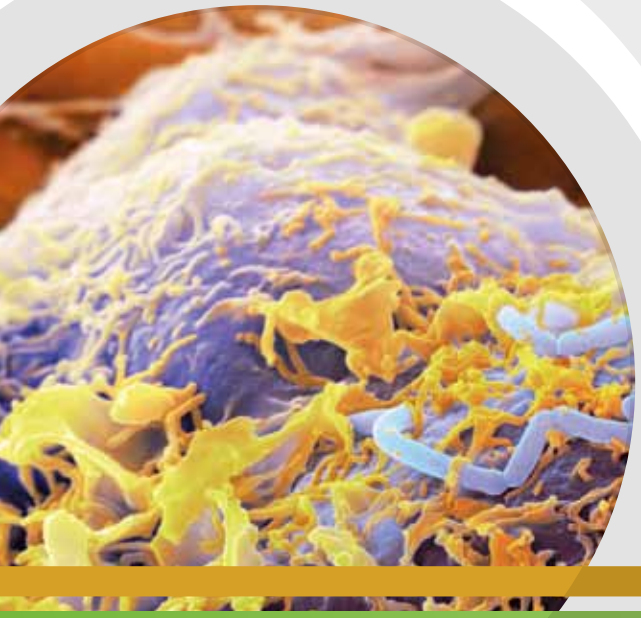


TECHNICAL REPORT

Second external quality assessment scheme for *Listeria monocytogenes* typing

www.ecdc.europa.eu

ECDC TECHNICAL REPORT

Second external quality assessment scheme for *Listeria monocytogenes* typing

This report was commissioned by the European Centre for Disease Prevention and Control (ECDC), coordinated by Taina Niskanen (ECDC Food- and Waterborne Diseases and Zoonoses programme) and produced by Susanne Schjørring, Jonas T. Larsson, Mia Torpdahl and Eva Møller Nielsen of the Foodborne Infections Unit at Statens Serum Institut, Artillerivej 5, DK-2300 Copenhagen S, Denmark.

Suggested citation: European Centre for Disease Prevention and Control. External quality assessment scheme for *Listeria monocytogenes* typing Stockholm: ECDC; 2014.

Stockholm, November 2014

ISBN 978-92-9193-610-6

doi 10.2900/11423

Catalogue number TQ-06-14-144-EN-N

Contents

Figures

Tables

Abbreviations

BN	BioNumerics
ECDC	European Centre for Disease Prevention and Control
EQA	External Quality Assessment
EU/EEA	European Union/European Economic Area
FWD	Food- and Waterborne Diseases and Zoonoses
PFGE	Pulsed Field Gel Electrophoresis
SSI	Statens Serum Institut
TESSY	The European Surveillance System
WGS	Whole genome sequence

Executive summary

This report presents the results of the second round of the *Listeria* External Quality Assessment (EQA) scheme for the typing of *Listeria monocytogenes* (further EQA-2). 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-2 which took place between October and December 2013.

Listeriosis is a relatively rare but serious foodborne disease, with 1 642 confirmed human cases reported in EU in 2012 (0.41 cases per 100 000). Compared to other foodborne infections under EU surveillance, listeriosis caused the most severe human disease, with 91.6% of the cases hospitalised.

Since 2007, ECDC's programme on Food- and Waterborne Diseases and Zoonoses (FWD) has been responsible for the EU-wide surveillance of listeriosis and 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 in using more advanced and discriminatory typing techniques for surveillance of foodborne infections. In 2012, ECDC initiated a pilot project on enhanced surveillance incorporating molecular typing data into TESSy (TESSy-MSS - 'molecular surveillance system').

The objectives of this EQA are to assess the quality of PFGE and serotyping and the comparability of the collected results produced by participating national public health reference laboratories in the European Union (EU), European Economic Area (EEA) and EU candidate countries. Strains for the EQA were selected from strains 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-2: 14 laboratories (78%) produced PFGE results, 17 laboratories (94%) participated in the serotyping exercise. Nine of these 17 laboratories performed conventional, phenotypic serotyping, while fourteen performed molecular, PCR-based serotyping.

The majority (86%) 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 BioNumerics software. 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 78%, a decrease from EQA-1 mainly attributed to one difficult strain. In the molecular (multiplex PCR-based) serotyping participants obtained an average score of 94%.

This EQA-2 scheme for typing of *Listeria* was the second EQA for laboratories participating in the FWD-Net. Their level of performance in the EQA was encouraging, although the numbers of participants were lower than in EQA-1. The molecular surveillance system being 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. Phenotypic serotyping is currently included in TESSy and PCR-based serotyping has been added for 2012. This data is being used for surveillance purposes by several EU countries. In general, countries demonstrated a high proficiency level for serotyping. Two laboratories shifted from the conventional method to the molecular and an additional five laboratories added molecular data to their submission of results. The results of the EQA-2 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. Only two laboratories produced results that need to be improved for inter-laboratory exchange of data. However, to achieve an acceptable quality, the technical issues identified could have been overcome by optimising laboratory procedures and providing trouble-shooting assistance and training.

1. Introduction

1.1. Background

The European Centre for Disease Prevention and Control (ECDC) is a European Union 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. As part of 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].

An external quality assessment (EQA) is a part of a quality management system that uses an external evaluator to assess the performance of laboratories on material that is 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 needs of improvement in laboratory diagnostic capacities relevant to surveillance of diseases listed in Decision No 2119/98/EC [2], and to ensure the reliability and comparability of results in laboratories from all EU/EEA countries. The main purposes of EQA schemes are:

- 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
- provision of continuing education
- identification of needs for training activities.

Since 2012 the Unit of Foodborne Infections at Statens Serum Institut in Denmark has been the EQA provider for the three lots covering *Salmonella*, Shiga toxin/verocytotoxin-producing *Escherichia coli* (STEC/VTEC) and *Listeria monocytogenes*. 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 second EQA-exercise under this contract (Listeria EQA-2).

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 2012, 1 642 confirmed human cases were reported in the EU corresponding to a notification rate of 0.41 cases per 100 000 population [3]. Compared to other foodborne infections under EU surveillance, listeriosis caused the most severe human disease, with 91.6 % of the cases hospitalised.

Since 2007, ECDC's programme on Food- and Waterborne Diseases and Zoonoses (FWD) has been responsible for the EU-wide surveillance of listeriosis and facilitation of 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, discriminatory typing techniques in the surveillance of foodborne infections. Therefore, in 2012 ECDC initiated a pilot project on enhanced surveillance incorporating molecular typing data ('molecular surveillance'). In the first pilot phase, three selected FWD-Net pathogens were included: *Salmonella*, *Listeria monocytogenes*, and Shiga toxin/verocytoxin-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 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 quality 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 covering these methods are needed. Therefore, since 2012 ECDC's FWD programme has been supporting EQA schemes focusing on expert assistance for mainly molecular typing methods. The focus organisms are: *Salmonella spp.*, Shiga toxin/verocytoxin-producing *Escherichia coli* (STEC/VTEC) and *L. monocytogenes.*

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-2 scheme

1.3.1 Pulsed-Field Gel Electrophoresis (PFGE) typing

The objective of the EQA-2 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-2 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-2 was funded by ECDC and arranged by Statens Serum Institut (SSI) in accordance with the International Standard ISO/IEC 17043:2010 [4]. The EQA-2 included PFGE and serotyping and was carried out between October and December 2013.

Invitations were emailed to ECDC contact points in the Food- and Waterborne Diseases Network (FWD-Net) (30 countries) by 3 September 2013. In addition, the ECDC coordinator sent invitations to the EU acceding and candidate countries; Montenegro, Serbia, the former Yugoslav Republic of Macedonia and Turkey.

Twenty–two public health national reference laboratories in EU/EEA and EU candidate countries accepted the invitation to participate but four laboratories later communicated that they were unable to perform the tests. 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 on 7 October 2013. The participants were asked to submit their results by e-mail to list.eqa@ssi.dk and complete an online Google form before 28 November 2013: (https://docs.google.com/forms/d/1OTreXO5Jqgih3EiUArO9dPm_1xYCGJZWoW6eBN59ZSk/viewform)

2.2 Selection of strains

Strains were selected for the EQA-2 scheme 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 17 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, 3a and 4b. A strain from EQA-1 was included to evaluate the improvement and strains from the EURLs EQA were included for comparison in the future (See Annex 8). The characteristics of the ten *L. monocytogenes* test strains used in the EQA-2 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-2 for PFGE could request the *Salmonella* Braenderup H9812 strain for use as a molecular size marker.

2.3 Carriage of strains

At the beginning of October all strains were blinded and packed and shipment was initiated on 7 October 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 damage to the shipment or errors in the specific strain number.

On 10 October 2013, instructions on how to submit results were e-mailed to participants. This included an updated version of the EQA protocol, the link to the online submission form and the zip files for the Bionumerics database experiment settings (PFGE part) as well as guidelines on how to export XML files from Bionumerics (Annex 9 and 10).

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 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) Standardised Laboratory Protocol for Molecular Subtyping of *Listeria monocytogenes* by Pulsed Field Gel Electrophoresis (PFGE)' [5].

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

2.5 Data analysis

When the results from the laboratories were received, the PFGE and serotyping results were added to a dedicated *Listeria* EQA-2 Bionumerics database at SSI. For PFGE, the gel quality was evaluated according to a modified version of the ECDC Food and Waterborne Disease MolSurv Pilot - SOPs 1.0 - Annex 5 - PulseNet US protocol PFGE Image Quality Assessment (TIFF Quality Grading Guidelines 2014 - Annex 3) by scoring the gel with respect to seven parameters (scores in the range 1–4, 4 being the top score). The scheme from EQA-1 'TIFF Quality Grading Guidelines 2013' is set out in Annex 4. In general, the difference between the schemes is that in the modified version, a score of 1 (poor) in any parameter is a non-acceptable gel that cannot be used for inter-laboratory comparisons. It was necessary to modify the scheme in order to have a category that clearly shows when a gel is not comparable with other gels. The Bionumerics analysis was evaluated according to a modified version of the BioNumerics Gel Analysis Quality Guidelines 2014 (Annex 5) with respect to five parameters (scores in the range 1–3, 3 being the top score). The scheme from EQA-1 'BioNumerics Gel Analysis Quality Guidelines 2013' is in Annex 6. In general, the main difference between the schemes is the reduction of categories from 4 to 3 (only excellent, fair and poor). 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, 14 laboratories (78%) participated in the PFGE part and 17 (94%) in the serotyping of *Listeria*. Conventional serotyping results were provided by nine laboratories (50%) and results of the PCR-based method were provided by 14 (78%) laboratories (six laboratories performed both methods). Both PFGE and serotyping were completed by 72% of the laboratories (Table 1).

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

Methods	PFGE		Serotyping		PFGE and serotyping
	TIFF	XML	Conventional	Molecular	
Number of participants	14	11	9	14	13
% of participants	78	79*	50	78	72

Eighteen laboratories participated in at least one of the methods.

* - % of the laboratories participating in the PFGE part

3.2 Pulsed Field Gel Electrophoresis (PFGE)

Fourteen laboratories participated in the *Listeria* PFGE, submitting raw gel images (TIFF files). Eleven of these laboratories had also analysed the gel using BioNumerics and submitted the results in the form of an XML-export 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 corrected TIFF Quality Grading Guidelines, with seven parameters being evaluated (Annex 3). In general, acceptable quality (fair – score of 2) should be achieved for each parameter. A score of 1 in just one category is obtained when a gel is not acceptable, making inter–laboratory comparison impossible.

In Table 2 the gel grading of both the *Apa*I and *Asc*I profiles are shown. A high average score of above 3.0 was obtained for all parameters (Table 2). For the parameters 'Cell suspension', 'Lanes', 'Restriction' and 'DNA degradation' – a high average score of 3.4-3.6 (between 'good' and 'excellent') was obtained (Table 2). The three remaining parameters – 'Gel Background', 'Bands' and 'Image acquisition and running conditions' – had lower average scores of 3.3-3.6, 3.3-3.4 and 3.1-3.0 respectively – still between 'good' and 'excellent'.

Table 2. Results of PFGE gel quality for 14 participating laboratories

The table shows the average scores and the percentage of laboratories obtaining scores 1–4 for the seven TIFF Quality Grading Guideline parameters. The numbers left/right correspond to the images from *Apa*I/*Asc*I.

