretation using BioNumerics.

In the serotyping part the participants were divided between the conventional serological (63%) and the molecular PCR multiplex (44%) methods. The correlation in results between these methods is good but the difference in time consumption and hence cost is considerable. Therefore, if serotyping results are required for EU-wide surveillance it is probably more realistic to expand the use of the PCR-based method. In principal, the capacity to use this method should be available in all laboratories with basic PCR capacity.

In the longer term, whole genome sequence (WGS)-based methods will most likely take over from both of the methods used in this EQA as laboratories begin to implement WGS. At the moment, no harmonised procedures for WGS data analysis exist for use in routine surveillance and international comparison of *Listeria* strains.

6.3 The EQA provider

The scheme used for grading the PFGE gel quality is part of the ECDC SOP for molecular typing data in TESSy, adopted from PulseNet USA. Applying the scheme for evaluation of gel quality in this EQA has demonstrated that in some cases there is no clear correspondence between the score and the suitability of the gel for inter-laboratory comparability. The EQA provider will therefore modify the interpretation of the scoring system so that a gel is always given a 'poor' score in a parameter where quality is so deficient that it is impossible to use the gel for reliable comparison with those obtained in other laboratories.

The feedback on the organisation of the EQA given by the FWD-Net laboratories indicates that the time schedule was tight for laboratories participating in the three EQAs for the typing of foodborne pathogens as well as other EQA schemes. It would therefore be preferable to separate the deadlines as much as possible for the next EQA and generally give more time to finish the results. Since results need to be evaluated individually due to the visual evaluation of the PFGE gels and analysis, it is also necessary to allow for reasonable time from receipt of results to

the individual evaluation reports and the final EQA report. Furthermore, individual feedback and troubleshooting regarding the molecular methods are part of the task for the EQA organiser. This can be quite time consuming and therefore the organisers should allow time for this, especially during the period after the participants have received the individual reports.

As mentioned above, a number of deviations were accepted in this first EQA on molecular typing methods. Some of these might have been caused by our guidelines to the participants not being sufficiently detailed (e.g. how to set up and use a BioNumerics database for the EQA data, how to create and name the relevant files, etc.) Before the next EQA, all guidelines will be reviewed in order to ensure that they provide sufficient detail. However, this also implies that deadlines for submission of results according to the guidelines will be enforced in the future. Furthermore, we will suggest a number of additional performance criteria related to the reporting of results (e.g. the use of correct numbering and file formats.)

7. References

1. Regulation (EC) No 851/2004 of the European Parliament and of the Council of 21 April 2004 establishing a European Centre for Disease Prevention and Control, Article 5.3. Available at: http://ecdc.europa.eu/en/aboutus/Key%20Documents/0404_KD_Regulation_establishing_ECDC.pdf
2. Decision No 2119/98/EC of the European Parliament and of the Council of 24 September 1998 setting up a network for the epidemiological surveillance and control of communicable diseases in the Community. Available at: http://ec.europa.eu/health/communicable_diseases/early_warning/comm_legislation_en.htm
3. The European Union Summary Report on Trends and Sources of Zoonoses, Zoonotic Agents and Food-borne Outbreaks in 2011; EFSA Journal 2013;11(4):3129. Available at: http://www.efsa.europa.eu/en/efsajournal/pub/3129.htm
4. Conformity assessment – General requirements for proficiency testing. ISO/IEC 17043:2010. http://www.iso.org/iso/catalogue_detail.htm?csnumber=29366
5. Standard PulseNet Listeria PFGE -One-Day (24-28 h) Standardized Laboratory Protocol for Molecular Subtyping of Listeria monocytogenes by Pulsed Field Gel Electrophoresis (PFGE). Available at: http://www.pulsenetinternational.org/SiteCollectionDocuments/pfge/5.3_2009_PNetStandProtLMonocytogenes.pdf.
6. Doumith M, Buchrieser C, Glaser P, Jacquet C, Martin P. Differentiation of the major *Listeria monocytogenes* serovars by multiplex PCR. JCM 2004;42,3819-3822.

