TECHNICAL REPORT

Fourth external quality assessment scheme for *Salmonella* typing

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Fourth external quality assessment scheme for *Salmonella* typing

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Suggested citation: European Centre for Disease Prevention and Control. Fourth external quality assessment scheme for *Salmonella* typing. Stockholm: ECDC; 2013.

Stockholm, December 2013

ISBN 978-92-9193-512-3

doi 10.2900/93836

Catalogue number TQ-02-13-615-EN-N

Contents

Abbreviations

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
MLVA	Multiple-Locus Variable number of tandem repeats Analysis
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 4th round of the *Salmonella* External Quality Assessment (EQA) scheme for typing of *Salmonella enterica* spp. *enterica* (further EQA-4). The EQA covers the methods Pulsed Field Gel Electrophoresis (PFGE), Multiple Locus Variable number of tandem repeat Analysis (MLVA) and phage typing. A total of 27 laboratories participated in the EQA-4 that took place in January–March 2013.

Salmonellosis is the second most commonly reported zoonotic disease with an EU notification rate of 20.7 cases per 100 000 population, and *Salmonella* is a common cause of foodborne outbreaks. Since 2007, ECDC's Programme on Food- and Waterborne Diseases and Zoonoses (FWD) has been responsible for the EU-wide surveillance of salmonellosis, 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). Besides this basic characterisation of the pathogens, there is a public health value of using more advanced and more 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-4 is to assess the quality of PFGE, MLVA and phage typing and the comparability of the collected test results between participating public health national reference laboratories in EU/EEA and EU candidate countries. Strains for the EQA were selected to cover strains of current public health relevance in Europe. Sets of ten strains were selected for each method, i.e. a mixture of *Salmonella* serovars for PFGE, *Salmonella* Typhimurium strains for MLVA, and *Salmonella* Typhimurium and Enteritidis strains for the corresponding two phage typing schemes.

A total of 27 laboratories participated in at least one part of the EQA-4: 25 laboratories (93%) produced PFGE results, 15 laboratories (56%) produced MLVA results, and 12 (44%) and 11 laboratories (41%) participated in the phage typing of *S.* Enteritidis and *S.* Typhimurium, respectively. Eight laboratories (30%) completed all parts of the EQA.

The majority (64%) of the laboratories were able to produce a PFGE gel of sufficiently high quality to allow for the profiles to be comparable to profiles obtained by other laboratories. The subsequent normalisation and interpretation of the profiles were performed using a specialised software (BioNumerics). Fifteen laboratories completed the gel analysis and this was generally performed in good accordance with the guidelines. Although MLVA is a relatively new method, 60% of the laboratories reported correct MLVA profiles for all strains and 87% found the correct profile for at least nine of the ten strains. The results indicate that the majority of the participating laboratories (93%) were able to perform the critical calibration of raw data and use the agreed nomenclature. Only 41–44% of the laboratories performed phage typing of the two major *Salmonella* serotypes, Typhimurium and Enteritidis. The results showed a fairly good quality of Typhimurium phage typing as 64% of laboratories reported correct results for all strains, and all laboratories found the correct phage type for at least eight of the ten strains. More incorrect phage types were reported for the Enteritidis scheme as 42% of laboratories only reported 50–80% correct types (58% reported correct results for all strains).

This EQA-4 scheme for typing of *Salmonella* is the first EQA specifically organised for laboratories participating in the European Food- and Waterborne Diseases and zoonoses network (FWD-Net) that includes molecular typing methods. The large number of participating laboratories as well as their performance in the EQA is encouraging. The molecular surveillance system that are 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 methods used for EU-wide surveillance are PFGE for all serovars and MLVA for Typhimurium. Furthermore, phage typing of Typhimurium and Enteritidis is included in TESSy and used for surveillance by several EU-countries. This EQA demonstrated that a majority of the participating laboratories were able to produce good typing results. Less than half of the laboratories produced results that need to be improved before inter-laboratory exchange of data; however, for the majority of the identified technical issues an acceptable quality is within reach by optimisation of 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 part of quality management systems and evaluates performance of laboratories, by an outside agency on material that is supplied specially for the purpose. ECDC's disease specific networks organise a series of EQA for EU/EEA countries. In some specific networks non-EU/EEA countries are also involved in the EQA activities organized by ECDC. The aim of the EQA is to identify needs of improvement in laboratory diagnostic capacities relevant to surveillance of disease listed in Decision No 2119/98/EC [2], and to ensure comparability of results in laboratories from all EU/EEA countries. The main purposes of external quality assurance 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, a framework service contract on 'Microbiological characterisation services to support surveillance of *Salmonella*, STEC/VTEC and Listeria infections' for the period 2012–2016 was put out to tender by ECDC. Unit of Foodborne Infections at Statens Serum Institut won the three lots covering *Salmonella*, Shiga toxin/verocytotoxin-producing *Escherichia coli* (STEC/VTEC) and *Listeria monocytogenes*, respectively. The contract for lot 1 (*Salmonella*) covers the organisation of an EQA exercise for PFGE, MLVA and phage typing of *Salmonella spp.,* reference material service for MLVA-typing of *S.* Typhimurium, and molecular typing services. The present report presents the results of the first EQA-exercise of this contract (*Salmonella* EQA-4).

1.2 Surveillance of salmonellosis

Salmonellosis is the second most commonly reported zoonotic disease in EU with a total of 97 897 salmonellosis cases reported by the 27 EU Member States in 2011 (EU notification rate 20.7 cases per 100 000 population). *Salmonella* is a common cause of foodborne outbreaks and in the EU, *Salmonella* is the most frequently detected causative agent in foodborne outbreaks reported to ECDC/EFSA (26.6 % of outbreaks in 2011, corresponding to 1 501 outbreaks.[3]

Since 2007, ECDC's Programme on Food- and Waterborne Diseases and Zoonoses (FWD) has been responsible for the EU-wide surveillance of salmonellosis, including the facilitation of the detection and investigation of foodborne outbreaks. One of the key objectives for the FWD programme is improving and harmonising the surveillance system in the EU in order to increase the 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. Besides this basic characterisation of the pathogens isolated from infections, there is a public health value of using more advanced and more discriminatory typing techniques for surveillance of foodborne infections. Therefore, in 2012 ECDC initiated a pilot project on enhanced surveillance through incorporation of molecular typing data ('molecular surveillance'). In the first pilot phase, three selected FWD-Net pathogens are included: *Salmonella*, *Listeria monocytogenes*, and Shiga toxin/verocytotoxin-producing *Escherichia coli* (STEC/VTEC). The overall goals of integrating molecular typing in 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 to identify new risk factors
- to aid in studying the characteristics of a particular pathogen and its behaviour in a community of hosts.

The molecular typing pilot project gives Member State users access to EU-wide molecular typing data for the included pathogens. 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, the ECDC Food- and Waterborne Programme has supported EQA schemes for serotyping and antimicrobial resistance testing for *Salmonella* and VTEC. These EQA schemes have contributed to strengthen the laboratory capacity in Member States and EEA countries 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 ascertain 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, from 2012, ECDC FWD Programme supported EQA schemes will focus on expert support for molecular typing, namely Pulsed Field Gel Electrophoresis (PFGE) and Multiple-Locus Variable-number tandem repeat analysis (MLVA) of *Salmonella,* PFGE of Shiga toxin/verocytotoxin -producing *Escherichia coli* (STEC/VTEC) and *L. monocytogenes* but includes also quality assurance activities for virulence gene detection, phage typing and serotyping of the selected pathogens. The EQA schemes were targeted to the national reference level laboratories that were expected to already perform the molecular surveillance at the national level.

1.3 Objectives of the EQA-4 scheme

1.3.1 Pulsed Field Gel Electrophoresis (PFGE) typing

The objective of the EQA-4 was to assess the quality of the standard PFGE molecular typing and comparability of the collected test results between 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 Multiple-Locus Variable number of tandem repeats Analysis (MLVA) typing of *Salmonella* Typhimurium

The aim of this EQA-4 was to determine and ensure the quality and integrity of the *S.* Typhimurium MLVA results in each participating laboratory. The MLVA loci of these strains should be sequenced by the organising laboratory so that the exact MLVA profile is known and can be compared to the results obtained by the participating laboratories. This ensured that the EQA covers both the laboratory procedure and the correct data analysis (calibration of raw data into MLVA profiles according to the nomenclature).