Parameters	1. Poor	2. Fair	3. Good	4. Excellent	Average
Image and running conditions	7%/7%	36%/36%	0%/7%	57%/50%	3.1/3.0
Cell suspension	0%/0%	14%/14%	7%/21%	79%/64%	3.6/3.5
Bands	7%/0%	14%/21%	21%/14%	57%/64%	3.3/3.4
Lanes	0%/0%	0%/7%	43%/50%	57%/43%	3.6/3.4
Restriction	0%/7%	0%/0%	57%/29%	43%/64%	3.4/3.5
Gel background	0%/0%	29%/14%	14%/14%	57%/71%	3.3/3.6
DNA degradation	0%/0%	7%/14%	21%/21%	71%/64%	3.6/3.5

An analysis of the parameter 'Image acquisition and running conditions' shows that scores vary considerably among the participating laboratories (Table 2). For example, for the *Apa*I gels, 57% of participants were graded as 'excellent', none as 'good', 36% obtained a low score 'fair', but only 7% (one laboratory) obtained the critical score of 'poor'. In the parameter 'Bands', 57%-64% of laboratories were graded with a top score (4) for *Apa*I/*Asc*I, while only 7% of participants were graded 'poor' (1) based on the *Apa*I profile, making a proper analysis of the gel impossible in Bionumerics.

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

A B

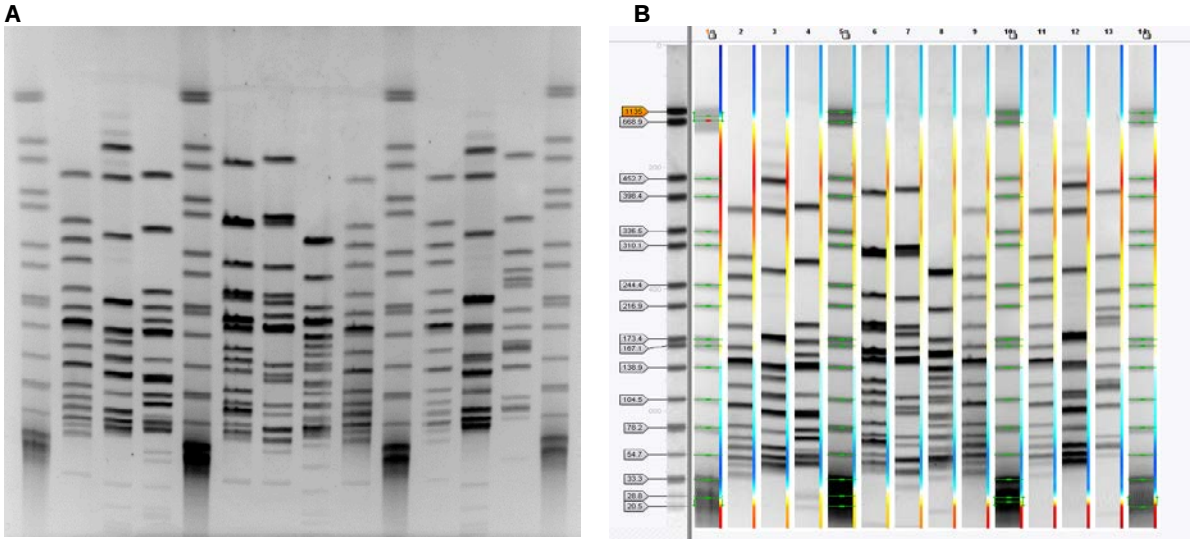
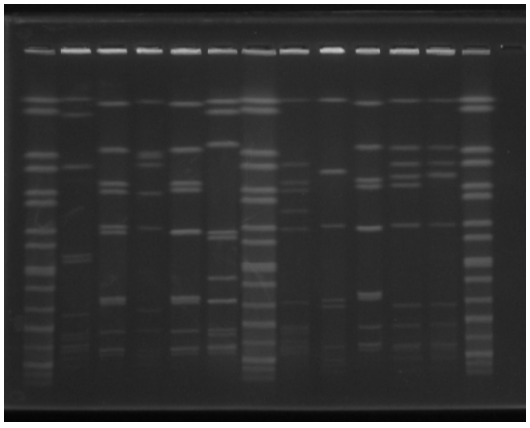


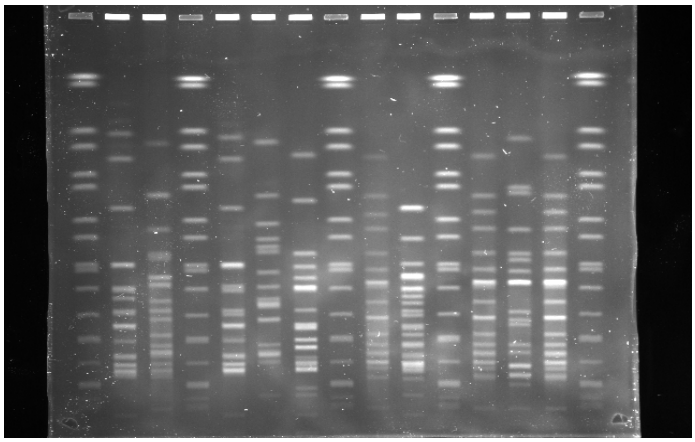
Figure 1A shows the actual gel profile and Figure 1B is a view of the normalisation in BioNumerics. The gel is scored as 'poor' (1) in the parameter 'Image acquisition and running conditions' since the running conditions are incorrect when compared to the PulseNet International protocol. This means that the strains were impossible to compare to other laboratories' strains and that the gel was impossible to analyse. Another noteworthy fact is that in Figure 1A the clear presence of shadow bands indicates incomplete DNA restriction in lanes 3 and 12 (marked with arrows).

Figure 2. A gel with a low score in 'Bands'



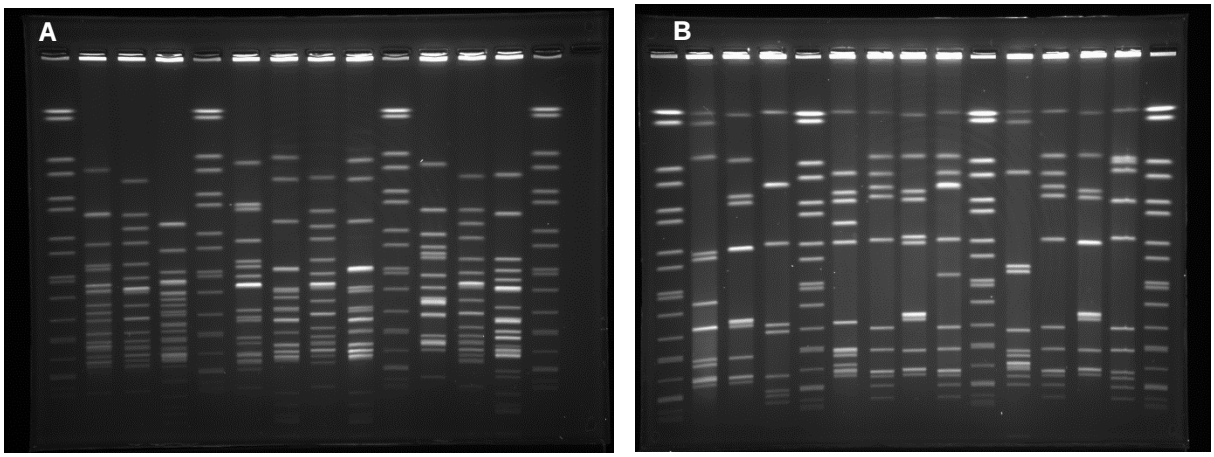
The gel shown in Figure 2 is scored 'poor' (1) in the parameter 'Bands'. The low score is due to the fuzziness of many of the bands and band distortion in several lanes, making it difficult to analyse the gel.

Figure 3. A gel with a 'fair' score in 'Gel background'



The gel shown in Figure 3 is scored 'fair' (2) in the parameter 'Gel Background' and 'Image acquisition and running conditions'. The score 'fair' in the parameter background is due to the overlay of fluorescent dye all over the gel. This layer makes all the bands a bit fuzzier and out of focus. De-staining the gel is important and has a great impact on the appearance and quality of the gel. Repeat the de-staining procedure in fresh water three times before the image capturing.

Figure 4. Gels with high scores in all seven parameters



Finally, two gels with high scores in all seven parameters are shown in Figure 4 (A and B). The images have been captured correctly, there is an even distribution of DNA, the bands are clear and there is no background and almost no debris and no shadow bands. However, the gel has run a bit too short. (A *Apa*I and B *Asc*I).

3.2.2 Gel analysis using BioNumerics

Eleven laboratories analysed the gels and were able to produce XML-export files according to the protocol attached to the invitation letter (Annexes 9 and 10). The Bionumerics analysis was graded according to the BioNumerics Gel Quality Grading Guidelines developed at SSI, which involves five parameters (Annex 5).

Table 3. Results of the Bionumerics analysis obtained by 11 laboratories

The table shows the five gel analysis parameters for the BioNumerics Quality Guidelines, the percentage of laboratories scoring 1–3 and the average score for all laboratories.

Parameters	1. Poor	2. Fair	3. Excellent	Average
Position of the gel	9%	18%	73%	2.6
Strips	0%	18%	82%	2.8
Curves	0%	18%	82%	2.8
Normalisation	9%	18%	73%	2.6
Band assignment	9%	36%	55%	2.5

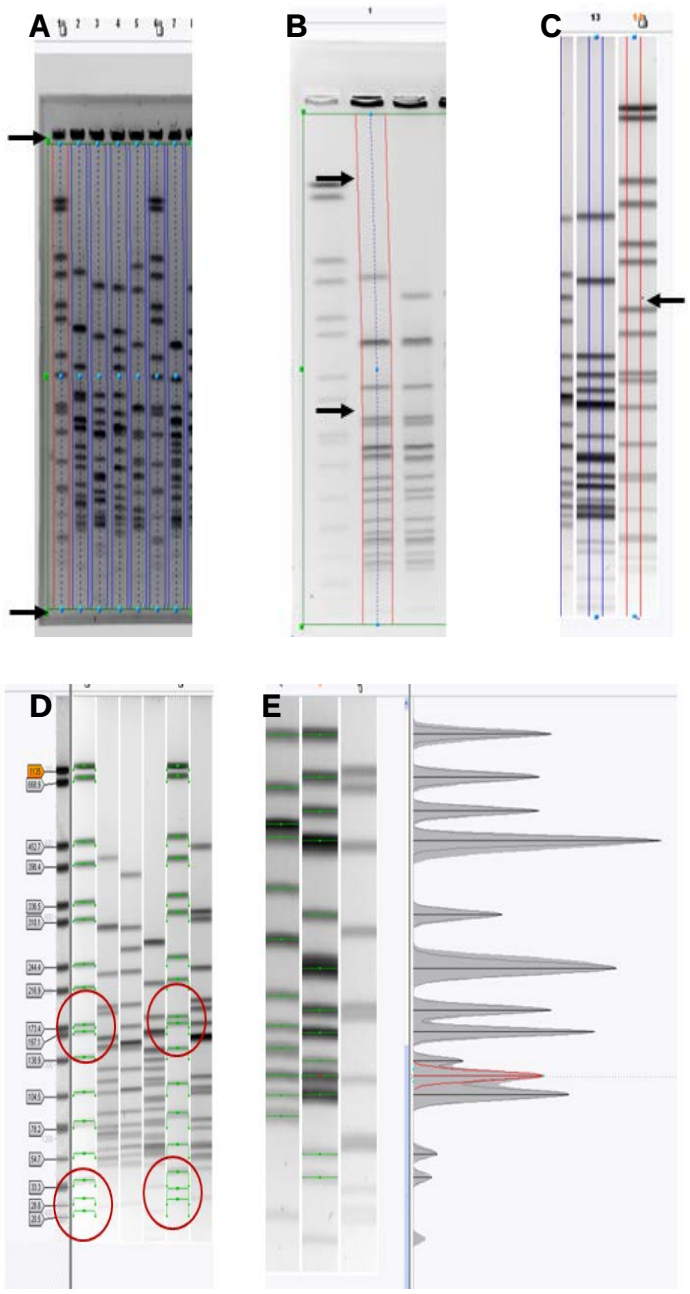
Two parameters – 'Strips' and 'Curves' – had a high average score of 2.8. Three parameters – 'Position of the gel', 'Normalisation' and 'Band assignment', were graded slightly lower with an average of 2.6, 2.6 and 2.5, respectively. Only 9% of the laboratories (not the same participant) were unable to correctly position the gel in the frame and perform normalisation and band assignment.

The BioNumerics software is well adapted to analysing PFGE profiles. One of the critical steps in the analysis is normalisation of the gel, but all steps in the analysis have an impact on the inter-laboratory comparison. The EQA arrangers distributed pre-configured Bionumerics databases to the participants – ready for use after unzipping. However some participants created their own non-compliant databases and submitted data which were not readily usable for inter-laboratory analysis.