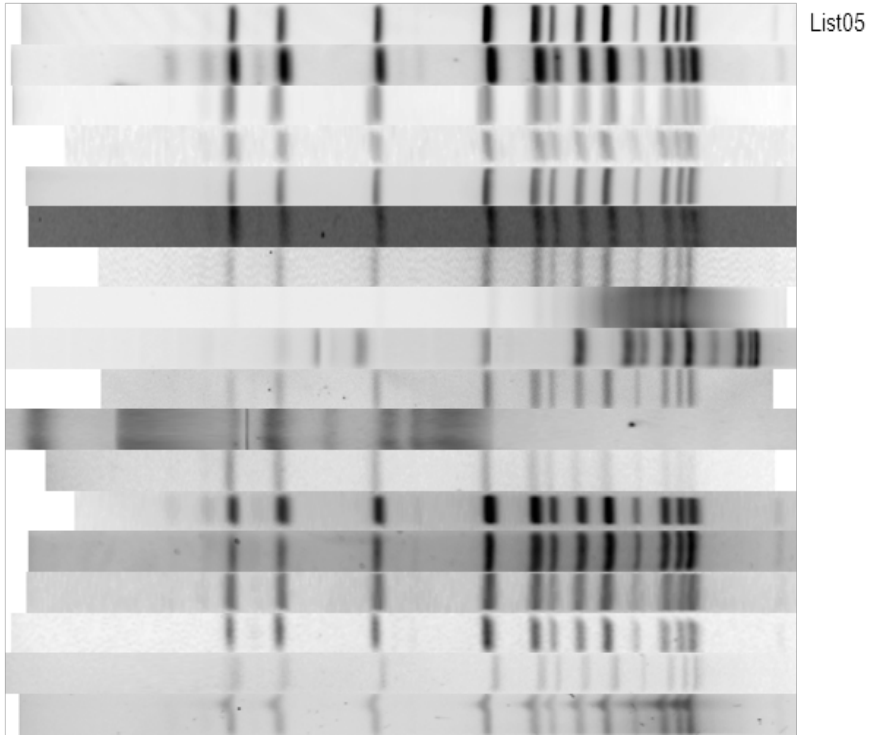
Annex 1. List of participants

Country	National institute	Laboratory
Austria	AGES – Österreichische Agentur für Gesundheit und Ernährungssicherheit	Listeria Reference Laboratory
Belgium	Institute of Public Health	National Reference Centre for Salmonella and Shigella
Denmark	Statens Serum Institut	Unit of Foodborne Infections
Finland	THL - Institute of Health and Welfare	Bacteriology Unit
France	Institut Pasteur	CNR/CCOMS listeria
Germany	Robert Koch Institute	NRC for Salmonella and other Bacterial Enterics
Greece	National School of Public Health CLBH/HCDCP	National Reference Centre for Salmonella and other enteropathogens
Hungary	National Public Health and Medical Officer Service	Department of Phage-typing and Molecular Epidemiology
Ireland	University Hospital Galway	National Salmonella, Shigella and Listeria Reference Laboratory
Italy	Istituto Superiore di Sanità	Department of Veterinary Public Health and Food Safety
Latvia	Riga East Clinical University Hospital	Bacteriology Department
Luxembourg	Laboratoire National de Santé	Department of Microbiology
Netherlands	National Institute for Public Health and the Environment	Laboratory for Infectious Diseases and Perinatal Screening
Slovenia	National Institute of Public Health	Department of Medical Microbiology
Spain	Institute of Health Carlos III	Reference Laboratory for Listeria
Sweden	Smittskydsinstitutet	Food and Water Unit
United Kingdom	Gastrointestinal Bacteria Reference Unit - Health Protection Agency	Foodborne Pathogens Reference Services
Turkey	Public Health Institution of Turkey	National Reference Laboratory for Enteric Pathogens

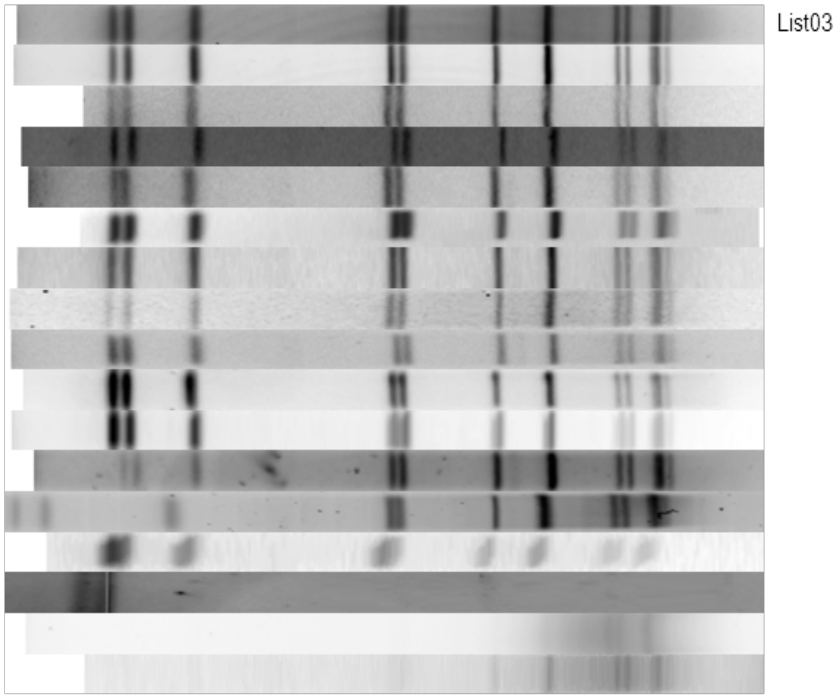
Annex 2. Examples of PFGE profiles

Profiles from the participants (17 profiles with ApaI and 16 with AscI)

PFGE_ApaI



PFGE_AscI



Annex 3. TIFF Quality Grading Guidelines

Parameter	TIFF Quality Grading Guidelines			
	Excellent	**Good**	**Fair**	**Poor**
Image acquisition and running conditions	By protocol, for example: - Gel fills whole TIFF - Wells included on TIFF - Bottom band of standard 1-1.5 cm from bottom of gel	Gel does not fill whole TIFF but band finding is not affected.	Not protocol - for example one of the following: - Gel does not fill whole TIFF and band finding is affected - Wells not included on TIFF - Bottom band of standard not 1-1.5 cm from bottom of gel - Band spacing of standards does not match global standard.	Not protocol –for example > one of the following: - Gel does not fill whole TIFF and this affects band finding - Wells not included on TIFF - Bottom band of standard not 1-1.5 cm from bottom of gel - Band spacing of standards does not match global standard.
Cell suspensions	The cell concentration is approximately the same in each lane	One or two lanes contain darker or lighter bands than the other lanes.	> two lanes contain darker or lighter bands than the other lanes, or At least one lane is much darker or lighter than the other lanes, making the gel difficult to analyse	The cell concentrations are uneven from lane to lane, making it impossible to analyse the gel.
Bands	Clear and distinct all the way to the bottom of the gel	- Slight band distortion in one lane but this does not interfere with analysis - Bands are slightly fuzzy and/or slanted - A few bands (≤ 3) are difficult to see clearly (i.e. DNA overload) especially at the bottom of the gel.	Some band distortion (i.e. nicks) in two to three lanes but can still be analysed. Fuzzy bands Some bands (four or five) are too thick Bands at the bottom of the gel are light but can be analysed.	- Band distortion that makes analysis difficult - Very fuzzy bands - Many bands too thick to distinguish - Bands at the bottom of the gel too light to distinguish
Lanes	Straight	- Slight 'smiling' (higher bands in outside lanes than inside) - Lanes gradually run longer towards the right or left (can still be analysed)	- Significant 'smiling' - Slight curves on the outside lanes - Can still be analysed	'Smiling' or curving that interferes with analysis
Restriction	Complete restriction in all lanes	One or two faint shadow bands on the gel	- One lane with many shadow bands - A few shadow bands spread out over several lanes	- More than one lane with several shadow bands - Lots of shadow bands over the whole gel.
Gel background	Clear	- Mostly clear background - Minor debris present that does not affect analysis	- Some debris present that may or may not make analysis difficult (e.g. auto band search finds too many bands) - Background caused by photographing a gel with very light bands (image contrast was 'brought up' in photographing gel (makes image look grainy).	Lots of debris present that may or may not make analysis difficult (i.e. auto band search finds too many bands).
DNA degradation (smearing in the lanes)	Not present	Minor background (smearing) in a few lanes but bands are clear.	Significant smearing in one to two lanes that may or may not make analysis difficult. Minor background (smearing) in many lanes.	Significant smearing in > two lanes that may or may not make analysis difficult. - Smearing so that a lane cannot be analysed unless untypeable (thiourea required).