1.3.3 Phage typing of *Salmonella* Typhimurium and Enteritidis

The objective was the provision of EQA exercise for phage typing of *Salmonella* Typhimurium and *S.* Enteritidis to assess comparability of results by used phage typing methods and potentially with other methods in the tender.

2. Study design

2.1 Organisation

The *Salmonella* EQA-4 was funded by ECDC and arranged by Statens Serum Insitut (SSI) according to the International Standard ISO/IEC 17043:2010 [4]. The EQA-4 was conducted from January 2013 through March 2013. The EQA-4 included PFGE (different serotypes), MLVA (Typhimurium), and two phage typing schemes (Typhimurium and Enteritidis).

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

Twenty–nine laboratories accepted the invitation to participate but two laboratories later communicated that they were not able to perform the tests. Therefore, a total of 27 laboratories are included in the result tables. The list of participants is presented in Annex 1. The EQA test-strains were sent to the participating laboratories at the end of January 2013. The participants were asked to submit their results by e-mail by the 25th of March 2013.

2.2 Selection of strains

Strains were selected for the EQA-4 programme based on the following criteria: a) they should represent commonly reported strains in Europe; b) they should remain stable during the preliminary testing period at the organising laboratory. SSI tested 42 strains and 30 of these were selected. The 10 strains for the PFGE part were selected based on their PFGE profiles, containing both 'easy' strains without difficult double bands and strains which were very similar. A variety of different serotypes relevant for the epidemiological situation in Europe were selected, including recent outbreak strains (Table 1). For the MLVA part, 10 *S.* Typhimurium were selected to cover common MLVA profiles and profiles with alleles that are not represented in the set of reference strains used for normalisation of fragment sizes. The characteristics of the 30 *Salmonella* test-strains used in the EQA-4 are listed as 'original' together with the participants' results in the tables (Annex 2, 5, 6 and 7).

Table 1. Number and serotypes of the EQA-4 test strains.

Method	No of test strains	Serotypes
PFGE	10	Mbandaka, Strathcona, Aberdeeen, Dublin, Poona, Infantis, Saintpaul, Typhimurium and Enteritidis
MLVA	10	Typhimurium*
Phage typing, *S.* Typhimurium	10	Typhimurium*
Phage typing, *S.* Enteritidis	10	Enteritidis

**The same set of 10 strains was used for the two Typhimurium specific methods.*

In addition to the 30 test strains, laboratories participating in the EQA for MLVA could request the set of 33 reference strains used for normalisation of the MLVA analysis (Annex 8) and the PFGE reference strain *S.* Branderup H9812.

2.3 Carriage of strains

By the end of January all strains were blinded and packed according to the International Standard ISO/IEC 17043:2010 [3] 'Conformity assessment – General requirements for proficiency testing', which has recently been issued (First edition 01/02/2010). The parcels were shipped from SSI labelled as UN 3373 Biological Substance, Category B.

The participants received their specific blinded strain numbers by email as an extra control. No participant reported damage to the shipment or errors in the specific strain numbers.

On 31 January, instructions on how to submit results were e-mailed to participants. This included an Excel sheet for calculating the MLVA alleles, and Excel sheets for submission of PFGE setup, MLVA results, and phage typing results. Included were also Zip files for the Bionumeric database experiment settings (PFGE part) and guidelines on how to export XML files from BioNumerics (Annex 9 and 10).

2.4 Testing

In the PFGE part, 10 *Salmonella* strains with mixed serotypes were tested and participants could choose to take part in the laboratory part only (submit the PFGE gel) or also take part in the additional analysis of the gel (submit normalised profiles with assigned bands). For the laboratory procedures, the participants were instructed to use the protocol *Standard PulseNet Salmonella PFGE -One-Day (24-28 h) Standardized Laboratory Protocol for Molecular Subtyping of Escherichia coli O157:H7, Salmonella serotypes, Shigella sonnei, and Shigella flexneri 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 involved emailing PFGE image either as TIFF file alone or as XML export files of the BioNumerics analysis.

In the MLVA part, 10 *S.* Typhimurium strains were tested to assess the participants' ability to obtain the true number of repeats in each of the five MLVA loci. The participants were instructed to use the *Standard protocol for S. Typhimurium MLVA Laboratory standard operating procedure for MLVA of Salmonella enterica serotype Typhimurium (ECDC, 2011)* [6]. An attached Excel sheet could be used to convert obtained fragment sizes to true allele numbers based on the results obtained for the 33 reference strains. The MLVA profiles (alleles) were submitted by email using the provided Excel sheet.

In the phage typing part, 10 *S.* Typhimurium and 10 *S.* Enteritidis strains were typed. The test evaluated the ability to perform phage typing of *S.* Typhimurium and *S.* Enteritidis using the two serotype specific phage typing systems that are maintained by Public Health England (Colindale). The results were submitted by e-mail using the provided excel sheet.

2.5 Data analysis

The PFGE, MLVA and phage typing results received from the laboratories were added to a dedicated *Salmonella* EQA BioNumerics database at SSI. For PFGE, the gel quality was evaluated according to ECDC FWD 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 BioNumerics analysis' were evaluated by scoring five parameters (scores in the range 1-4, 4 being the top score) using the BioNumerics Gel Analysis Quality Guidelines (Annex 4). The MLVA and phage typing results were scored as correct/incorrect result for each strain and the percentage of correct answers was used as the score for each method.

3. Results

3.1 Participation

The laboratories could choose to participate in the full scheme or only a selection of the methods. Of the 27 participants, 25 laboratories (93%) participated in the PFGE part, 56% performed MLVA typing, 44% phage typing of *S.* Enteritidis, and 41% phage typing of *S.* Typhimurium. Eight laboratories (30%) completed all three parts of the EQA (Table 2).

Table 2. Number of FWD-Net laboratories submitting results for each method. Twenty-seven laboratories participated in at least one method.

Methods	PFGE	MLVA	Phage typing		All methods
			S. T	*S.*E	
Number of participants	25	15	11	12	8
% of participants	93	56	41	44	30

3.2 Pulsed Field Gel Electrophoresis (PFGE)

Twenty five laboratories participated in the PFGE by submitting raw gel images (TIFF files). Fifteen of these laboratories had also analysed the gel by the use of BioNumerics and submitted the results in the form of an XML-file. One data file was not possible to evaluate due to compatibility troubles between BioNumerics version 6 and 7.0.

3.2.1 Gel quality

All participating laboratories were able to produce profiles that were recognisable as the profile for the relevant EQA strain. The gels – and consequently the profiles for individual strains – were of highly variable quality (Table 3). The gels were graded according to the TIFF Quality Grading Guidelines, where seven parameters are used for the grading (Annex 3). In general, an acceptable quality should be obtained for each parameter since a low quality score in just one category can have a high impact on the ability to further analyse the image and compare to other profiles.

For three parameters a high average score, above 3.5, was obtained, i.e. between good and excellent (Table 3). The parameters were Cell Suspension, Lanes and Restriction. Two parameters, DNA Degradation and Gel Background both had an average score of 3.3, also between good and excellent. The two parameters Bands and Image Acquisition and Running conditions had an average score below 3 (2.9 and 2.6 respectively), i.e. between fair and good.

Table 3. Results of PFGE gel quality for 25 participating laboratories

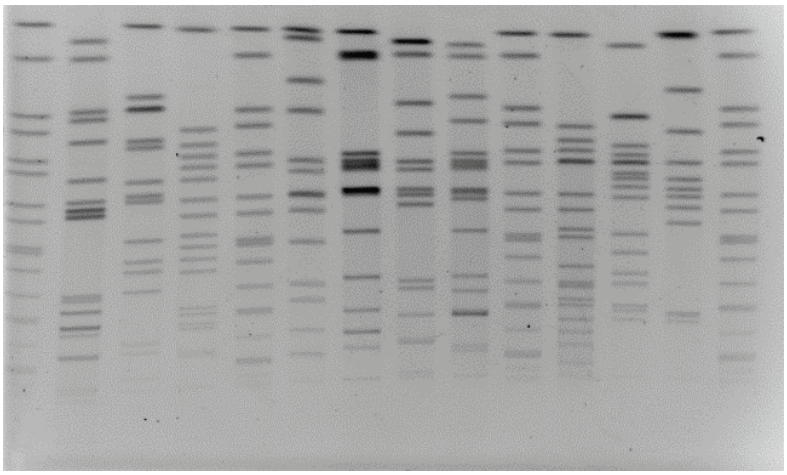
Parameters	1 – Poor (%)	2 – Fair (%)	3 – Good (%)	4 – Excellent (%)	Average (%)
Image acquisition and running conditions	16	32	28	24	2.6
Cell suspension	0	4	4	92	3.9
Bands	28	8	8	56	2.9
Lanes	4	8	4	84	3.7
Restriction	4	8	8	80	3.6
Gel background	0	24	24	52	3.3
DNA degradation	0	20	32	48	3.3

The average score and the percentage of laboratories obtaining scores 1-4 for the seven TIFF Quality Grading Guidelines parameters.