In order to grade the profiles and BioNumerics analysis quality, all data were imported into the same EQA Bionumerics database using the correct experiment names with underscores ('PFGE_ApaI' and not 'PFGE-ApaI') and 8-bit TIFF files. It should be noted that the correct gel reference - H9812Lm - is necessary for compliance both with the ECDC TESSy MSS database and the ECDC EQAs. Figure 5 demonstrates areas where the participants are often careless. Figure 5A shows the correct positioning of the gel in the frame. Large variations in the position of the top/bottom frame may have an impact on further analysis – we recommend the top frame just beneath the wells. Figure 5B illustrates the correct width and shape of the lane definitions (strips). The band should fit snugly within the width of

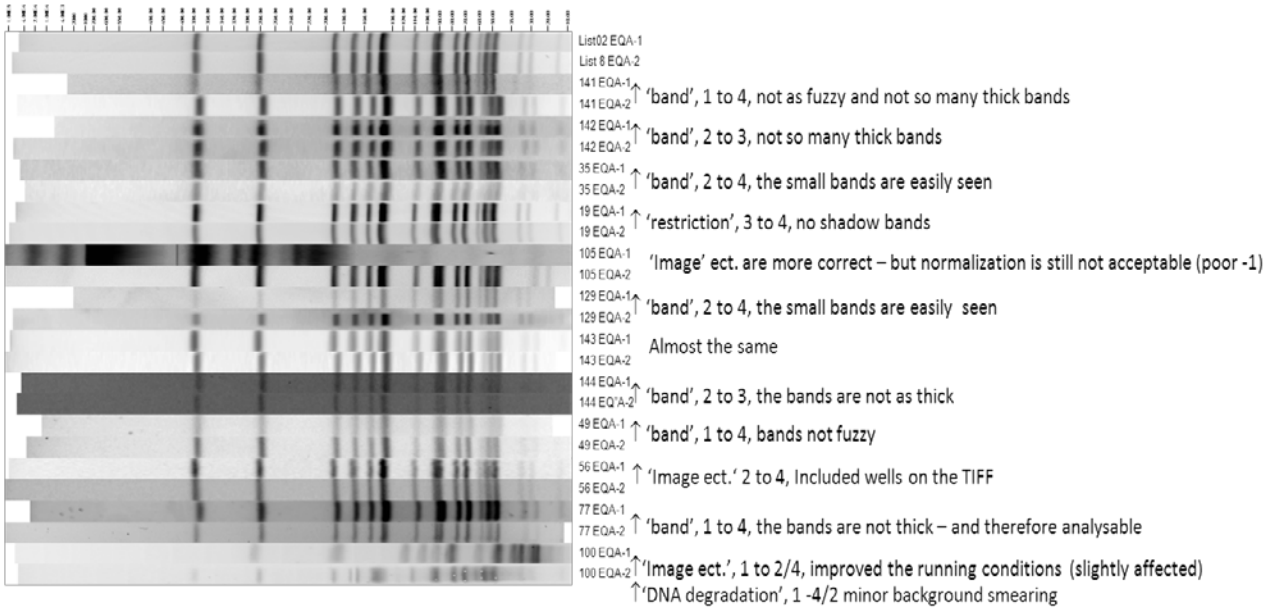
the lane and be corrected for occurrences of smiling lanes. Figure 5C illustrates the proper width of curve extraction in the lane. The width should be approximately 1/3 of the width of the lane and the curve should be positioned horizontally to avoid debris etc. Figure 5D is the normalising step. Here it is crucial to have remembered to place the *Salmonella* Braenderup (H9812Lm) in every sixth lane on the gel. All bands in the reference strain should be assigned, including the doublet at 167/173 kb and the two small bands below 33 kb. Figure 5E illustrates the band assignment. Bands in doublets are assigned when white space can be observed between them but the intensity curve located to the right can be a help when defining double bands.

Figure 5. Check point when using BioNumerics



A: Frame position, B: Strip definition, C: Curves extraction, D: Normalisation, E: Band assignment

Figure 6. Improvement of gel quality from EQA-1 to EQA-2



Comparison of strain 2 (EQA-1) and strain 8 (EQA-2) for each of the 12 participants are represented by arbitrary numbers. ↑(improved) from score x to y. The numbers left/right correspond the images from ApaI/AscI.

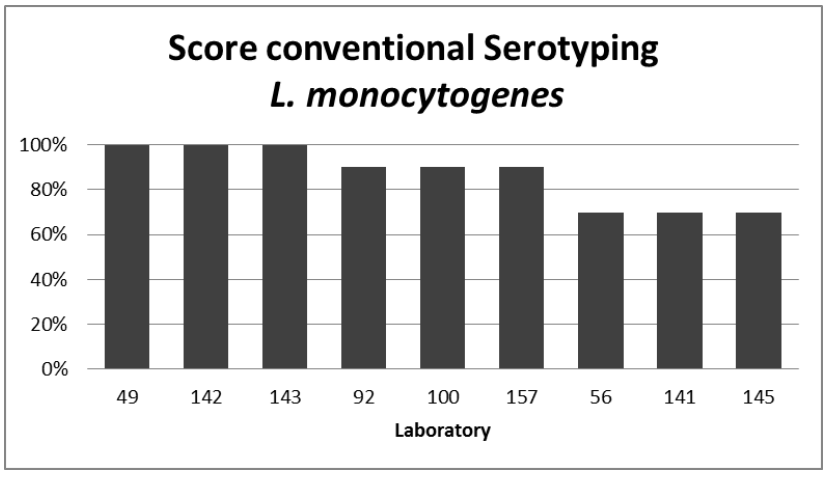
3.3 Serotyping

3.3.1 Conventional serotyping

Nine laboratories performed the conventional serotyping of *L. monocytogenes* (Figure 7). Strain 6 caused some additional problems since the serotyping was not clear when tested with serum from Denka Seiken. The agglutination with the polyvalent O:V/VI was clear but the monovalent agglutinations with O:VI, O:VII, O:VII and O:IX were not clear. This would lead to an invalid result for the O-antigens in strain 6. The strain (which is from 1996) was originally characterised as a 4a. For the evaluation of the EQA the results 4ab, 4b, 4c, 4d, 4e and no somatic antigen are considered satisfactory due to the non-clear reactions.

Three of the participants were able to correctly serotype all ten EQA test strains. Three participants mistyped one strain. Three laboratories mistyped three isolates.

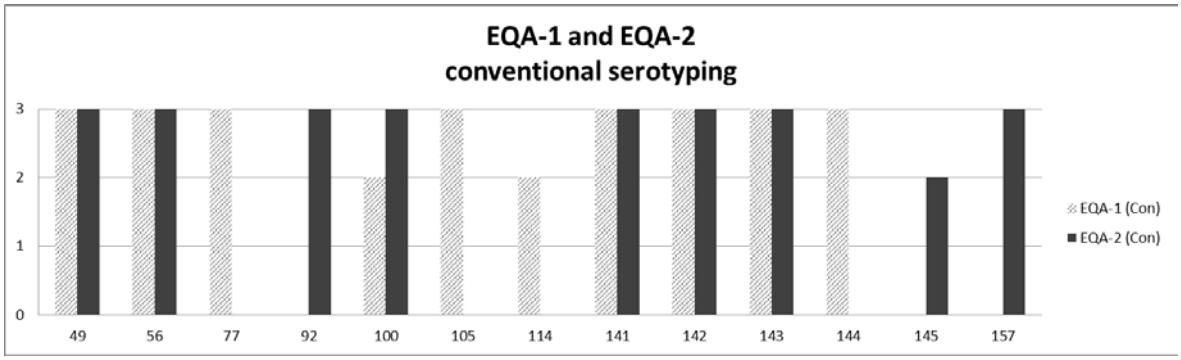
Figure 7. Results of conventional serotyping of *L. monocytogenes*



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

To show the exact progress of the laboratory's performances, three strains from EQA-1 were included in EQA-2. Strain 2 (1/2a), 3 (4b) and 4 (1/2c) from EQA-1 are numbered 8, 1 and 9 respectively in the EQA-2. Figure 8 shows the performances based only on the three isolates.

Figure 8. Comparing EQA-1 and EQA-2 conventional serotyping of *L. monocytogenes*

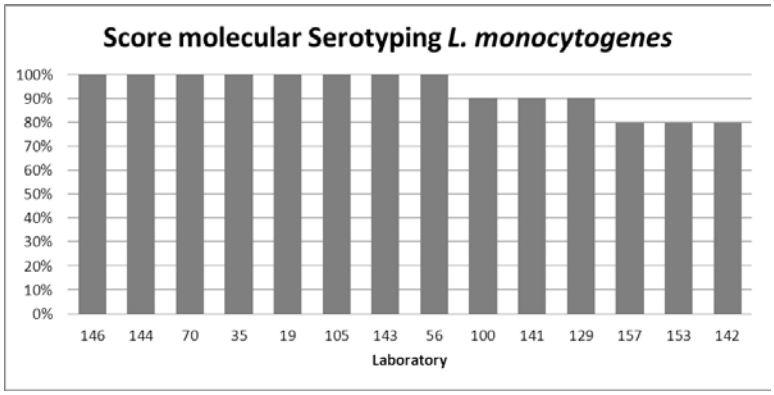


The participating laboratories are represented by arbitrary numbers. Bars represent the number of correctly assigned serotypes of the three strains (2, 3 and 4 from EQA-1 and 8, 1 and 9 from EQA-2).

3.3.2 Molecular serotyping

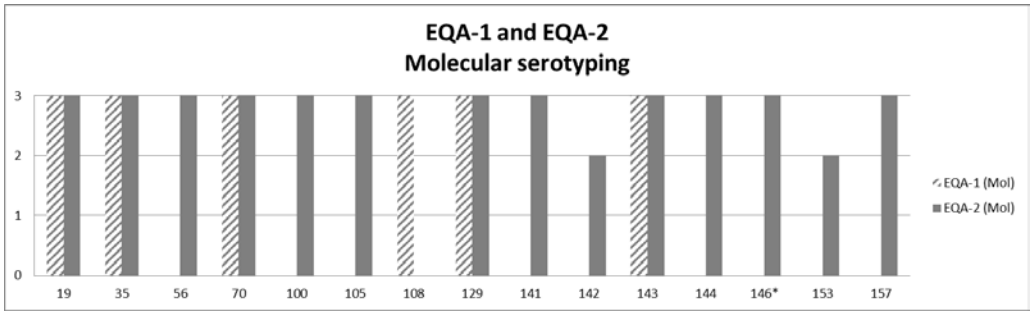
Fourteen laboratories performed the molecular serotyping of *L. monocytogenes* (Figure 9). Eight of these were able to correctly serotype all 10 EQA test strains, including the two participants whose results were translated by the EQA provider from an older version of Doumith's nomenclature [7] instead of the newest Doumith *et al.* [6]. Three laboratories had 90% correct results, and three laboratories scored 80%.

Figure 9. Results of molecular serotyping for *L. monocytogenes*



The 14 participating laboratories are represented by arbitrary numbers. Bars represent the percentage of correctly assigned serotypes.

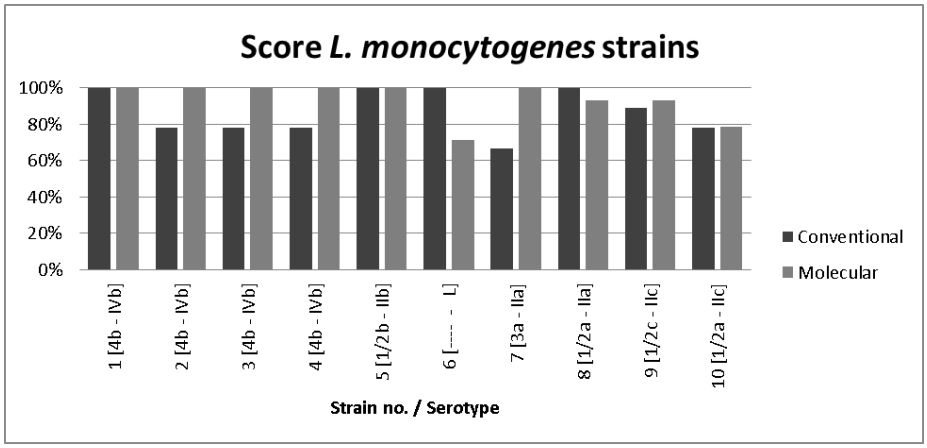
Figure 10. EQA-1 and EQA-2 molecular serotyping for *L. monocytogenes*



The participating laboratories are represented by arbitrary numbers. Bars represent the number of correctly assigned serotypes of the three strains (2, 3 and 4 from EQA-1 and 8, 1 and 9 from EQA-2). * indicates that the laboratory participated in EQA-1, but none of the three strains were correctly serotyped.