Annex 4. BioNumerics Gel Analysis Quality Guidelines

Parameters/scores	Excellent	Good	Fair	Poor
Position of gel	Excellent placement of frame and gel inverted.	The image frame is positioned too low. Too much space framed at the bottom of the gel. Too much space framed on the sides of the gel. (Guidelines recommend framing just beneath the wells).		Frame includes wells Gel not with light bands of dark background
Strips	All lanes correctly defined.	A single lane is not correctly defined.	Lanes defined too narrowly (users should include the whole gel lane).	Lanes not defined correctly – Too wide/not following the actual gel lanes
Curves	1/3 or more of the lane is used for averaging curve thickness.	Curves defined either as very narrow strip or encompassing almost the whole lane. (Average thickness recommended to be reduced/increased to ~ 1/3 of the lane).		
Normalisation	All bands assigned correctly in all reference lanes.	Bottom band at 20.5 kb was not assigned in some of the reference lanes.		Missing assignment of bands in the reference lanes 5,10 and 15. The references were not included in the submitted XML file (follow the XML export guide).
Band assignment	Excellent band assignment with regard to the quality of the gel.	Some double bands are assigned incorrectly.	Some shadow bands are assigned. (Guidelines require control of band assignment after using auto search).	The positions are correct but double bands assigned at the exact same positions. Band assignment incorrect (commonly caused by thickness of the bands/overexposure). Only used auto search to find bands, no manual corrections. (Guidelines require control of band assignment after using auto search).

Annex 5. Scoring of the PFGE results

Gel quality

Parameters\laboratory	141	142	35	19	105	143	129	49	56	70	77	130	114	100	108	144	138
Image and running conditions	2	2	3	3	1	3	2	2	2	2	2	3	2	1	2	2	2
Cell suspension	3	4	4	4	4	4	4	4	4	4	2	4	4	4	3	4	4
Bands	1	2	2	4	2	4	2	1	4	4	1	2	4	4	3	2	1
Lanes	4	4	4	4	4	4	4	2	4	4	3	3	4	4	4	4	4
Restriction	4	2	4	3	4	3	4	4	4	4	2	4	4	4	4	3	3
Gel background	2	3	3	3	3	3	3	3	3	4	3	4	4	3	4	4	3
DNA degradation	3	4	4	4	3	3	2	4	4	4	4	4	1	1	4	4	1

BN analysis

Parameters\laboratory	141	142	35	19	105	143	129	49	56	70	108	77	130
Position of the gel	3	3	4	4	1	4	3	4	4	1	3	3	3
Strips	4	4	4	4	2	4	4	4	4	4	4	2	2
Curves	3	4	3	4	3	4	4	4	4	3	4	3	3
Normalisation	4	3	4	4	1	4	3	4	3	1	4	3	3
Band assignment	3	4	4	4	0	3	4	3	4	4	4	3	3

Annex 6. Serotyping results

Conventional serotyping

Strain (Serotype)/Laboratory	141	142	105	143	144	49	56	77	100	114
1 (1/2c)	1/2c	1/2c	1/2c	1/2c	1/2c	1/2c	1/2c	1/2c	1/2c	1/2a
2 (1/2a)	1/2a	1/2a	1/2a	1/2a	1/2a	1/2a	1/2a	1/2a	1/2a	1/2a
3 (4b)	4b	4b	4b	4b	4b	4b	4b	4b	4e	4b
4 (1/2c)	1/2c	1/2c	1/2c	1/2c	1/2c	1/2c	1/2c	1/2c	1/2c	1/2a
5 (1/2c)	1/2c	1/2c	1/2c	1/2c	1/2c	1/2c	1/2c	1/2c	1/2c	1/2b
6 (1/2a)	1/2a	1/2a	1/2a	1/2a	1/2a	1/2a	1/2a	1/2a	1/2a	1/2a
7 (1/2b)	1/2b	1/2b	1/2b	1/2b	1/2b	1/2b	1/2b	1/2b	1/2b	1/2a
8 (4b)	4b	4b	4b	4b	4b	4b	4b	4b	4b	4b
9 (4b)	4b	4b	4b	4b	4b	4b	4b	4b	4b	4b
10 (4a/4c)	4a	4a	4a	4a	4	4c	4c	4c	4c	4a

Molecular serotyping

Strain (Serotype)/Laboratory	35	129	143	70	108	19	146 *
1 (IIc)	IIc	IIc	Iic	IIc	IIc	IIc	1/2c
2 (Iia)	IIa	IIa	Iia	IIa	IIa	IIa	1/2a
3 (IVb)	IVb	IVb	Ivb	IVb	IVb	IVb	4
4 (IIc)	IIc	IIc	Iic	IIc	IIc	IIc	1/2c
5 (IIc)	IIc	IIc	Iic	IIc	IIc	IIc	1/2c
6 (IIa)	IIa	IIa	Iia	IIa	IIa	IIa	1/2a
7 (IIb)	IIb	IIb	Iib	IIb	IIb	IIb	1/2b
8 (IVb)	IVb	IVb	Ivb	IVb	IVb	IVb	4
9 (IVb)	IVb	IVb	Ivb	IVb	IVb	IVb	4
10 (L)	L	0	L	L	IVa	L	4a