The laboratories obtained very diverse scores for the parameter Image Acquisition and Running Conditions (Table 3). Only 52% of participants were graded good [3] or excellent [4] for this parameter thereby grading 48% of participants at a critical score (1 and 2). For the parameter Bands, 56% of laboratories were graded with a top score 4 (Table 3). Unfortunately, 28% of participants only obtained the score 1 for the parameter Bands, making the further analysis of the gel impossible. Nine (36%) laboratories produced gels that were graded 1 (poor) in at least one of the two parameters Bands and Image Acquisition and Running Conditions. Profiles obtained on gels with poor quality in these parameters will generally be very hard or impossible to compare with profiles produced on other gels. All the participants Gel Quality scores are listed in Annex 5.

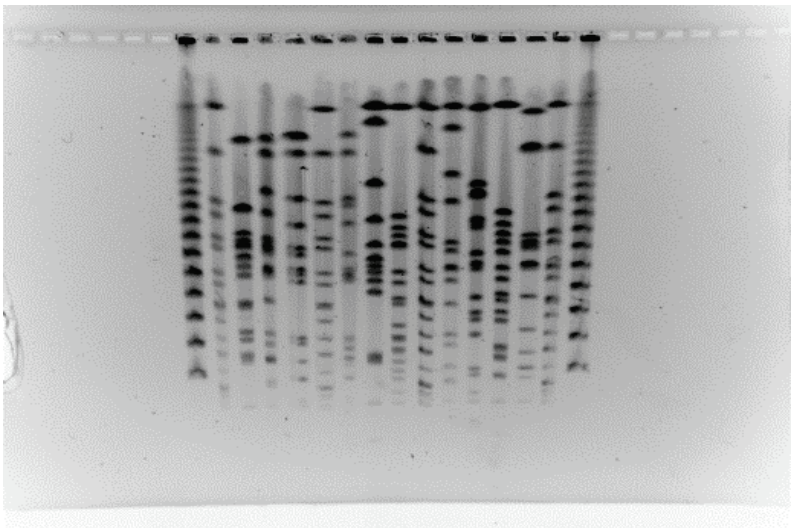
Figure 1 shows a gel that scored 1 (poor) in the parameter Image Acquisition and Running Conditions. The low score was caused by a combination of several factors. The picture was taken without including the wells on the gel, the gel was not run far enough and as can be seen from the next section (BioNumerics analysis), running conditions were not according to protocol.

Figure 1. A gel with low score in Running Conditions.



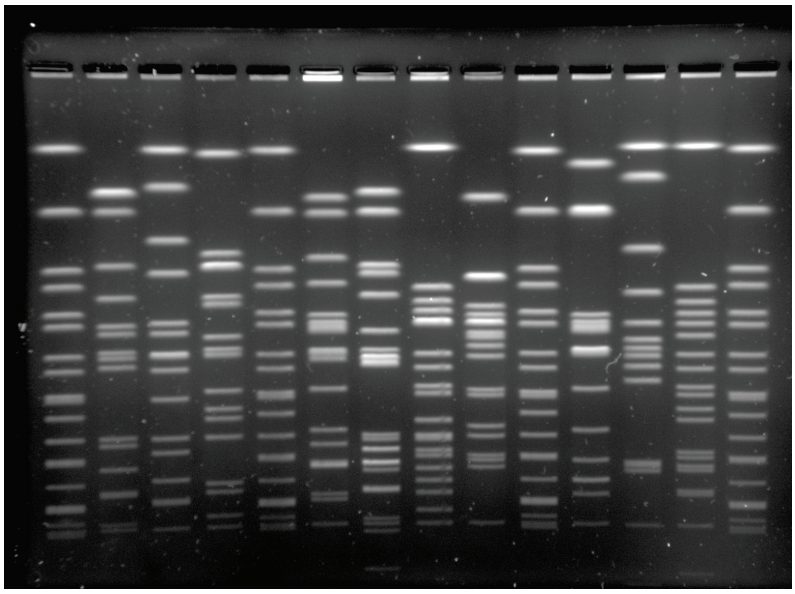
The gel shown in Figure 2 scored 1 (poor) in the parameter Bands (Figure 2). The low score was caused by many bands being too thick to distinguish. The gel also scored 1 in Image Acquisition and Running Conditions for several reasons: the gel was not correctly cropped and the running conditions were not done according to protocol.

Figure 2. A gel with low score in Bands and Image Acquisition and Running Conditions.



Finally, a gel that was scored high for all seven parameters is shown in Figure 3. The image was captured and cropped correctly, there is an even distribution of DNA, the bands are clear and there are no background or shadow bands and only minor debris.

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



3.2.2 Gel analysis by the use of BioNumerics

Fifteen laboratories had analysed the gel and were able to produce XML files according to the protocol attached to the invitation letter (Annex 9 and 10). Unfortunately, we were not able to import one of the XML files due to problems with the version of the software. The presented results are therefore based on fourteen analysed gels. Gel analysis was graded according to the BioNumerics Gel Analysis Quality Guidelines developed at SSI, including five parameters in the grading (Annex 4). All the BioNumerics Gel Quality scores are listed in Annex 5.

Table 4. Results of the BioNumerics analysis obtained by 14 laboratories.

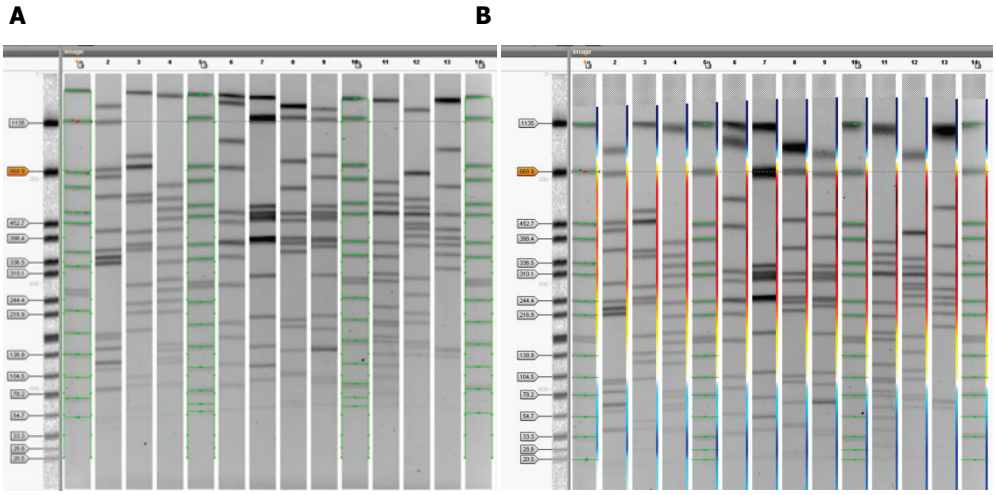
Parameters	1 – Poor (%)	2 – Fair (%)	3 – Good (%)	4 – Excellent (%)	Average
Position of gel	7	0	29	64	3.5
Strips	0	0	0	100	4.0
Curves	0	0	43	57	3.6
Normalisation	7	0	36	57	3.4
Band assignment	14	7	14	64	3.3

The five BioNumerics gel analysis Quality Guidelines parameters and the percentage of laboratories scoring 1-4. Also shown is the average score for all laboratories.

Three parameters, Position of the Gel, Strips, and Curves had a very high average score, above 3.5 (Table 4). Two parameters, Normalisation, and Band Assignment were graded a bit lower with an average of 3.4 and 3.3, respectively. Only 7% of laboratories were unable to correctly position the gel in the frame and perform normalisation, whereas 14% had problems with Band Assignment.