Figure 10 shows the performance based only on the three isolates. Strain 2 (1/2a), 3 (4b) and 4 (1/2c) from EQA-1 are numbered 8, 1 and 9 respectively in EQA-2.

Figure 11. EQA strains and average percentage score for each of the ten strains



Bars represent the percentage of serotypes correctly assigned by the participants.

An analysis of each individual strain in Figure 11 shows that two strains (strain 1 and 5) were serotyped 100% correctly using either molecular or conventional serotyping. Strains 1-5 and no. 7 were all serotyped 100% correctly using molecular serotyping, whereas only strains 6 and 8 were serotyped 100% correctly using conventional serotyping. Strain 6 stands out – the non-agglutinable strain – since no clear reactions influenced the result of this strain. Overall, participants performing conventional serotyping identified 87% of the isolates correctly, while participants using molecular serotyping identified 94% correctly.

4. Conclusions

A total of 18 laboratories participated in the EQA-2 scheme. Of these 14 (78%) produced PFGE results and 17 (94%) performed serotyping. Nine laboratories (50%) serotyped using the conventional method, while fourteen laboratories (78%) used molecular serotyping. This corresponds to an increase of 50% for the molecular serotyping against EQA-2. Thirteen laboratories (72%) completed both PFGE and serotyping.

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 (93%) of the laboratories were able to produce PFGE gels of sufficiently high quality to enable the profiles to be compared with those obtained by other laboratories. This comparability primarily relies on the use of correct running conditions, distinct bands and a good quality image acquisition. The profiles were normalised and gel profiles interpreted using the specialised software BioNumerics. Eleven of the laboratories (79%) carried out this software analysis of their gel, and 91% of them performed well in accordance with the guidelines.

Serotyping of *L. monocytogenes* was included in EQA-2, 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 of correct serotypes obtained with the molecular (multiplex PCR-based) serotyping was high, with the eight participating laboratories achieving a score of 100%. However, two participants submitted results using a nomenclature from an older Doumith [7] article than the intended nomenclature in Doumith [6]. These older nomenclatures were translated by the EQA providers. The test strains were chosen to cover most of the serotypes present in isolates causing human disease. Compared to the multiplex PCR method the conventional, phenotypic serotyping is much more laborious, slower and requires experienced personnel. This is reflected in the 50% increase in molecular participants. 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-2 scheme for typing *L. monocytogenes* is the second EQA organised for laboratories participating in FWD-Net. The performance of the laboratories EQA showed improvement since EQA-1, but 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. Phenotypic serotyping is currently included in TESSy and used for surveillance purposes by several EU countries. PCR-based serotyping is currently in the process of being added to TESSy. In general, the quality of serotyping was high. This second EQA for PFGE typing of *Listeria* demonstrates that the majority of participating laboratories were able to produce good results. Only 14% of the laboratories produced results that were too poor for the inter-laboratory comparison of data. For the software analysis, 18% need to improve their Bionumerics analysis. However, trouble-shooting and assistance to the laboratories has resulted in improvement in the quality.

5. Discussion

5.1 Pulsed Field Gel Electrophoresis (PFGE)

Fourteen laboratories participated in the PFGE part of the EQA-2. 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).

A large majority (83%) of the laboratories were able to produce gels of acceptable quality. Only one participant scored 'poor' in the 'Image acquisition and running conditions' and the 'Restriction' parameters (*Apa*I) and one in the parameter 'Bands' (*Asc*I). The rest of the participants scored 'fair' or better for all seven parameters (Table 2). However 36% of participants scored 'fair' in the 'Image acquisition and running conditions'. This is not as critical as 'poor' but emphasises the need to validate the running conditions in order to insure comparability of the profiles. Compared to last year, the portion of participants scoring 'fair' in this parameter decreased from 10/17 (59%) in EQA-1 to 5/14 (36%) in EQA-2. 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 not filling the whole image with the gel, including wells, or not leaving 1–1.5 cm below the smallest band on the gel. Although these are less critical than not using the correct running conditions, they can still have a major impact on the ability to assign bands correctly.

In the category 'Bands', one laboratory was given the lowest score (1 – 'poor') while 14% and 21% were given the second lowest (2 – 'fair') in either *Apa*I or *Asc*I. Most of the 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 in relation to exposure time and focus. Many laboratories seemed to have enhanced the contrast at image acquisition in order to boost weak bands. Unfortunately, this results in fewer grey levels, saturated pixels and thicker bands, all of which make it harder to distinguish double bands. This and the overloading of plugs with DNA are the main reasons for a low score in the category 'Bands'.

In the parameters 'Lanes' and 'Restriction', only one single participant scored 'fair' (2) or 'poor' (1) in either of the *Apa*I or *Asc*I profiles. Consequently there is no need to focus on quality issues.

For the parameter 'DNA degradation', none of the participants' gels had so much smearing that it was impossible to analyse them, although two gels only scored 'fair' (2). Compared to EQA-1 it is a reduction from 24% to 14% for the scores '2' and below. For a highly sensitive method such as PFGE it is important to follow the protocol. In order to reduce DNA degradation, significant improvements can be made by carefully following the instructions regarding plug preparation. This includes the lysis step, recommended time of restriction for the relevant enzyme, and washing plugs six times, as recommended.

Only 79% of the laboratories that performed PFGE also did the subsequent gel analysis (i.e. the normalisation and band assignment that provides the actual PFGE profiles for comparison). This analysis had to be done using the software BioNumerics, and some laboratories may not have access or may only have limited experience in using Bionumerics 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 (79%) that submitted gel analysis data performed well in accordance with the guidelines. Only one laboratory obtained a 'poor' score in both the 'Position of the gel' and 'Normalisation' categories while one laboratory scored 'poor' in the 'Band assignment' category. However, it was not the same two laboratories that obtained a ´poor´ score in these categories in EQA-2.

5.2 Serotyping

Seventeen laboratories participated in the EQA-2 serotyping exercise. Nine of these submitted results from conventional phenotypic serotyping and fourteen submitted multiplex PCR-based results. Six of them participated in both methods. In general the results were very good, with 87% correct from conventional and 94% correct from multiplex PCR-based serotyping. Compared to EQA-1 the performance improved using the PCR-based serotyping (from 83% to 94%) whereas the number of correctly assigned types in the conventional serotyping decreased from 94% to 87%, despite the acceptance of all results for strain 6.

5.2.1 Conventional serotyping

Due to the non-clear reactions in the conventional serotyping of strain 6, all results were accepted however single O-groups were mistyped:

- One 4b mistyped as 4d or 4e
- The 3a isolate was mistyped as 1/2a, 1/2c or untypeable
- One 1/2a isolate was mistyped as 1/2b
- One 1/2a was mistyped as 1/2c.

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. Included in these 57% were two participants who had their submitted results translated from the nomenclature in Doumith et al. 2004 [7] to that in Doumith et al. 2005 [6]. Three participants' results were only incorrect in the nomenclature – the L strain was submitted as blank, not established or untypeable. The three other participants that did not achieve the 'L' result, reported the strain as IVa, IVb or IIa respectively. Three results from one IIc strain were reported as IIa and one IVb was reported as IIa. It is not surprising that strain 6 caused some problems, since among the serotypes in the EQA, this is the rarest one found in humans. The multiplex PCR reaction for this strain should be negative in all amplifications, with exception of the positive control.

A comparison of progress in performance shows very encouraging results. The comparison of the three strains used in both EQAs shows that all laboratories performed better or at the same level as in the previous EQA-1 (2012) even though two laboratories changed their method to molecular serotyping. Out of the eight participants that submitted both conventional and molecular results, five laboratories were new to the molecular method.

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 and adhere strictly 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 familiarise themselves with the image acquisition and electrophoresis equipment and ensure that this is maintained.

Most laboratories participated in the serotyping exercise. The contributions were relatively evenly divided between conventional phenotypic and PCR multiplex-based methods, with six of the laboratories participating in both methods. The results indicate that two of the problems are nomenclature problems with the PCR-based serotyping and the unexpected non-agglutinable strain. ECDC will standardise the TESSy system using the original Doumith et al. 2005 [6] nomenclature for PCR multiplex serotyping, which was also the indicated nomenclature for this EQA. This report therefore uses the Doumith nomenclature shown in Table 4.

A number of other errors were made, many of which could easily have been avoided by simply 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.

6.2 ECDC and FWD-Net

A total of 18 laboratories participated in the EQA-2 scheme, which was only half of those invited, but equals the number that participated in the first EQA. Future EQAs should aim to have a higher number of participating laboratories, although an assessment of the capacity to perform molecular typing of *Listeria* could be valuable. It is encouraging, however, that 13 of the 18 laboratories performed both PFGE and serotyping.

Only 14% of the laboratories did not produce PFGE profiles of sufficiently high quality for inter-laboratory comparison, while 18% of the laboratories were not able to perform the data analysis at the accepted level. These results indicate that there still is a need for capacity-building in laboratory procedures and gel analysis and interpretation using BioNumerics.

In the serotyping part the participants were divided between the conventional serological (50% of all participants) and the molecular PCR multiplex (78% of all participants) 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 encourage usage of the PCR-based method. In principal, the capacity to use this method should be available in all laboratories with basic PCR capacity and the increased participation in the molecular serotyping is reassuring.

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, there are no harmonised procedures for WGS data analysis in routine surveillance and international comparison of *Listeria* strains, but some laboratories are beginning to proceed with this idea.

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. The scheme for evaluation of gel quality in this EQA was modified to ensure correspondence between the score and the suitability of the gel for inter-laboratory comparability. The score ´poor´(1) in any of the parameters corresponds to images being impossible to use for reliable comparison with those obtained in other laboratories.

There was no negative feedback regarding the rearrangement of the time schedule for the three EQAs. The FWD-Net laboratories indicated last year that the time schedule was too tight for laboratories participating in all three EQAs for the typing of foodborne pathogens as well as other EQA schemes. Since results have to be evaluated

individually due to the need for a 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. However some countries are reluctant to ask the EQA provider questions – we will try harder to encourage them to use the trouble shooting team.

Although the EQA provider improved the guidelines for participants by providing additional details and an online submission form, there were still a number of problems. Pictures were still submitted as 16-bit; reference lanes were missing; the specific number of the strains was not added to the Key field in Bionumerics and wrong nomenclatures were used for the serotyping. Table 4 in this report should improve the nomenclature issue and the serotyping fields in Bionumerics will be limited to a selected list which is already implemented in the online submission form.

Additionally, a standard comment will be included in the overall summary of PFGE performance in the next EQA.

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 2012; EFSA Journal 2014;12(2):3547.
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) Standardised Laboratory Protocol for Molecular Subtyping of *Listeria monocytogenes* by Pulsed Field Gel Electrophoresis (PFGE). Available at: http://www.cdc.gov/pulsenet/PDF/listeria-pfge-protocol-508c.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.
7. Doumith M, Jacquet C, Gerner-Smidt P, Graves LM, Loncarevic S, Mathisen T, Morvan A, Salcedo C, Torpdahl M, Vazquez JA, Martin P. Multicenter validation of a multiplex PCR assay for differentiating the major *Listeria monocytogene*s serovars 1/2a, 1/2b, 1/2c, and 4b: towards an international standard. J Food Prot. 2005 Dec;68(12):2648-50.