Incorrect result

*Incorrect nomenclature

Annex 7. Guide to BN database

Guide for setting up your EQA database

There are two ways to set up an EQA database. If you have BioNumerics Version 6 or above you can just use the ready-made database(s) that have been sent out together with these instructions. The database is packaged in the zip archive called “Listeria EQA db.zip” or “Salmonella EQA db.zip”. If you have an older version of BioNumerics (prior to 6.0) or wish to set up the database yourself, please use the instructions below.

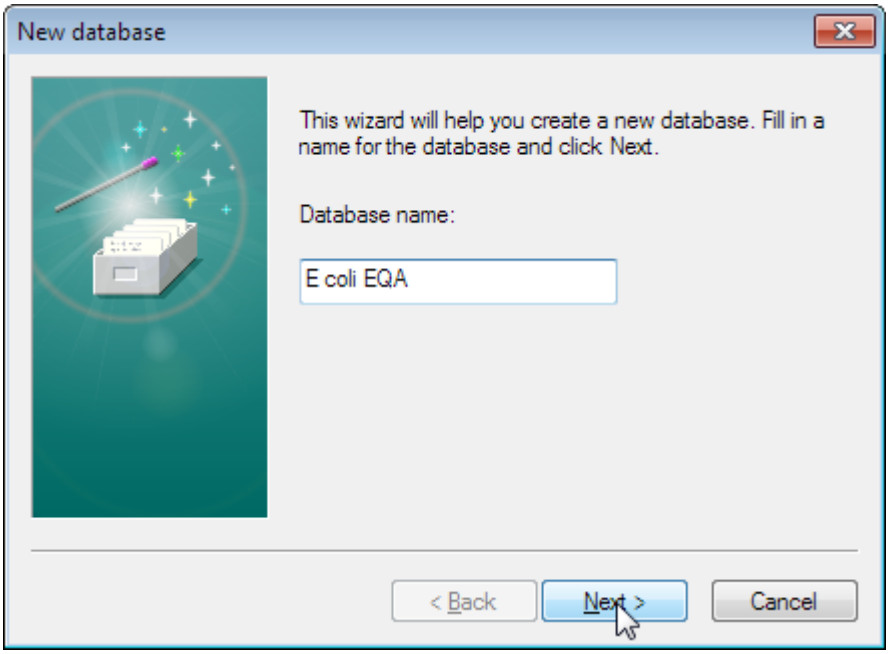
Setting up a database from scratch

All the images in this instruction refer to *E. coli* so just exchange ‘E coli’ for either ‘Salmonella’ or ‘Listeria’ when setting up the databases.

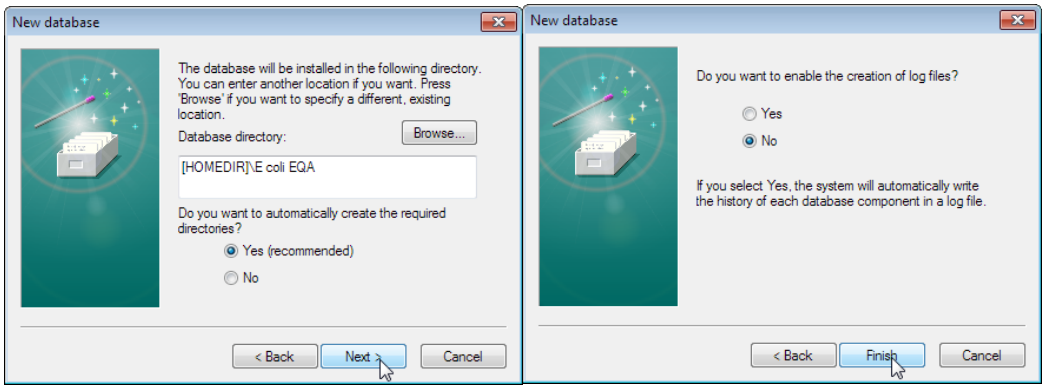
The database is created by first setting up an empty database and then importing an XML file containing experiment settings and field definitions.

Setting up the empty database

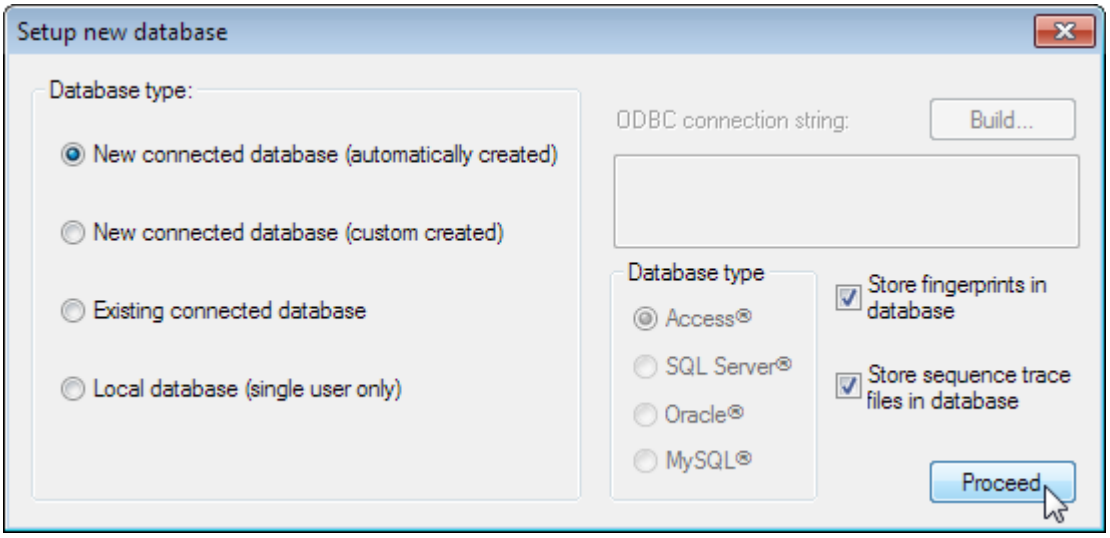
Choose ‘Create a new database’.