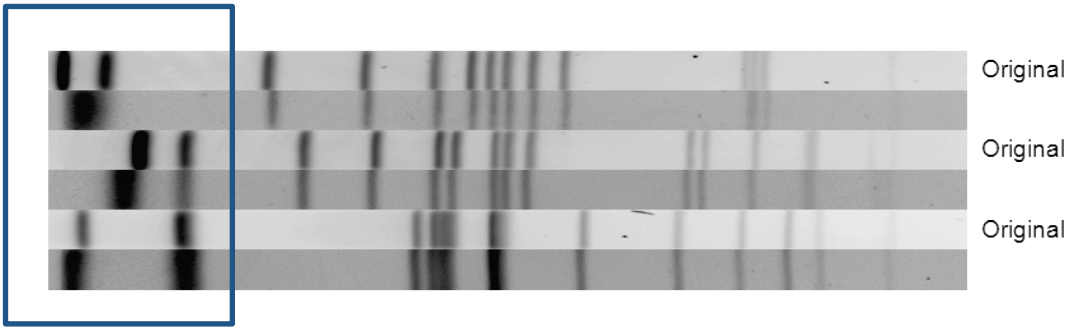
One of the very critical steps is normalisation of the gel in BioNumerics, which is a parameter that is dependent on the use of correct running conditions, i.e. the score from the TIFF Quality Grading Guidelines (Annex 4). Looking at Figure 4, it is clear that the normalisation of the gel made the top bands very thick and fuzzy thereby creating problems in deciding the exact position of the bands and comparing this with other isolates (Figure 5). It also seems like the running conditions used for this particular gel caused an original double band to merge into one band (Figure 5).

Figure 4. Normalisation of a gel by the use of BioNumerics.



A, Showing the band assignment on the reference lanes. B, Colours showing how critical the normalisation is on the gel (darker colours indicate critical normalisation).

Figure 5. Comparison of two profiles for each of three strains.



The original high-quality profile is compared to the profile obtained by the laboratory producing the gel shown in Figure 4. The blue box is framing the critical area caused by inadequate running conditions.

3.3 Multiple-Locus Variable number of tandem repeats Analysis (MLVA)

Fifteen laboratories performed the *Salmonella* Typhimurium MLVA (Annex 6). Nine (60%) of these were able to correctly MLVA type all ten EQA strains (Figure 6). Four laboratories reported the correct MLVA profile for nine of the strains and one laboratory had correct results for seven strains. The typical error accounting for the vast majority of the incorrect profiles by these laboratories was to either replace an NA-locus with a repeat number or vice versa. One of these laboratories seemed to have analysed/reported the MLVA profile for one EQA strain under two strain numbers, thereby getting incorrect profile for one strain. One laboratory had multiple allele errors in several MLVA profiles and these were probably caused by incorrect or lack of calibration of the measured fragment sizes.

For one of the EQA-strains, strain 19, the STTR6 locus seemed to have changed immediately before shipment to the participants resulting in the presence of two alleles in some of the culture tubes. This is clear from the variability in results obtained for this locus of strain 19 (Annex 6). Both alleles were evaluated as correct. In addition, the reporting of one repeat change was evaluated as an acceptable result when observed in one of the highly discriminatory and therefore less stable loci: STTR5, STTR6 or STTR10. The results for each strain are summarised in Table 5.

Table 5. Results of the MLVA analysis (15 laboratories).

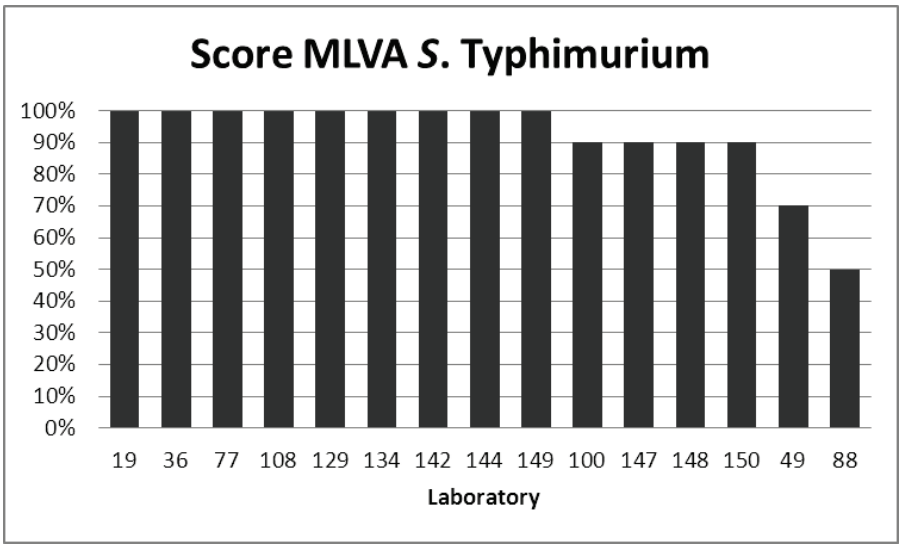
Strain	Correct	Accepted[1]	Incorrect
11	87%	0%	13%
12	93%	7%	0%
13	80%	7%	13%
14	100%	0%	0%
15	80%	7%	13%
16	80%	13%	7%
17	93%	0%	7%
18	93%	0%	7%
19[2]	40%	67%	13%
20	93%	0%	7%

[1] Accepted profiles have one repeat change in one of the loci STTR5, STTR6 or STTR10.

[2] The results for strain 19 gives a total of 120% as three laboratories identifying two MLVA profiles for this strain.

Shown as the percentage of correct profiles, accepted profiles (one repeat change in one of the loci, STTR5, STTR6 or STTR10) and incorrect profiles (error in at least one locus, except the accepted one-repeat changes in the highly variable loci).

Figure 6. Results of MLVA typing of 10 S. Typhimurium strains by 15 laboratories.



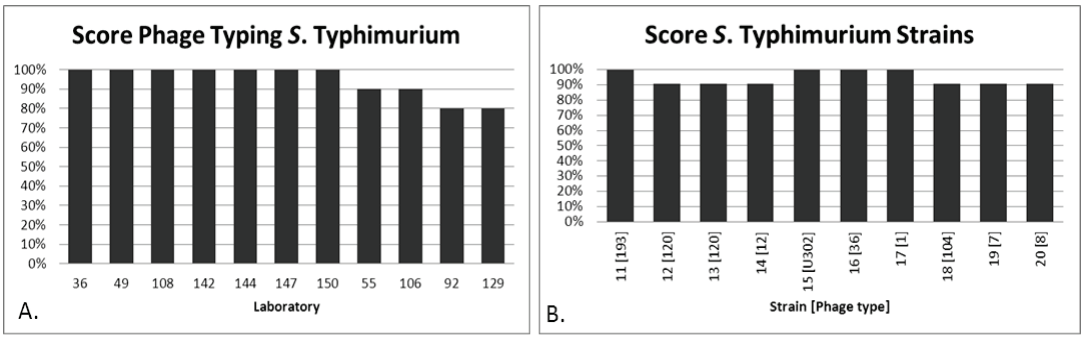
Laboratories are represented by an arbitrary number and their performance is shown as percentage correct or accepted MLVA profiles.

3.4 Phage typing

3.4.1 *Salmonella* Typhimurium

Eleven laboratories performed the *Salmonella* Typhimurium phage typing (Figure 7A). Seven (64%) of these were able to correctly phage type all ten EQA strains. Two laboratories had 90% of the strains correct and two 80%. When looking at each individual strain (Figure 7B), four of the strains were phage typed 100% correctly and six strains were typed incorrectly by one laboratory each. The results obtained by each laboratory are presented in Annex 7.

Figure 7. Results of phage typing of 10 S. Typhimurium strains by 11 laboratories.