Annex 1. List of participants

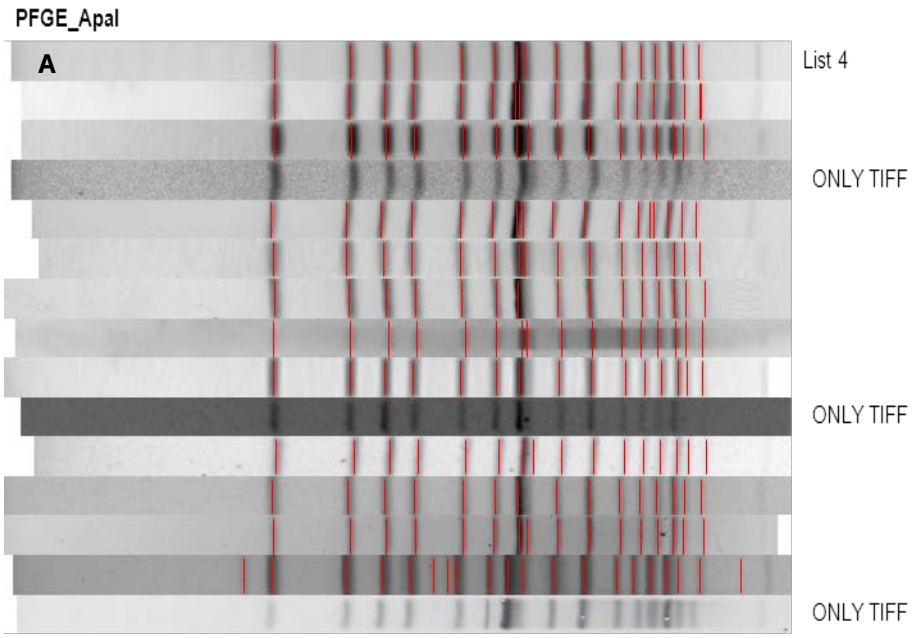
Country	National institute	Laboratory
Austria	AGES – Österreichische Agentur für Gesundheit und Ernährungssicherheit	National Reference Laboratory *Listeria*
Belgium	WIV-ISP	National Reference Centre for Listeria
Czech Republic	VRI	Zoonoses unit
Denmark	Statens Serum Institut	Unit of Foodborne Infections
Finland	THL - Institute of Health and Welfare	Unit of Bacteriology
France	Institut Pasteur	French NRC and WHOCC for Listeria
Germany	Robert Koch Institute	NRC for Salmonella and other Bacterial Enterics
Hungary	National Center for Epidemiology	Department of Phage-typing and Molecular Typing
Ireland	University Hospital Galway	National Salmonella, Shigella and Listeria Reference Laboratory
Italy	Istituto Superiore di Sanità	Microbiological Foodborne Hazard Unit
Lithuania	National Public Health Surveillance Laboratory	Microbiological Testing Department
Luxembourg	Laboratoire National de Santé	Surveillance Epidemiologique
Netherlands	RIVM	IDS-BSR
Poland	National Institute of Public Health	Laboratory of Enteric Rods
Romania	Cantacuzino NIRDMI	Zoonoses
Slovenia	National Institute of Public Health	Department of Medical Microbiology
Spain	Institute of Health Carlos III	Reference Laboratory for Listeria
United Kingdom	Gastrointestinal Bacteria Reference Unit - Health Protection Agency	Foodborne Pathogens Reference Services

Annex 2. Examples of PFGE profiles

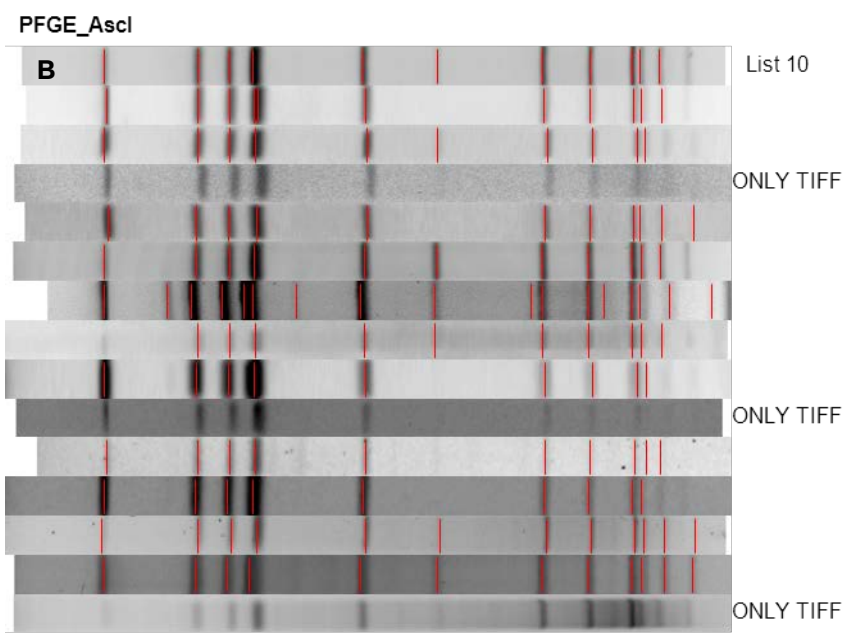
Profiles from the 14 participants

A: 14 profiles of strain 4 cut with ApaI (11 with band assignment) B: 14 profiles of strain 10 cut with AscI (11 with band assignment)

Strain 4



Strain 10



Annex 3. TIFF Quality Grading Guidelines 2014

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.	- Gel does not fill whole TIFF and band finding slightly affected - Wells not included on TIFF - Bottom band of standard not 1-1.5 cm from bottom of gel and analysis is slightly affected. - Band spacing of standards does not match global standard and analysis is slightly affected.	- Gel does not fill whole TIFF and band finding is highly affected. - Bottom band of standard not 1-1.5 cm from bottom of gel and analysis is highly affected. - Band spacing of standards does not match global standard and analysis is highly affected.
Cell suspensions	The cell concentration is approximately the same in each lane	Up to two lanes contain darker or lighter bands than the other lanes.	More than 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 (three or less) 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 analysable.	- 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 make the analysis impossible.
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.	- Smearing so that several lanes are not analysable (except of untypeable thiourea required).

Annex 4. TIFF Quality Grading Guidelines 2013

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 doesn't fill whole TIFF but band finding is not affected	Not protocol; for example, one of the following: - Gel doesn't 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 doesn't match global standard	Not protocol; for example, >1 of the following: - Gel doesn't 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 doesn't match global standard
Cell Suspensions	The cell concentration is approximately the same in each lane	1-2 lanes contain darker or lighter bands than the other lanes	- >2 lanes contain darker or lighter bands than the other lanes, or - At least 1 lane is much darker or lighter than the other lanes, making the gel difficult to analyze	The cell concentrations are uneven from lane to lane, making the gel impossible to analyze
Bands	Clear and distinct all the way to the bottom of the gel	- Slight band distortion in 1 lane but doesn't interfere with analysis - Bands are slightly fuzzy and/or slanted - A few bands (e.g., ≤3) difficult to see clearly (e.g., DNA overload), especially at bottom of gel	- Some band distortion (e.g., nicks) in 2-3 lanes but still analyzable - Fuzzy bands - Some bands (e.g., 4-5) are too thick - Bands at the bottom of the gel are light, but analyzable	- 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 the outside lanes vs. the inside) - Lanes gradually run longer toward the right or left - Still analyzable	- Significant smiling - Slight curves on the outside lanes - Still analyzable	- Smiling or curving that interferes with analysis
Restriction	Complete restriction in all lanes	- One to two faint shadow bands on gel	- One lane with many shadow bands - A few shadow bands spread out over several lanes	- Greater than 1 lane with several shadow bands - Lots of shadow bands over the whole gel
Gel Background	Clear	- Mostly clear background - Minor debris present that doesn't 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 1-2 lanes that may or may not make analysis difficult - Minor background (smearing) in many lanes	- Significant smearing in >2 lanes that may or may not make analysis difficult - Smearing so that a lane is not analyzable (except if untypeable [thiourea required])

Annex 5. BioNumerics Gel Analysis Quality Guidelines 2014

Parameters/scores	Excellent	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.	Wells wrongly included when placing the frame Gel is not inverted
Strips	All lanes correctly defined.	Lanes are defined to narrow (or wide) Lanes are defined outside profile A single lane is not correctly defined.	Lanes not defined correctly
Curves	1/3 or more of the lane is used for averaging curve thickness.	Curve extraction defined either to narrow or including almost the whole lane.	Curve set so that artefacts will cause wrong band assignment
Normalisation	All bands assigned correctly in all reference lanes.	Bottom bands <33kb were not assigned in some or all of the reference lanes	Many bands not assigned in the reference lanes The references were not included when submitting the XML-file
Band assignment	Excellent band assignment with regard to the quality of the gel.	Few double bands assigned as single bands or single bands assigned as double bands. Few shadow bands are assigned.	Band assignment not done correctly, making it impossible to make an inter-laboratory comparison.

Annex 6. BioNumerics Gel Analysis Quality Guidelines 2013

Parameters \scores	Excellent	Good	Fair	Poor
Position of gel	Excellent placement of frame, and gel inverted	The image frame is positioned to low		Frame includes wells
		Too much space framed at the bottom of the gel.		Gel not with light bands on dark background
		Too much space framed on the sides of the gel.		
		(Guidelines recommend framing just beneath the wells)		
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 a very narrow strip or encompassing almost the whole lane		
		(Average thickness is 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 not assigned in some of the reference lanes.		Missing assignments of bands in the reference in lane 5, 10 and 15
				The references were not included in the submitted XML file (follow the XML export guide).
Band assignment	Excellent band assignment in relation to the quality of the gel.	Some double bands are assigned wrongly.		The positions are correct, but double bands assigned at the exact same positions.
			Some shadow bands are assigned	Band assignment not correct, (commonly caused by thickness of the bands/overexposure)
			(Guidelines require control of band assignment after using auto search)	Only used auto search to find bands, no manual corrections.
				(Guidelines require control of band assignment after using auto search).

Annex 7. Scoring of the PFGE results

Gel quality

Parameters\Laboratory	141	142	157	35	19	105	129	143	144	56	77	100	49	153
Image and Running Conditions	2/2	4/4	4/2	4/3	4/4	1/1	4/2	2/4	2/2	4/4	4/4	2/4	4/4	2/2
Cell Suspension	4/4	4/4	4/4	4/4	4/4	2/3	4/4	4/2	4/4	4/4	4/4	2/2	4/3	3/3
Bands	4/4	3/4	1/2	4/4	4/4	2/2	4/4	4/2	3/3	4/4	4/4	2/4	4/4	3/3
Lanes	3/3	4/4	3/3	4/4	4/4	2/4	4/3	4/4	3/3	4/3	4/3	3/3	4/4	4/4
Restriction	3/4	3/3	4/4	3/4	4/4	3/1	4/4	4/3	3/4	4/4	4/4	3/4	3/3	3/3
Gel Background	4/4	4/4	2/3	4/4	4/4	4/4	2/2	4/3	2/4	4/4	3/4	2/2	3/4	4/4
DNA Degradation	4/4	4/4	4/3	4/4	4/4	2/2	3/3	3/4	4/4	4/4	4/4	4/2	4/4	3/3

BioNumerics analysis

Parameters\Laboratory	141	142	35	19	105	129	143	56	77	49	153
Position of Gel	3	3	2	3	2	3	3	3	3	3	1
Strips	3	3	3	3	2	2	3	3	3	3	3
Curves	2	3	3	3	3	3	3	3	3	3	2
Normalization	2	3	3	3	2	3	3	3	3	3	1
Band Assignment	2	3	3	3	2	1	3	2	3	3	2

The participating laboratories are represented by arbitrary numbers.

The numbers left/right correspond the images from ApaI/AscI.

Difference in ApaI/AscI

Annex 8. Serotyping results

Conventional serotyping

Strain (Serotype) / Laboratory	Original	157	100	92	142	141	143	56	145	49
1	4b	4b	4b	4b	4b	4b	4b	4b	4b	4b
2	4b	4b	4e	4b	4b	4b	4b	4d	4b	4b
3	4b	4b	4b	4b	4b	4d, 4e	4b	4d	4b	4b
4	4b	4d	4b	4b	4b	4b	4b	4d	4b	4b
5	1/2b	1/2b	1/2b	1/2b	1/2b	1/2b	1/2b	1/2b	1/2b	1/2b
6	-------	4a	4c	4c	4a	4c	4a	-------	4a	4c
7	3a	3a	3a	Untypable	3a	1/2a	3a	3a	1/2c	3a
8	1/2a	1/2a	1/2a	1/2a	1/2a	1/2a	1/2a	1/2a	1/2a	1/2a
9	1/2c	1/2c	1/2c	1/2c	1/2c	1/2c	1/2c	1/2c	1/2b	1/2c
10	1/2a	1/2a	1/2a	1/2a	1/2a	1/2c	1/2a	1/2a	1/2c	1/2a

Molecular serotyping

Strain (Serotype) / Laboratory	Original	157	146	100	144	153	70	142	141	35	19	105	129	143	56
1	IVb	IVb	IVb	IVb	IVb	IVb	IVb	IVb	IVb	IVb	IVb	4b-4d-4e	IVb	IVb	4b-4d-4e
2	IVb	IVb	IVb	IVb	IVb	IVb	IVb	IVb	IVb	IVb	IVb	4b-4d-4e	IVb	IVb	4b-4d-4e
3	Ivb	IVb	IVb	IVb	IVb	IVb	IVb	IVb	IVb	IVb	IVb	4b-4d-4e	IVb	IVb	4b-4d-4e
4	IVb	IVb	IVb	IVb	IVb	IVb	IVb	IVb	IVb	IVb	IVb	4b-4d-4e	IVb	IVb	4b-4d-4e
5	IIb	IIb	IIb	IIb	IIb	IIb	IIb	IIb	IIb	IIb	IIb	1/2b-3b-7	IIb	IIb	1/2b-3b-7
6	L	IVb	L	L	L	IIa	L	L	IVa	L	L	*L. monocytogenes* 4a-4c; *Listeria* spp.	-	L	*L. monocytogenes* 4a-4c; *Listeria* spp.
7	IIa	IIa	IIa	IIa	IIa	IIa	IIa	IIa	IIa	IIa	IIa	1/2a-3a	IIa	IIa	1/2a-3a
8	IIa	IIa	IIa	IIa	IIa	IVb	IIa	IIa	IIa	IIa	IIa	1/2a-3a	IIa	IIa	1/2a-3a
9	IIc	IIc	IIc	IIc	IIc	IIc	IIc	IIa	IIc	IIc	IIc	1/2c-3c	IIc	IIc	1/2c-3c
10	IIc	IIa	IIc	IIa	IIc	IIc	IIc	IIa	IIc	IIc	IIc	1/2a-3a	IIc	IIc	1/2a-3a

Correct result

Incorrect result

Result accepted after nomenclature translation

Strains that are included in the EURL EQA

Strains that are included in the EQA-1

Results accepted – due to non-clear agglutination

Annex 9. Guide to Bionumerics database

Guide for setting up your EQA database

There are two ways to set up the BioNumerics database necessary for the EQA. If you have BioNumerics Version 6 or above you 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.