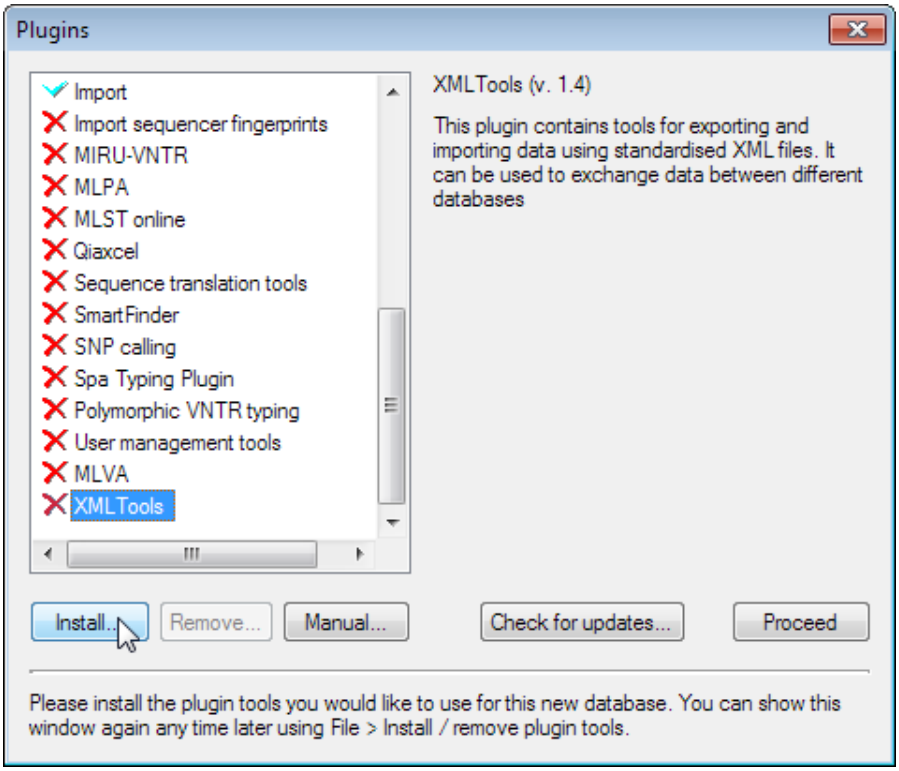
Enter a database name, ‘Salmonella EQA’ or ‘Listeria EQA’.



Use default values.



Choose a new connected database (Access type).