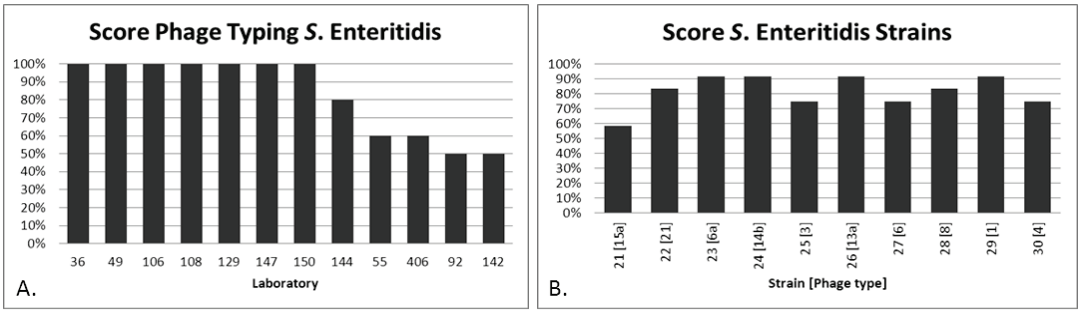


A, laboratories are represented by an arbitrary number and their performance is shown as percentage correct phage types. B, The EQA strains and the percentage correct score.

3.4.1 *Salmonella* Enteritidis

Twelve laboratories performed the *Salmonella* Enteritidis phage typing (Figure 8A). Seven (58%) of these were able to correctly phage type all ten EQA strains. One laboratory had 80% correct, two had 60% and two laboratories only had 50% correct. None of the strains were phage typed correctly by all laboratories (Figure 8B). Only 58% found the correct page type 15a for strain 21 whereas the remaining strains were phage typed correctly by 75%–91% of the laboratories.

Figure 8. Results of phage typing of 10 S. Enteritidis strains by 12 laboratories.



A, Laboratories are represented by an arbitrary number and their performance is shown as percentage correct phage types. B, The EQA strains and the percentage correct score.

4. Conclusion

A total of 27 laboratories participated in at least one part of the EQA-4: 25 laboratories (93%) produced PFGE results, 15 laboratories (56%) produced MLVA results, and 12 (44%) and 11 laboratories (41%) participated in the phage typing of *S.* Enteritidis and *S.* Typhimurium, respectively. Eight laboratories (30%) completed all parts of the EQA.

PFGE is still the gold standard for high discriminatory typing of *Salmonella* and the only generic method for typing of all *Salmonella* serovars. The majority (64%) of the laboratories were able to produce a PFGE gel of sufficiently high quality to allow for the profiles to be comparable to profiles obtained by other laboratories. This comparability primarily relies on the use of correct running conditions, good quality image acquisition, and distinct bands. The subsequent normalisation and interpretation of the profiles were performed using the specialised software BioNumerics. Fifteen (60%) of the laboratories did this analysis of their gel and generally this was performed in good accordance with the guidelines.

MLVA for typing of *S.* Typhimurium is a fairly new method that has been increasingly used over the last decade as this is a fast, low-cost method that gives a high discrimination within one of the most prevalent *Salmonella* serovars. Considering the few years as an internationally recognised method and the need for access to specialised equipment (capillary electrophoresis), it is promising that half of the laboratories choose to participate in the EQA. Although the interpretation of MLVA data is simpler and less prone to subjective interpretation than the band-based PFGE profiles, it is important to calibrate ('normalise') the measured fragment sizes to obtain inter-laboratory comparability of MLVA results. The results indicate that the vast majority (93%) of the laboratories were able to obtain a good calibration of raw data and use the agreed nomenclature. To be sure that the calibration is correct in all cases, it is necessary to have access to the raw data for the test strains as well as the reference strains. Sixty per cent of the laboratories reported correct MLVA profiles for all strains and 87% found the correct profile for at least nine of the ten strains. Most of the errors were probably due to minor problems with the laboratory procedures, which should be possible to overcome by optimising the procedure in each laboratory. To our knowledge, several of the laboratories participating in the EQA are not performing the MLVA method on a routine basis and we therefore expect that the performance could be even higher with more experience and better optimisation in each laboratory. It should be considered for the next EQA to make it mandatory to submit the MLVA raw data to ensure the optimal evaluation of results, including the occurrence of one-repeat changes among test strains and to allow for specific trouble shooting and feed-back to the laboratories.

Phage typing of the two major *Salmonella* serotypes, Typhimurium and Enteritidis, was included in the EQA as the only phenotypic method. The phage typing schemes have been used for surveillance in some parts of Europe for many decades. Only 41–44% of the laboratories participated in this part of the EQA. A rather high level was obtained for phage typing of Typhimurium as all laboratories found the correct phage type for at least eight of the ten strains (64% of laboratories had all correct). More incorrect phage types were reported for the Enteritidis scheme as 42% of laboratories only found 50–80% correct types (58% had all correct). Test strains in both schemes were chosen to be common and well-established phage types. The comparability of phage typing relies on several critical factors such as access to the reference phage stock suspensions, correct dilution and quality check of the phage working dilutions, correct use of the updated phage reaction scheme for assigning the phage type, and experienced personal that preferable are trained at a reference centre. The correct interpretation of the phage reactions is critical and challenging as even minor variations in the phage reactions are in some cases used for assigning the correct phage type.

This EQA-4 scheme for typing of *Salmonella* is the first EQA specifically organised for laboratories participating in FWD-Net, that includes molecular typing methods. The large number of participating laboratories as well as their performance in the EQA is encouraging. The molecular surveillance system that are 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 methods used for EU-wide surveillance are PFGE for all serovars and MLVA for Typhimurium. Furthermore, phage typing of Typhimurium and Enteritidis is included in TESSy and used for surveillance by several EU-countries. This first EQA for molecular typing demonstrates that a majority of participating laboratories were able to produce good typing results. Less than half of the laboratories produced results that need to be improved for inter-laboratory exchange of data; however, for the majority of the identified technical issues, an acceptable quality is within reach by optimisation of procedures in laboratories, trouble-shooting assistance, and training.

5. Discussion

5.1 Pulsed Field Gel Electrophoresis (PFGE)

Twenty-five laboratories participated in the PFGE part of the EQA-4. All laboratories were able to produce a PFGE gel and generate an image of the gel (TIFF file). The gel quality was assessed according to the TIFF Quality Grading Guidelines which involve evaluation of a gel by seven parameters. The majority of the laboratories were able to produce gels with sufficiently high quality for three of the parameters: Cell Suspension, Lanes and Restriction. Therefore, specific focus on these gel quality issues is not required. For the parameters Gel Background and DNA Degradation, no laboratory was given the lowest score 1 (poor). Approximately half the participants were given the highest score (excellent) and the remaining a score of 2 (fair) or 3 (good). These parameters are therefore not the most problematic in this EQA, but it is still desirable to improve the laboratories' capacity in these areas. In general, for a highly sensitive method such as PFGE, it is very important to follow the protocol. In order to improve the categories Gel Background and DNA Degradation, major improvements can be made by carefully following the instructions regarding the lysis step, recommended time of restriction for the relevant enzyme, washing plugs six times as recommended, and de-staining the gel adequately after dying.

In general, major improvements could be made when capturing the image and producing a TIFF file. Many laboratories seemed to enhance the contrast at image acquisition in order to enhance weak bands. Unfortunately, that results in thicker bands and makes it hard to distinguish double bands. This together with overloading plugs with DNA are major contributors to the low score in the category Bands. Many laboratories had problems with the critical category Image Acquisition and Running Conditions. This is problematic as incorrect running conditions will make the gel impossible to compare with other gels run with correct conditions. It is important to use running conditions as described for the relevant organism as these vary significantly between species. It is also important to have equipment that are running properly as well as making sure that the running temperature are as described in protocol. The grading guidelines indicate that the score 2 (fair) can be obtained for the parameter Image Acquisition and Running Conditions even when the band spacing of the standard does not match the global standard. In such cases, the score depends on other criteria included in the evaluation of this parameter. This is, however, inconvenient as it gives the impression that a gel that cannot be normalised correctly is still acceptable. Therefore in this EQA, some of the gels that have obtained at least the score 2 in all parameters are not suitable for inter-laboratory comparison. Other common deviations from protocol is seen for image acquisition where many laboratories forget to fill the whole image with the gel, include wells and leave 1 to 1.5 cm below the smallest band on the gel. This is less critical than using incorrect running conditions, but can still have major impact on the ability to assign bands correctly.

Only 60% 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 by the use of specialised software, BioNumerics, and some laboratories might not have access to this or limited experience in using BioNumerics databases for PFGE analysis. However, to be able to perform national surveillance as well as to submit profiles to the EU-wide Molecular Surveillance System within TESSy, it is important to have the capacity to analyse and interpret the PFGE gels. Most of the 15 laboratories that submitted gel analysis data had performed this in good accordance with the guidelines.