- Set up a new database; do not use any of your existing databases. This is important in order to be able to submit correctly formatted results (A).
- If (and only if) you have a BioNumerics version prior to 6.0, follow the instructions on setting up a database from scratch (B).

A. Setting up a database if you have BioNumerics 6.0 – 7.x

- The database is packaged in the zip archive called 'Listeria EQA-2 BN<6/7>.zip' 'E coli EQA-5 BN<6/7>.zip' or 'salmonella EQA-5 BN<6/7>.zip'. Note that there are two versions of each, one for Version 6 and one for Version 7 of BioNumerics.
- Choose the correct file and unzip it into the folder where you would like to have your database. The archive contains the complete ready-made database (one file and one folder).
- Open Bionumerics and change the home directory to where you placed your database.

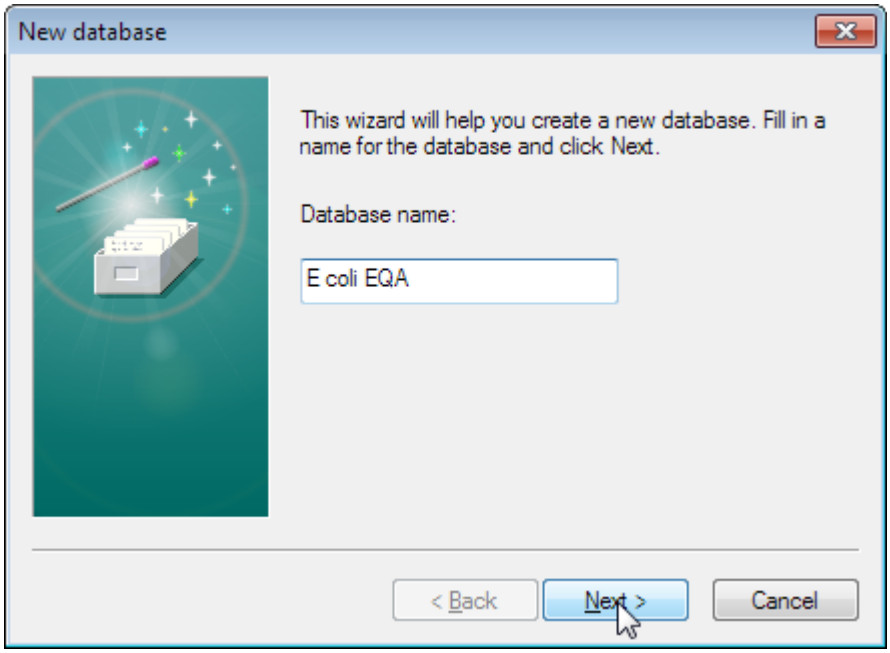
B. Setting up a database from scratch

- All the images in this instruction refer to *E. coli* so just substitute 'E coli' for either 'Salmonella' or 'Listeria' when setting up the databases.
- The database is set up by first creating an empty database and then importing an XML file containing experiment settings and field definitions.

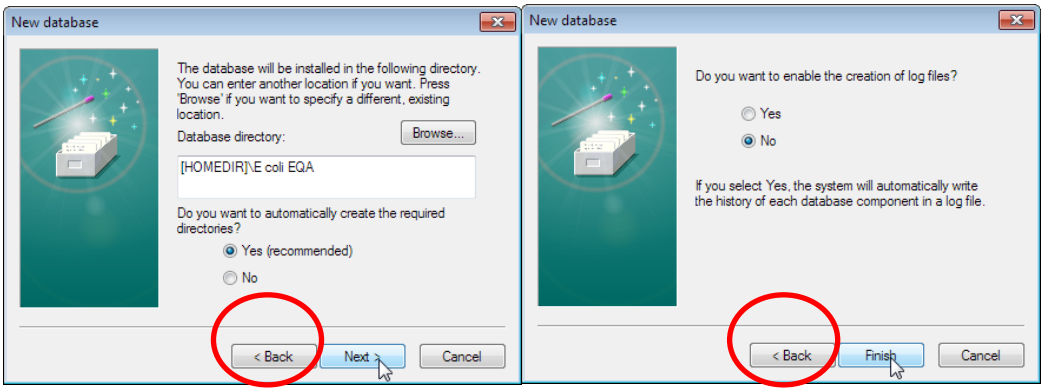
Setting up the empty database

1. Choose 'Create a new database'

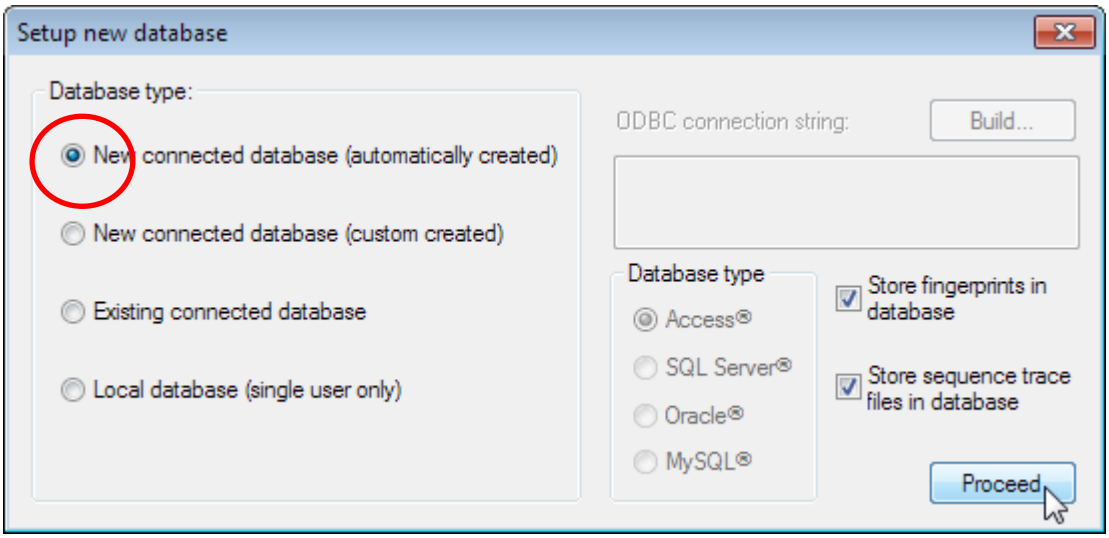
2. Enter a database name ('Salmonella', 'Listeria' or 'E coli EQA').