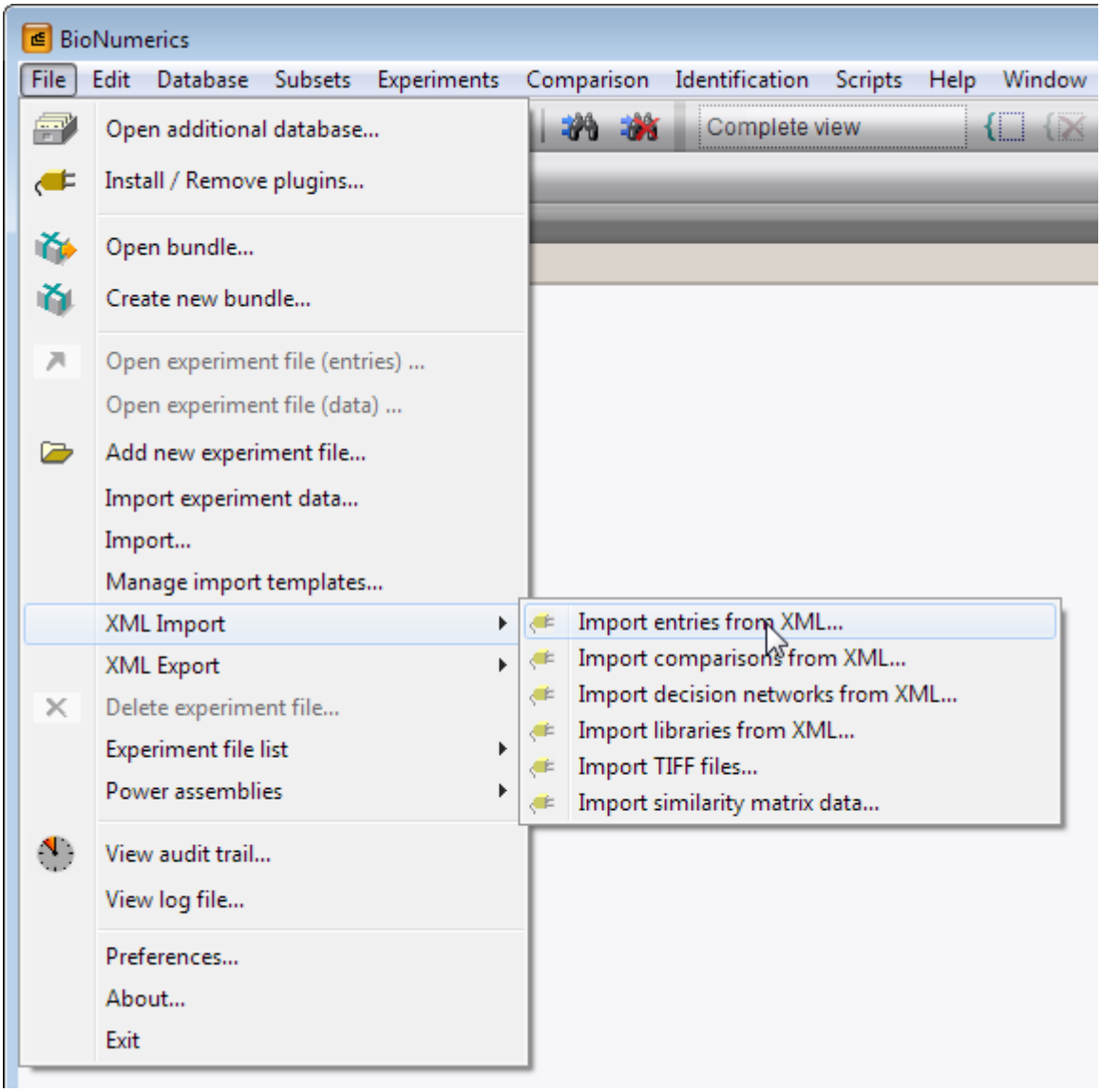


When choosing plugins, add the XML Tools plugin by selecting the plugin from the list and pressing 'Install...'

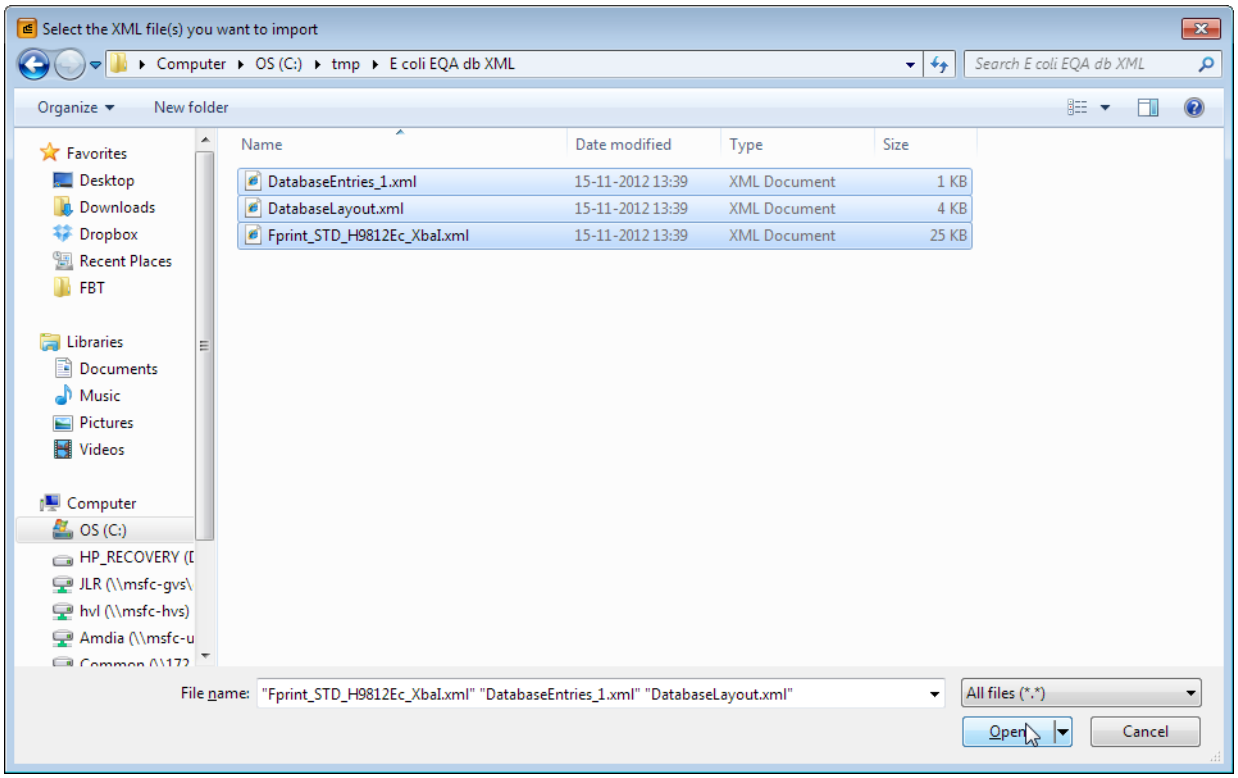
Proceed to the next window. The database is now set up and ready to import the database definitions.

Importing the XML structure

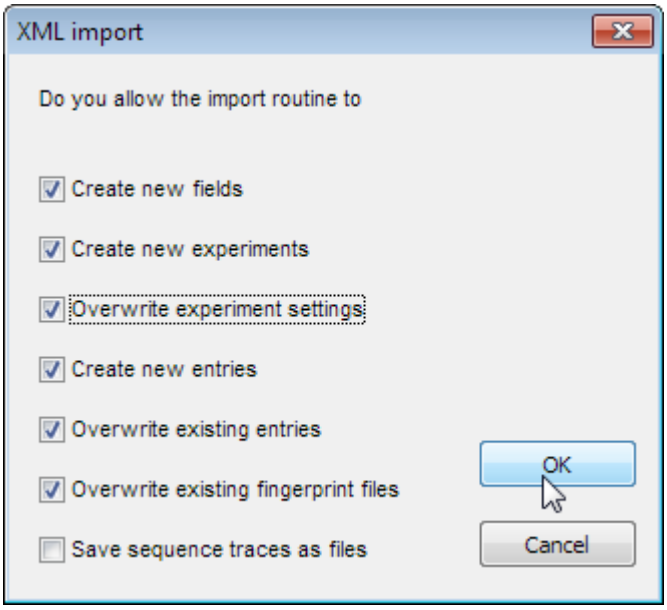
Unzip the contents of the supplied file 'Listeria EQA db XML.zip' or 'Salmonella EQA db XML.zip'.



Select 'Import entries from XML' in the menu.