5.2 Multiple-Locus Variable number of tandem repeats Analysis (MLVA)

Fifteen laboratories participated in the MLVA part of the EQA, which consisted of ten strains of *S.* Typhimurium and monophasic variants of this serovar. Of the 15 laboratories, 60% MLVA typed all strains correctly and 87% reported correct MLVA profiles for at least nine strains.

One laboratory had major problems with the correct allele calling. The many errors suggest problems with analysing the reference strains or problems with the use of the results of the reference strains for correct calibration of the fragment sizes obtained for the EQA strains. The problems with the MLVA analysis at this laboratory should be solved before continuing to use MLVA for external comparisons. We have asked for raw data as well as the reference run from this laboratory to explore the reasons for the deviating results. SSI will support with trouble shooting if that is agreed by the participant.

Except for this one laboratory that seems to have general problems with the calibration of fragment sizes, most other errors were related to missing the presence of a locus (reporting as absent allele (NA) where a fragment should have been detected) or vice versa, i.e. a false positive allele number for an absent locus. This can be due to the use of unbalanced primer mix resulting in very different peak heights and thereby either missing a peak or identifying background noise as a signal. Another explanation can be that the samples for capillary electrophoresis were overloaded, which can cause large peaks to pick up other primer dyes used in the mix and thereby be mistaken for a peak representing another locus. A common laboratory mistake is failure to add primers to one tube and therefore fail to detect a locus.

One of the EQA test strains had changed in one locus during the overnight growth before making stabs for sending strains to participants. This resulted in a mix of alleles in the cultures sent to at least some of the laboratories. Three highly skilled laboratories were very impressively able to find both alleles and send results for this. The changes in the fast changing loci are unfortunately impossible to avoid and it is not possible to foresee when such change will appear. The test strains were passaged ten times and re-tested to check for stability before sending out; however, it might be possible in future EQAs to perform a last test on the actual cultures to be sent out to avoid a situation with mixed alleles. However, for a high discriminatory method as MLVA, there is always a risk of changes in the profile during the transport and culturing taking place in the laboratories before testing. In general, changes only occur in the fast changing loci, STTR5, STTR6 and STTR10 and changes in these loci were therefore accepted when evaluating the results of this EQA. This implies a risk for higher scores than justified as some of the reported one-locus variants could be due to sub-optimal calibration of measured fragment sizes. Except for strain 19 with mixed alleles, an acceptable one-repeat variant was reported in only one instance by one of the high-performing laboratories. It can be deduced from analysis of the raw data if a strain has actually changed or if the variant is due to calibrations problems. Therefore, it could be considered to ask for raw data from all participants in future EQAs.

5.3 Phage typing

Eleven and twelve laboratories participated in the EQA schemes for Enteritidis and Typhimurium, respectively. Five laboratories had a 100% correct phage typing of both Enteritidis and Typhimurium. Only two laboratories made errors in both the Enteritidis and the Typhimurium phage typing scheme. Approximately 40% of the participants made mistakes in phage typing, indicating that it is possible to improve the phage typing capacity among participants. Test strains in both schemes were chosen to be common and well-established phage types.

5.3.1 *Salmonella* Typhimurium

Eleven laboratories performed the *S.* Typhimurium phage typing. Seven of these (64%) were able to correctly phage type all ten EQA strains compared to 23% in the EQA-3 [7]. The remaining laboratories had only error in one strain each. All errors were unique to that laboratory. We therefore conclude that there was not a problem with the distributed strains, although there is a theoretical possibility of strains changing phage type during transportation. Four of the phage types were typed correctly by all laboratories (phage types 193, 302, 36 and 1). Some of the errors made could indicate that the problems are when interpreting the phage reaction. This step is very critical since the phage reaction is important for assigning the correct phage type and because some phage types are different by only one or a few phage reactions. For two strains in particular (phage types 120 and 104), it seems that the misinterpretation was done for the additional phages that are important for distinguishing some Typhimurium phage types.

5.3.2 *Salmonella* Enteritidis

Twelve laboratories participated in this part of the EQA. Seven (58%) laboratories performed 100% correctly compared to 47% in the EQA-3 [7]. The remaining laboratories typed several strains incorrectly. Two laboratories had only 50% of the 10 EQA strains correct. Errors were made on all ten EQA strains and the errors were very different from one laboratory to another. The incorrect phage types were likely to occur due to misinterpretation of phage reactions or possibly mistakes in reading the phage typing scheme when translating a phage reaction into a phage type.

6. Recommendations

6.1 Laboratories

By evaluating the results obtained by the FWD-Net laboratories in this EQA, a number of technical issues that have an impact on the quality of typing results were identified. For each method, improvements of the performance can be expected to be achieved by a range of measures.

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

A little more than half of the laboratories participated in the MLVA exercise, and the results indicate that a few of these laboratories are probably not using this method routinely and they could benefit from getting more experience by regular use of the method. Most of the rather minor mistakes made can probably be attributed to lack of optimisation of the procedures in each laboratory, e.g. primer mix for the multiplex PCR reaction and the load of DNA in samples for capillary electrophoresis.

Phage typing is a method that is highly dependent on access to, and use of, standardised phage solutions which are provided by a single supplier (Public Health England, Colindale). Furthermore, trained and experienced personnel are important for the results. As a variety of misinterpretations were made, no specific technical issues can be pointed out based on the reported phage types (raw data was not included).

A number of other types of errors were made which could easily be avoided, e.g. by reading the instructions for how to create and send TIFF and XML files of the PFGE results, by keeping track on numbering and strains to avoid mixing up strains, and by proofreading the results before submission. For this first EQA on molecular typing, some errors in procedures were accepted and forwarding extra results/information/corrections after deadline was accepted in some instances. This cannot be expected to be the case in future EQA rounds.

6.2 ECDC and FWD-Net

The PFGE part of the EQA-4 had a high participation rate and many laboratories were able to produce fairly good gels. However, the fact that 60% of these laboratories were only capable of submitting the raw gel image and did not perform the data analysis part indicates that there is a need for capacity building in the area of gel analysis and interpretation by the use of BioNumerics.

Only few laboratories participated in the phage typing part of the EQA. Over the last years, the number of laboratories that perform phage typing has decreased and the applicability for international surveillance and outbreak investigations is therefore reduced. Although the results were fairly good, the few participants and the obtained results indicate that more support is probably needed to expand the use of phage typing and to improve the quality, especially of the Enteritidis scheme. As the general trend goes towards molecular methods, it should be considered if an expansion of the use of phage typing is feasible. Alternatives to phage typing of these two common *Salmonella* serovars is the well-established MLVA for Typhimurium (the method already included in this EQA and in the EU Molecular Surveillance System), and one of the MLVA protocols described and validated for typing of Enteritidis. In the longer perspective, whole genome sequence (WGS) based methods will most likely take over, as laboratories over time implement WGS and WGS-based methods, and are harmonised and made applicable for international comparison.

Since 44% of the laboratories did not participate in the MLVA exercise and probably have not implemented this method, there is a potential for much more use of MLVA for typing of Typhimurium, the second most common serovar in Europe. For new laboratories wishing to take up this method as well as some of the less experienced laboratories, a training course might be the way to increase the capacity. No training courses specifically for MLVA typing have previously been provided for the FWD-Net laboratories. In this context, the implementation of MLVA for Enteritidis as a standard method in the surveillance should also be considered. To further explore the relevance of this, a survey among the FWD-Net laboratories could be initiated to map the present use, the epidemiological usefulness, and the interest in implementing MLVA for Enteritidis.

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 and adopted from PulseNet USA. By using this scheme for evaluation of gel quality in this EQA, it is our experience that in some cases there is an unclear correspondence between the score and the suitability of the gel for inter-laboratory comparability. Therefore, the EQA provider will modify the way the scoring system is interpreted so that a gel is always given the score ´poor´ in a parameter that has such severe quality deficiencies that it is impossible to use the gel for reliable comparison to gels obtained in other laboratories.

The EQA provider recommends that the laboratories in all cases are asked to provide the raw MLVA data for the reference strains and the EQA strains. It is usually necessary in order to evaluate the validity of possible one-repeat changes in the fast changing loci of the test strains. Furthermore, access to the raw data allows for relevant feedback to laboratories on improvements and trouble shooting.