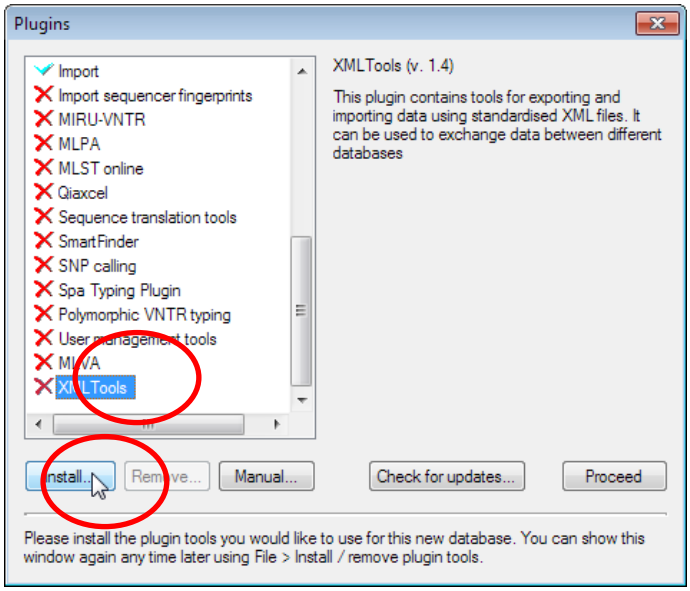
3. Use default values



4. Choose a new connected database (Access type).



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

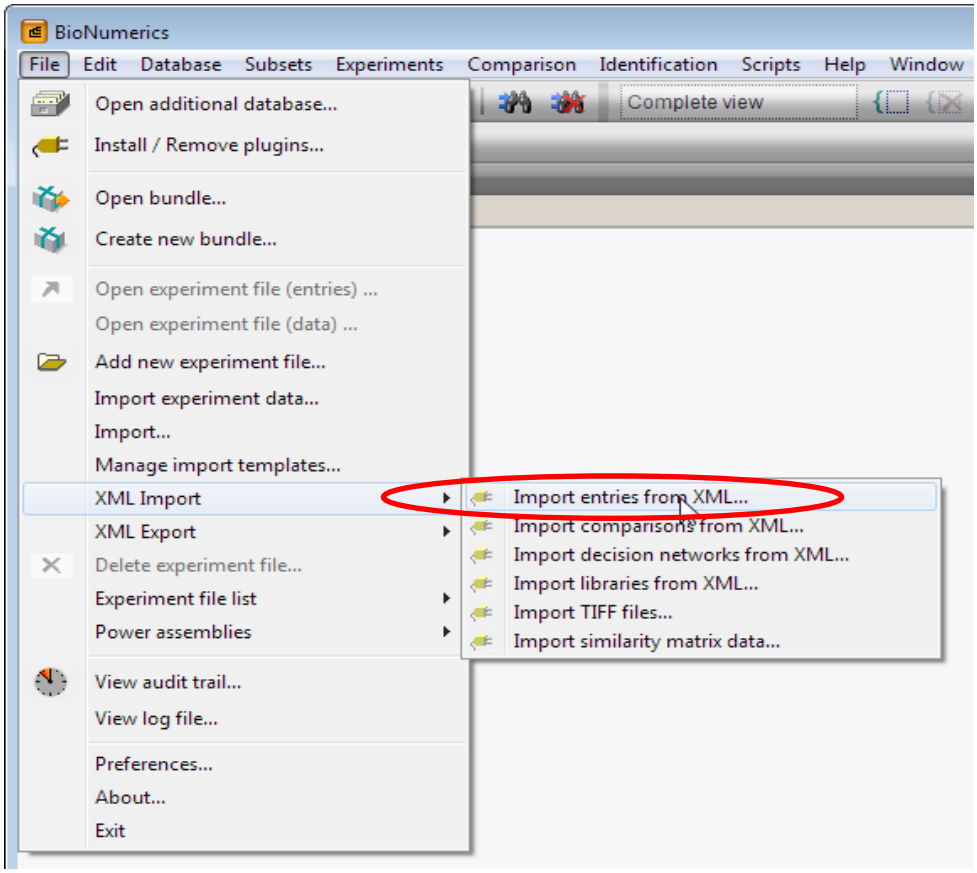


6. Proceed to the next window. The database is now set up and ready for the database definitions to be imported.

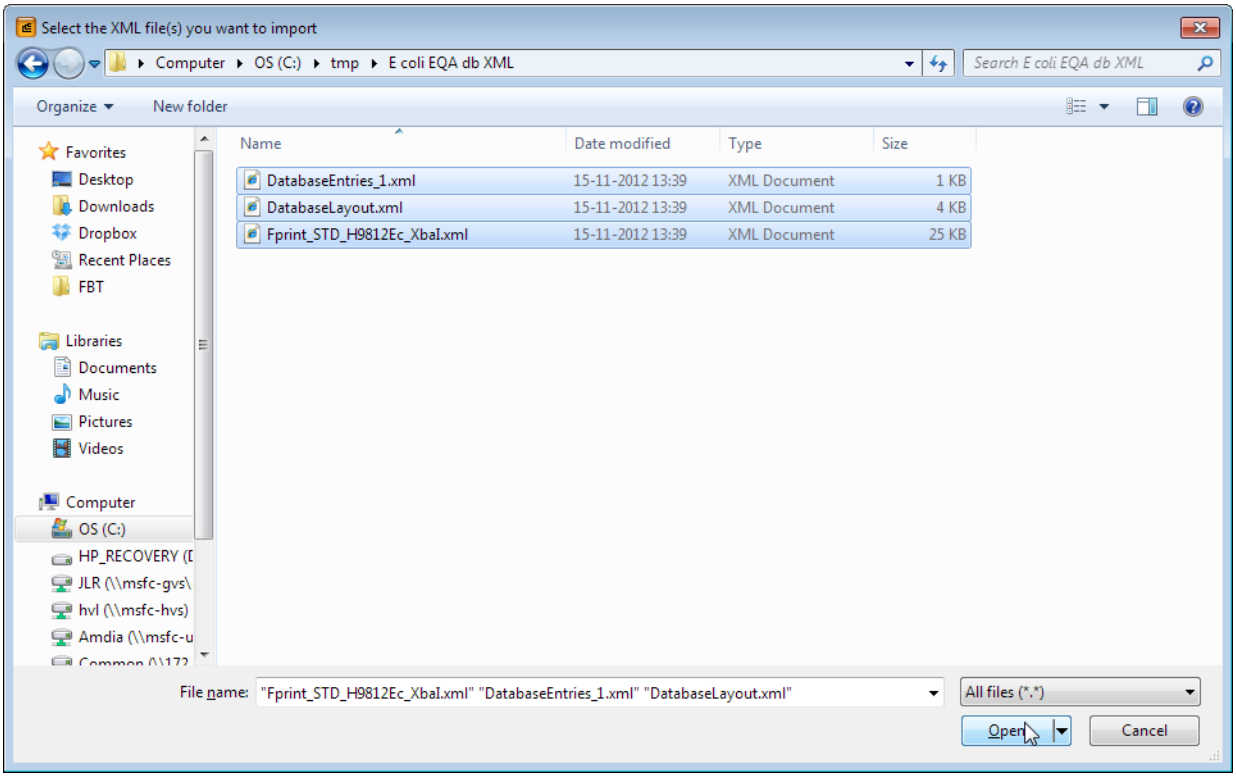
Importing the XML structure

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

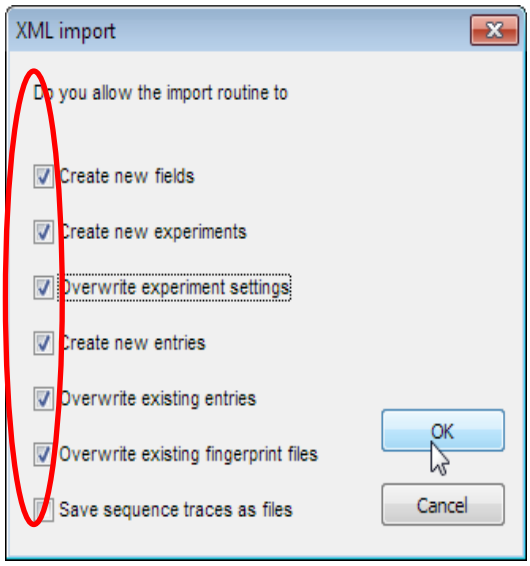
8. Select 'Import entries from XML' in the menu.



9. Locate your newly unzipped files. Select all of them and click on ‘Open’.



10. Mark the box ‘Overwrite experiment settings’ and click ‘OK’.



11. Restart the database.

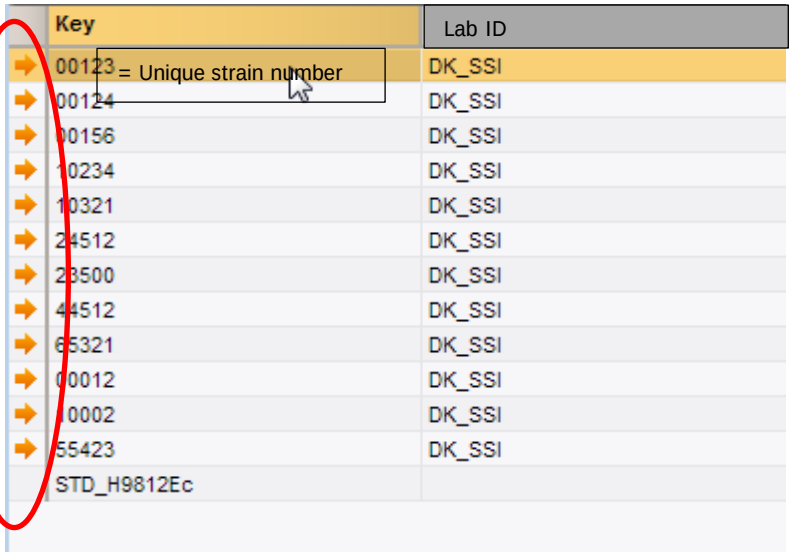
Annex 10. Guide to XML export

After analysing the data, export all results in XML format. The procedure looks slightly different in BioNumerics Version 6 (A) and 7 (B). If you have an older version of the software, the instructions for Version 6 are quite similar.

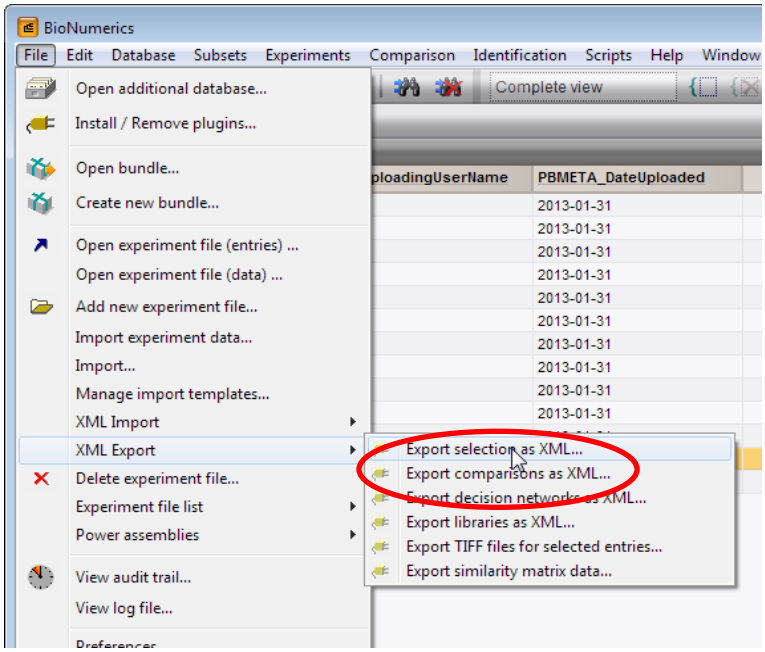
A. Exporting XML data from your database BioNumerics Version 6

In BioNumerics Version 6 and earlier it is necessary to export TIFF files separately from the analysed data. Follow all the steps in this guide.

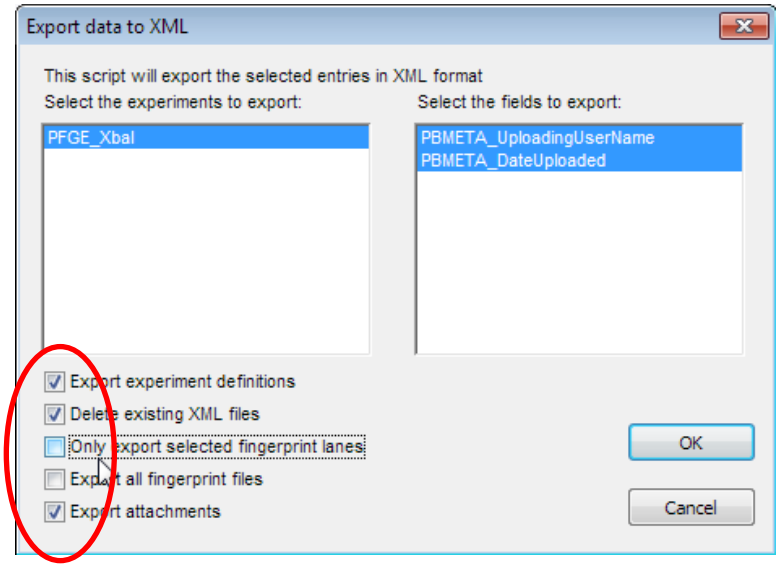
1. After analysing your data, select all the isolates to be exported.



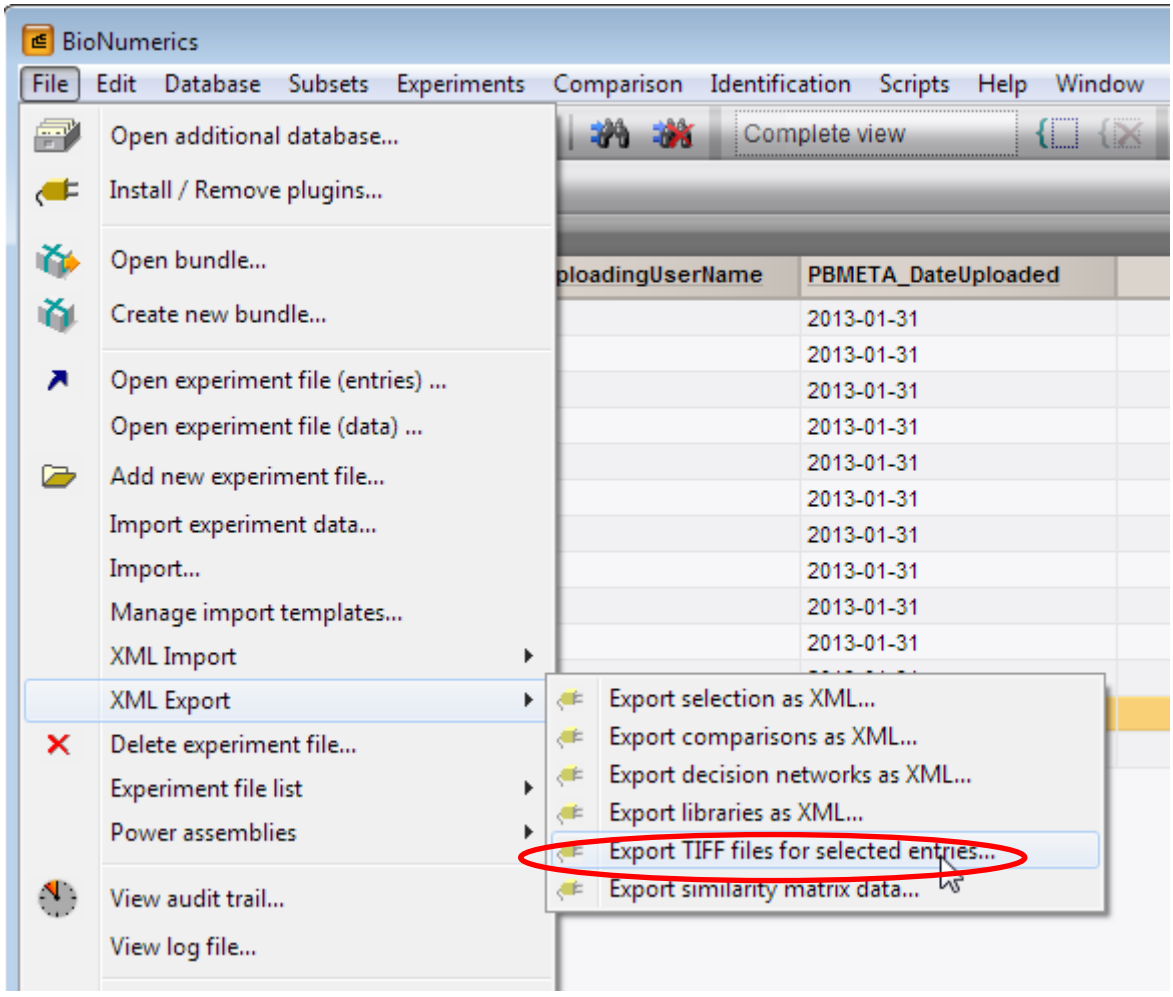
2. Export selection as XML.