Locate your newly unzipped files. Select all of them and click on 'Open'.



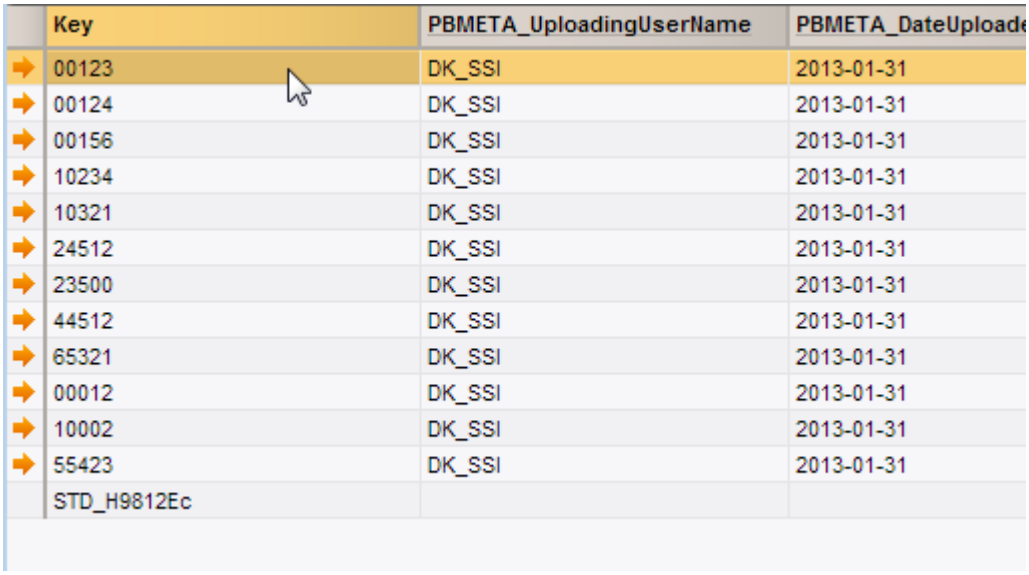
Mark the box 'Overwrite experiment settings' and click 'OK'.

Restart the database.

Annex 8. Guide to XML export

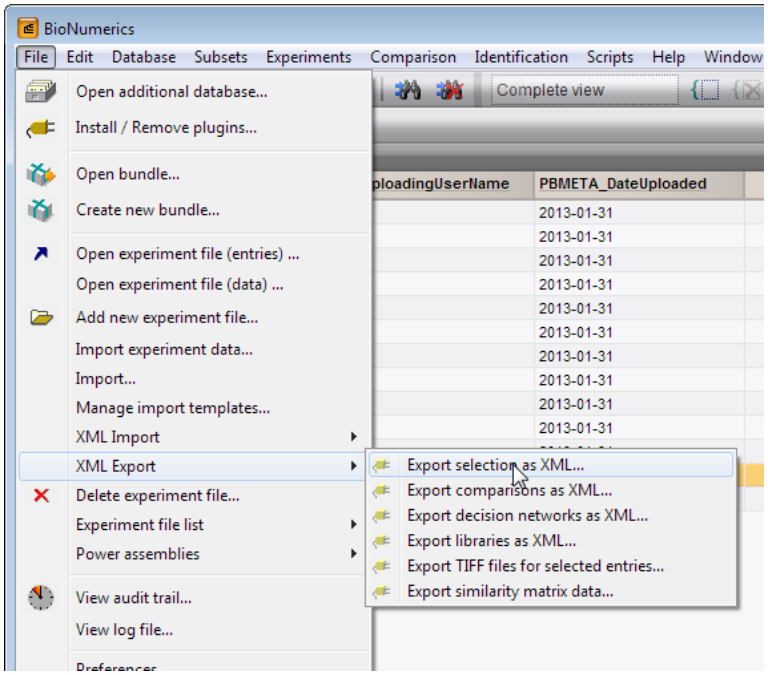
Exporting XML data from your database

After analysing your data, select all the isolates that you would like to export.

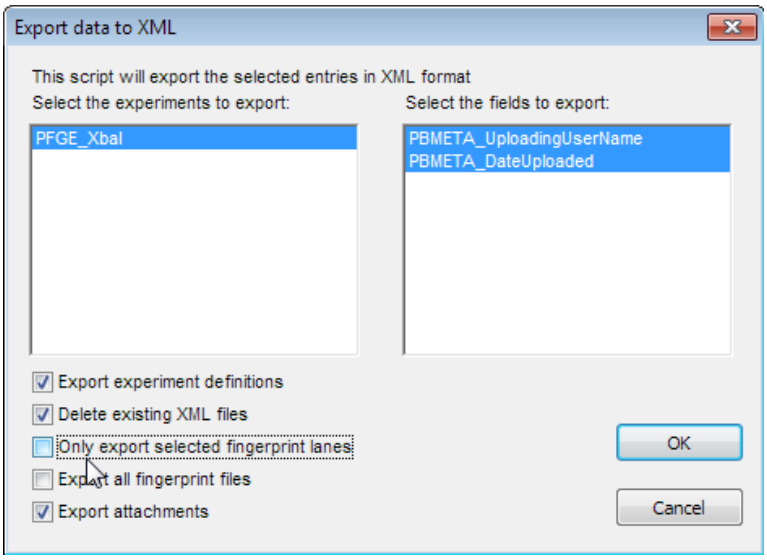


Key	PBMETA_UploadingUserName	PBMETA_DateUploade
00123	DK_SSI	2013-01-31
00124	DK_SSI	2013-01-31
00156	DK_SSI	2013-01-31
10234	DK_SSI	2013-01-31
10321	DK_SSI	2013-01-31
24512	DK_SSI	2013-01-31
23500	DK_SSI	2013-01-31
44512	DK_SSI	2013-01-31
65321	DK_SSI	2013-01-31
00012	DK_SSI	2013-01-31
10002	DK_SSI	2013-01-31
55423	DK_SSI	2013-01-31
STD_H9812Ec		