The feedback on the organisation of the EQA given by the FWD-Net laboratories points at the tight time schedule for laboratories participating in the three EQA's for typing of foodborne pathogens as well as other EQA schemes. Therefore, it would be preferable to separate the deadlines as much as possible for the next EQA and generally give more time to finish the results. Since the evaluation of results needs to be done individually and cannot be automated due to the visual evaluation of the PFGE gels and analysis, it is also necessary to allow for a reasonable time from receipt of results to the individual evaluation reports and the final EQA report. Furthermore, individual feedback and trouble-shooting regarding the molecular methods are part of the task for the organiser of this EQA. This can be quite time consuming and therefore the organisers should reserve time for this, especially in the time 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 be attributed to our guidelines to the participants that for some subjects seemed not to be 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 provide sufficient details. However, this also implies that deadlines for submission of results according to the guidelines will be enforced. 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.

References

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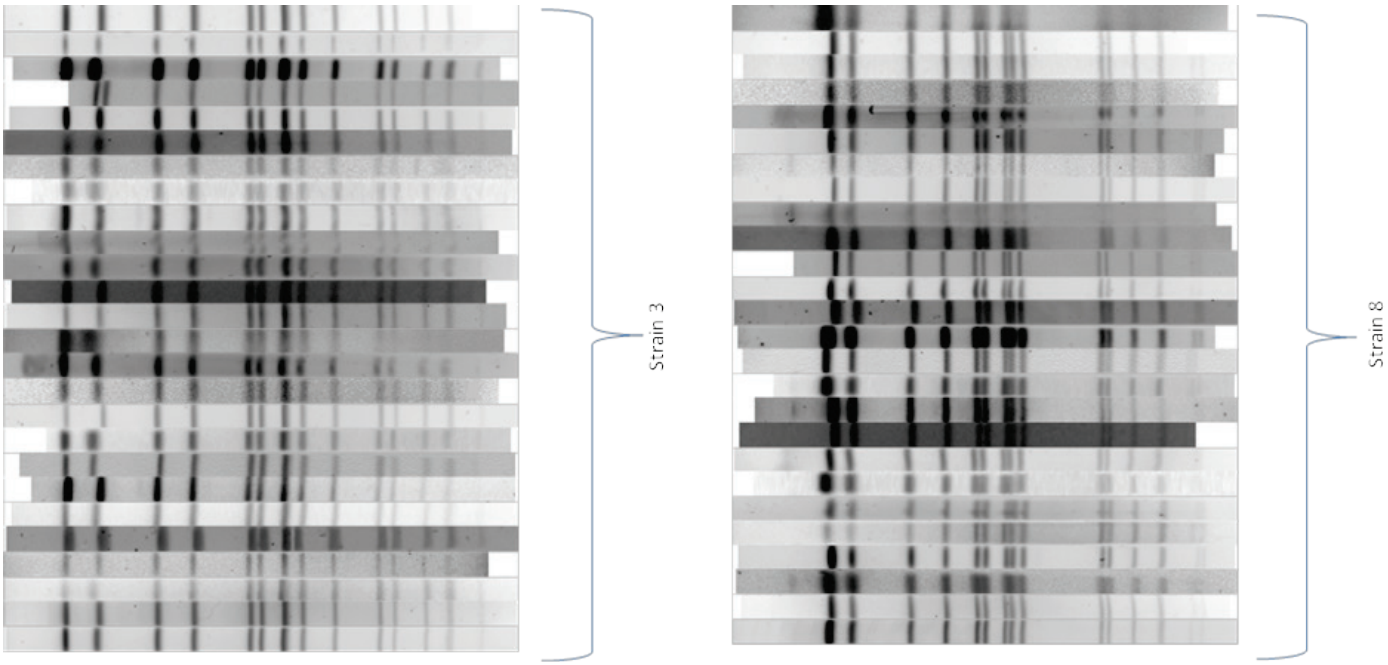
Annex 1. List of participants

Country	ECDC_Institut	ECDC_Laboratory
AUSTRIA	INSTITUTE FOR MEDICAL MICROBIOLOGY AND HYGIENE GRAZ	NATIONAL REFERENCE CENTRE FOR SALMONELLA AUSTRIA
BELGIUM	INSTITUTE OF PUBLIC HEALTH	NATIONAL REFERENCE CENTRE FOR SALMONELLA AND SHIGELLA
BULGARIA	NATIONAL CENTER OF INFECTIOUS AND PARASITIC DISEASES	NRL FOR ENTERIC PATHOGENS
DENMARK	STATENS SERUM INSTITUT	UNIT OF FOODBORNE INFECTIONS
ESTONIA	HEALTH BOARD	CENTRAL LABORATORY OF COMMUNICABLE DISEASES
FINLAND	THL - INSTITUTE OF HEALTH AND WELFARE	BACTERIOLOGY UNIT
FRANCE	INSTITUT PASTEUR	FRENCH NATIONAL REFERENCE CENTER FOR SALMONELLA
GERMANY	ROBERT KOCH - INSTITUT	NATIONAL REFERENCE CENTRE FOR SALMONELLAE AND OTHER 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
ICELAND	LANDSPITALI UNIVERSITY HOSPITAL	DEPT. OF CLINICAL MICROBIOLOGY
IRELAND	UNIVERSITY HOSPITAL GALWAY	NATIONAL SALMONELLA, SHIGELLA AND LISTERIA REFERENCE LABORATORY
ITALY	ISTITUTO SUPERIORE DI SANITÀ	DEPARTMENT OF INFECTIOUS, PARASITIC AND IMMUNE MEDIATED DISEASES
LATVIA	RIGA EAST CLINICAL UNIVERSITY HOSPITAL	BACTERIOLOGY DEPARTMENT
LUXEMBOURG	LABORATOIRE NATIONAL DE SANTE	DEPARTMENT OF MICROBIOLOGY
NETHERLANDS	NATIONAL INSTITUTE FOR PUBLIC HEALTH AND THE ENVIRONMENT	LABORATORY FOR INFECTIOUS DISEASES AND PERINATAL SCREENING
NORWAY	NORWEGIAN INSTITUTE OF PUBLIC HEALTH	NATIONAL REFERENCE LABORATORY FOR ENTEROPATHOGENIC BACTERIA
POLAND	NATIONAL INSTITUTE OF PUBLIC HEALTH - NATIONAL INSTITUTE OF HYGIENE	DEPARTMENT OF BACTERIOLOGY
PORTUGAL	INSTITUTO NACIONAL DE SAÚDE DR. RICARDO JORGE	DEPARTAMENTO DE DOENÇAS INFECCIOSAS
ROMANIA	CANTACUZINO NATIONAL INSTITUTE OF RESEARCH - DEVELOPMENT FOR MICROBIOLOGY & IMMUNOLOGY	MOLECULAR EPIDEMIOLOGY LABORATORY
SERBIA	INSTITUTE OF PUBLIC HEALTH OF SERBIA "DR MILAN JOVANOVIC BATUT"	REFERENCE LABORATORY FOR SALMONELLA, SHIGELLA AND VIBRIO CHOLERAE
SLOVAK REPUBLIC	PUBLIC HEALTH AUTHORITY OF THE SLOVAK REPUBLIC	LABORATORY FOR MOLLECULAR DIAGNOSTICS
SLOVENIA	NATIONAL INSTITUTE OF PUBLIC HEALTH	DEPARTMENT OF MEDICAL MICROBIOLOGY
SPAIN	INSTITUTO DE SALUD CARLOS III	UNIDAD DE ENTEROBACTERIAS
SWEDEN	SMITTSKYDSINSTITUTET	FOOD AND WATER UNIT
TURKEY	PUBLIC HEALTH INSTITUTION OF TURKEY	NATIONAL REFERENCE LABORATORY FOR ENTERIC PATHOGENS
UNITED KINGDOM	HEALTH PROTECTION AGENCY - MICROBIOLOGY SERVICES, COLINDALE	GASTROINTESTINAL BACTERIA REFERENCE UNIT

Annex 2. Examples of PFGE profiles



Profiles from the participants.

Annex 3. TIFF quality grading guidelines quality guidelines[1]

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	- 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])

[1] ECDC FWD MolSurv Pilot - SOPs 1.0 - Annex 5 - PulseNet US protocol PFGE Image Quality Assessment.