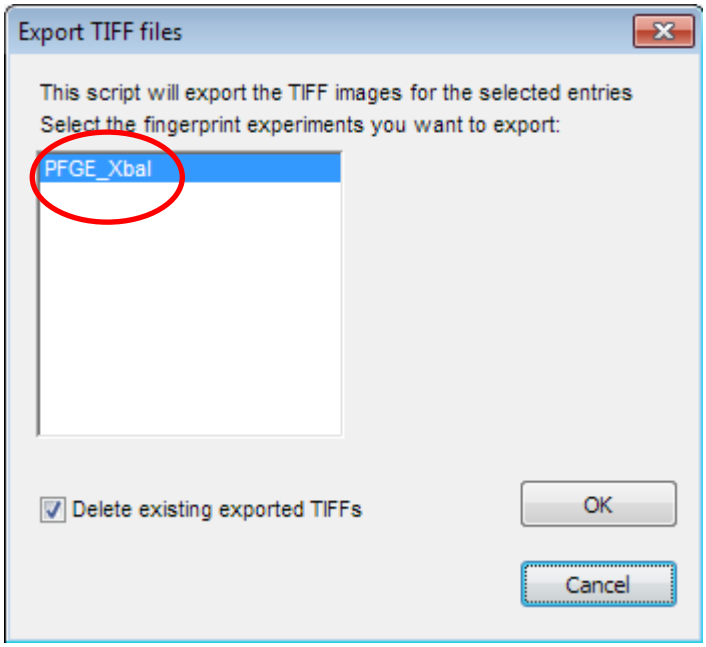
3. De-select the check box 'Only export selected fingerprint lanes'



4. Now export the TIFF file(s).



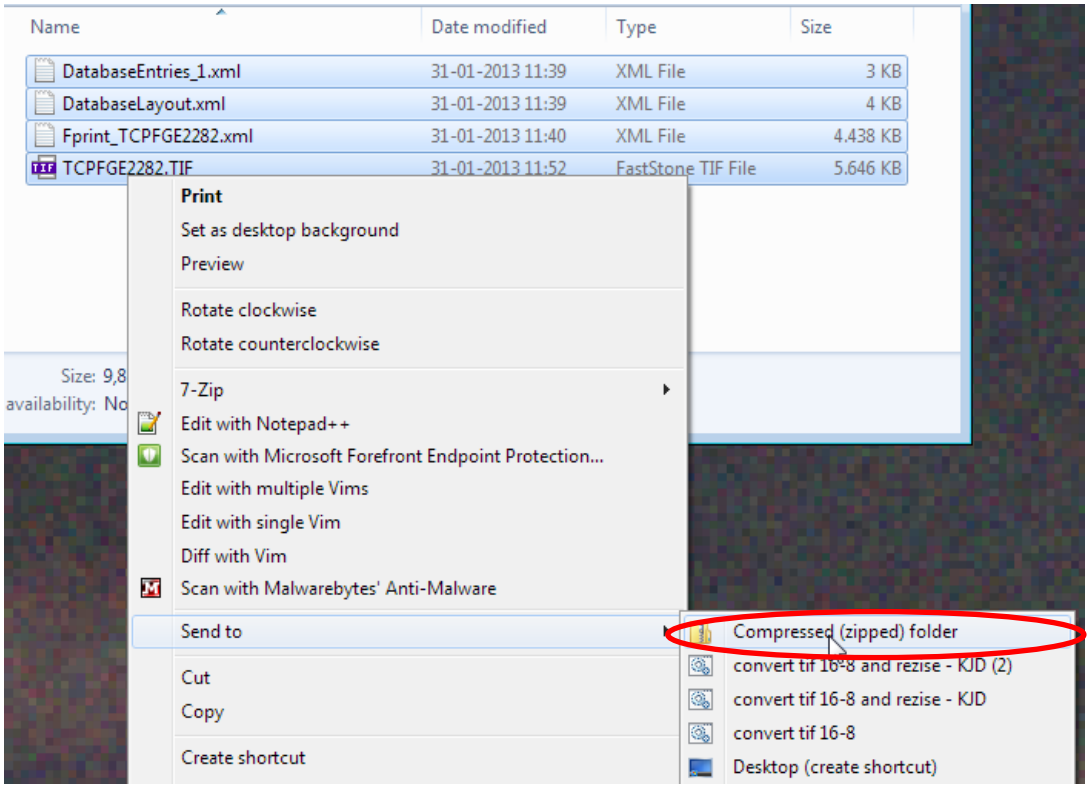
5. Select which experiments to export. In the case of *Listeria* it is possible to export both enzymes at the same time.



6. Now locate the EXPORT directory in your database directory. **Remember to check that the TIFF file is included.**

7. Send all XML and TIFF files located there via mail.

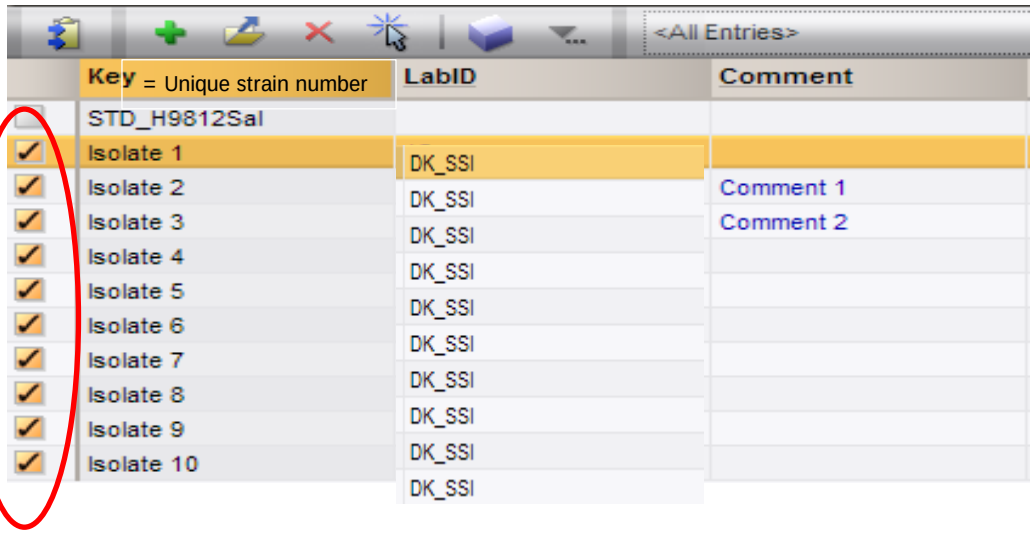
8. Please compress them into a zip archive. One way of creating the zip archive is to mark all the XML and TIFF files, right click on them and choose 'Send to → Compressed (zipped) folder'.



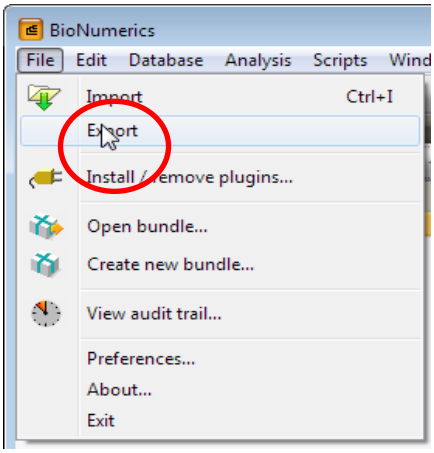
B. Exporting XML data from your database Bionumerics version 7

In BioNumerics 7 all data is exported in a single step.

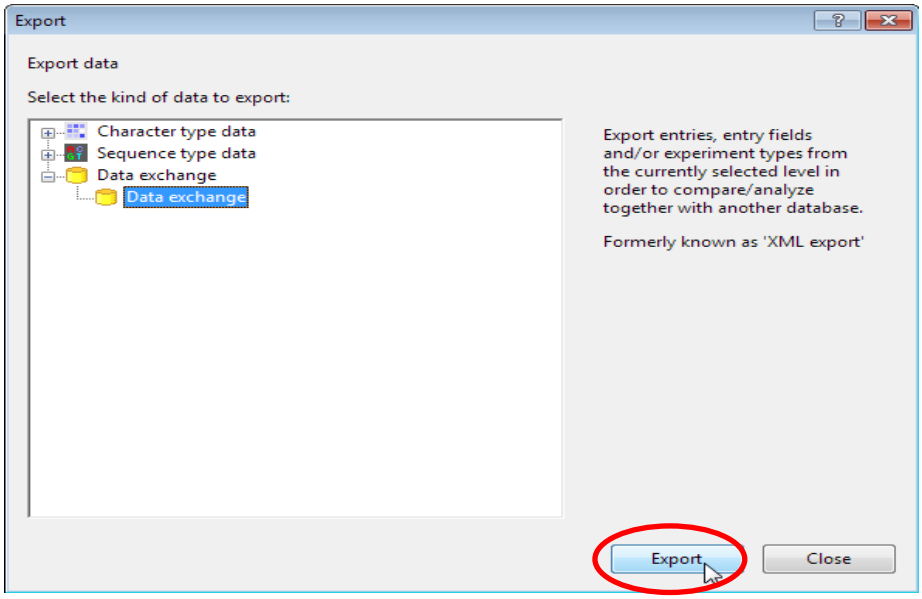
1. Select all the isolates to be exported.



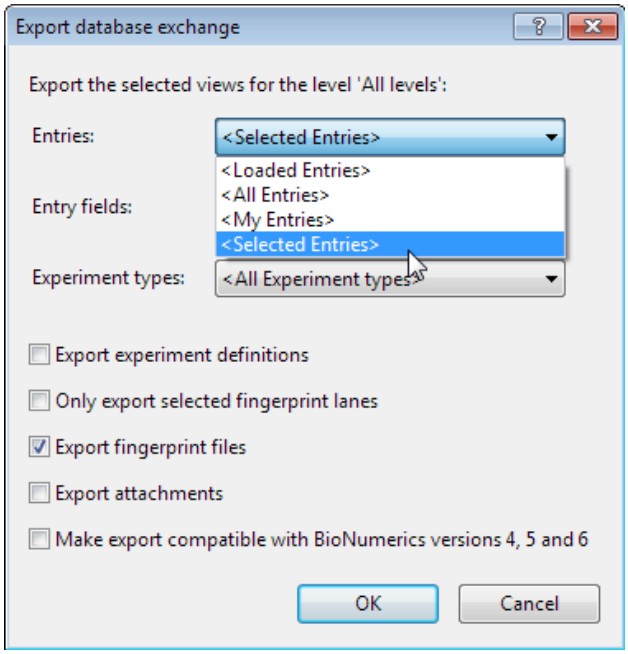
2. Click 'File → Export', choose Data exchange.



3. Click 'Export'



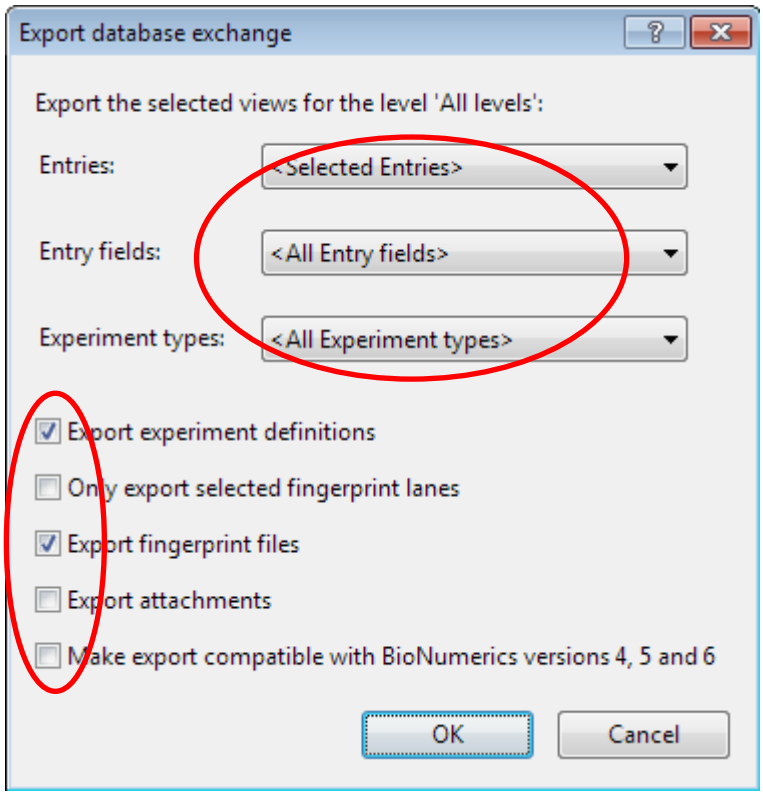
4. Under the 'Entries' drop-down menu choose 'Selected entries'.



5. Under the 'Entry fields' drop-down menu select <All Entry Fields>.

6. Under the 'Experiment types' drop-down menu select <All experiment types>.

7. In the checkboxes tick **ONLY** the alternative 'Export fingerprint files'



8. Now locate the EXPORT directory in your database directory.

9. The export described will yield a file called export.zip that contains all data.

10. Rename the file with your Lab_ID (e.g. DK_SSI).

11. Submit this file to the EQA providers by email.