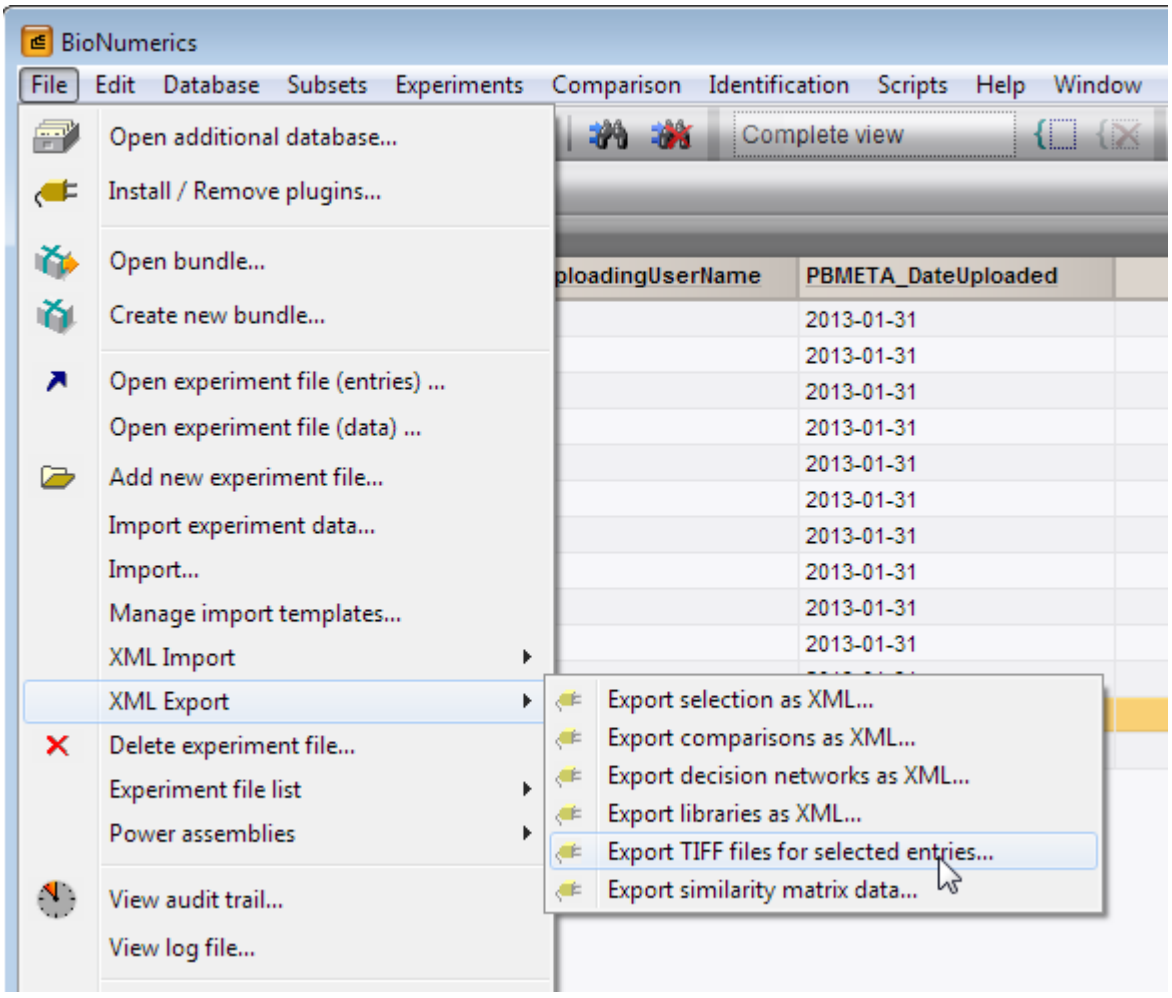
Export selection as XML.



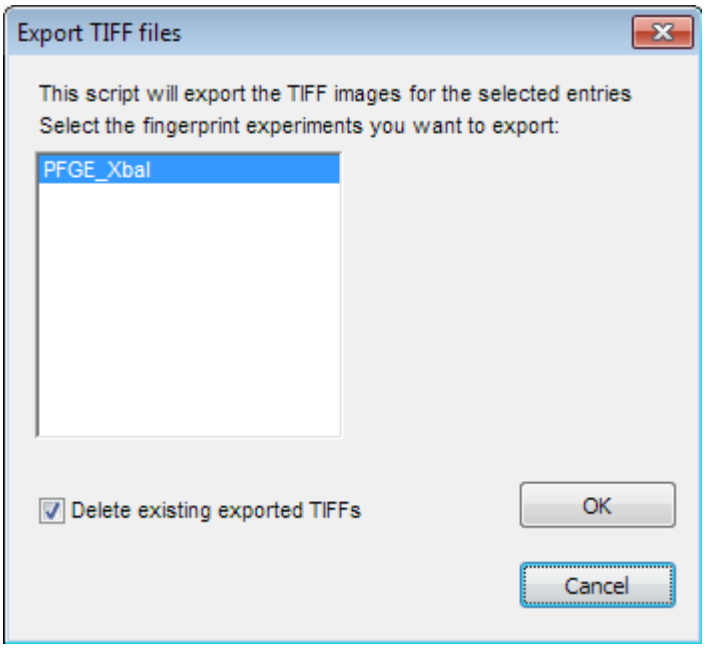
De-select the check box 'Only export selected fingerprint lanes'.



Now export the TIFF file(s).



Select which experiments to export. In the case of Listeria you can export both enzymes at the same time.



Now locate the EXPORT directory in your database directory. Send all XML and TIFF files located there via mail. Please compress them into a zip archive. One way of creating the zip archive is to mark all the XML and TIFF files, right click on them and choose 'Send to → Compressed (zipped) folder'.