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 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 to frame 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 very narrow strip or encompassing almost the whole lane		
		(Average thickness is recommended to be reduced/ increased to ~1/3 of the lane)		
Normalization	All bands assigned correctly in all reference lanes	Bottom band at 20,5 kb were 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 regards to the quality of the gel	Some double bands are assigned wrong		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 requires control of band assignment after using auto search)	Only used auto search to find bands, no manual corrections
				(Guidelines requires control of band assignment after using auto search)

Annex 5. Scores of the PFGE results

Gel Quality

Parameters\Laboratory	147	125	142	36	19	128	106	129	148	130	144	49	132	55	134	138	77	406	92	140	108	100	96	114	150
Image and Running Conditions	2	2	2	4	3	1	4	3	3	2	3	4	1	2	2	3	1	1	4	2	3	3	4	4	2
Cell Suspension	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	3	4	4	4	4	2
Bands	1	1	4	4	4	1	4	4	1	2	3	4	1	4	2	4	4	4	4	4	4	4	1	3	1
Lanes	4	4	4	4	4	2	4	4	4	4	4	4	1	4	4	4	4	3	4	2	4	4	4	4	4
Restriction	4	4	4	4	4	3	4	2	4	4	4	1	4	4	4	2	4	4	4	4	4	4	4	4	3
Gel Background	4	2	3	3	3	4	2	2	2	4	4	2	4	4	4	4	4	3	3	4	4	3	2	4	4
DNA Degradation	3	2	3	4	4	4	3	4	4	3	4	4	2	4	2	2	3	4	4	3	4	3	2	4	3

Scored by Annex 3 (TIFF Quality Grading Guidelines)

BioNumerics analysis

Parameters\Laboratory	147	142	36	19	129	130	49	132	55	134	406	92	108	150
Position of Gel	4	1	4	4	4	4	4	3	4	3	3	4	3	4
Strips	4	4	4	4	4	4	4	4	4	4	4	4	4	4
Curves	4	4	3	4	4	3	3	4	4	3	3	4	4	3
Normalization	4	3	4	4	3	3	4	4	4	4	1	3	4	3
Band Assignment	4	4	1	4	2	4	4	3	4	3	1	4	4	4

Scores by Annex 4 (BioNumerics Gel Analysis Quality Guidelines)

Annex 6. MLVA results

	11					12					13					14					15					16					17					18					19					20				
Laboratory/Strain no. /Allele ID	STTR9-Real	STTR5-Real	STTR6-Real	STTR10-Real	STTR3-Real	STTR9-Real	STTR5-Real	STTR6-Real	STTR10-Real	STTR3-Real	STTR9-Real	STTR5-Real	STTR6-Real	STTR10-Real	STTR3-Real	STTR9-Real	STTR5-Real	STTR6-Real	STTR10-Real	STTR3-Real	STTR9-Real	STTR5-Real	STTR6-Real	STTR10-Real	STTR3-Real	STTR9-Real	STTR5-Real	STTR6-Real	STTR10-Real	STTR3-Real	STTR9-Real	STTR5-Real	STTR6-Real	STTR10-Real	STTR3-Real	STTR9-Real	STTR5-Real	STTR6-Real	STTR10-Real	STTR3-Real	STTR9-Real	STTR5-Real	STTR6-Real	STTR10-Real	STTR3-Real	STTR9-Real	STTR5-Real	STTR6-Real	STTR10-Real	STTR3-Real
Original	2	9	15	5	212	3	12	9	-2	211	3	13	-2	-2	211	4	14	12	8	211	3	16	15	23	311	4	18	-2	10	212	3	16	-2	-2	311	3	13	16	14	311	4	8	19	10	211	2	17	-2	15	212
19	2	9	15	5	212	3	12	9	-2	211	3	13	-2	-2	211	4	14	12	8	211	3	16	15	23	311	4	18	-2	10	212	3	16	-2	-2	311	3	13	16	14	311	4	8	19	10	211	2	17	-2	15	212
36	2	9	15	5	212	3	12	9	-2	211	3	13	-2	-2	211	4	14	12	8	211	3	16	15	23	311	4	18	-2	10	212	3	16	-2	-2	311	3	13	16	14	311	4	8	18	10	211	2	17	-2	15	212
49	2	9	15	5	212	3	12	9	-2	211	3	13	-2	-2	211	4	14	12	8	211	3	16	15	-2	311	4	18	9	10	212	3	16	-2	-2	311	3	13	16	14	311	4	8	18	-2	211	2	17	-2	15	212
77	2	9	15	5	212	3	12	9	-2	211	3	13	-2	-2	211	4	14	12	8	211	3	17	15	23	311	4	18	-2	10	212	3	16	-2	-2	311	3	13	16	14	311	4	8	18	10	211	2	17	-2	15	212
88	2	28	15	5	212	3	12	8	-2	211	3	12	-2	-2	211	4	14	12	8	211	3	16	16	23	410	4	18	-2	9	212	3	16	-2	-2	410	3	12	15	13	410	4	8	18	9	211	2	16	-2	14	212
100	2	9	15	5	212	3	12	9	-2	211	3	13	-2	-2	211	4	14	12	8	211	3	16	15	23	311	4	18	-2	10	212	3	16	-2	-2	311	3	13	16	14	311	4	8	-2	10	211	2	17	-2	15	212
108	2	9	15	5	212	3	12	9	-2	211	3	13	-2	-2	211	4	14	12	8	211	3	16	15	23	311	4	18	-2	10	212	3	16	-2	-2	311	3	13	16	14	311	4	8	18/19	10	211	2	17	-2	15	212
129	2	9	15	5	212	3	12	9	-2	211	3	13	-2	-2	211	4	14	12	8	211	3	16	15	23	311	4	18	-2	10	212	3	16	-2	-2	311	3	13	16	14	311	4	8	18	10	211	2	17	-2	15	212
134	2	9	15	5	212	3	12	9	-2	211	3	13	-2	-2	211	4	14	12	8	211	3	16	15	23	311	4	18	-2	10	212	3	16	-2	-2	311	3	13	16	14	311	4	8	19	10	211	2	17	-2	15	212
142	2	9	15	5	212	3	12	9	-2	211	3	13	-2	-2	211	4	14	12	8	211	3	16	15	23	311	4	18	-2	10	212	3	16	-2	-2	311	3	13	16	14	311	4	8	18/19	10	211	2	17	-2	15	212
144	2	9	15	5	212	3	12	9	-2	211	3	13	-2	-2	211	4	14	12	8	211	3	16	15	23	311	4	18	-2	10	212	3	16	-2	-2	311	3	13	16	14	311	4	8	19	10	211	2	17	-2	15	212
147	2	9	15	5	212	3	12	9	-2	211	3	13	16	14	311	4	14	12	8	211	3	16	15	23	311	4	18	-2	10	212	3	16	-2	-2	311	3	13	16	14	311	4	8	18	10	211	2	17	-2	15	212
148	2	9	15	5	212	3	12	9	-2	211	3	13	12	-2	211	4	14	12	8	211	3	16	15	23	311	4	18	-2	10	212	3	16	-2	-2	311	3	13	16	14	311	4	8	18	10	211	2	17	-2	15	212
149	2	9	15	5	212	3	12	9	-2	211	3	13	-2	-2	211	4	14	12	8	211	3	16	15	23	311	4	18	-2	10	212	3	16	-2	-2	311	3	13	16	14	311	4	8	18/19	10	211	2	17	-2	15	212
150	2	9	14	-2	212	3	12	9	-2	211	3	13	-2	-2	211	4	14	12	8	211	3	16	15	23	311	4	18	-2	11	212	3	16	-2	-2	311	3	13	16	14	311	4	8	18	10	211	2	17	-2	15	212

Incorrect result

Accepted result

Annex 7. Phage typing results

Salmonella Typhimurium

Laboratory/Strain no.	11	12	13	14	15	16	17	18	19	20
Original	193	120	120	12	U302	36	1	104L	7	8
36	193	120	120	12	U302	36	1	104L	7	8
49	193	120	120	12	U302	36	1	104L	7	8
55	193	120	120	12	U302	36	1	120	7	8
92	193	120	151	109	U302	36	1	104	7	8
106	193	104b	120	12	U302	36	1	104L	7	8
108	193	120	120	12	U302	36	1	104	7	8
129	193	120	120	12	U302	36	1	104	59	66
142	193	120	120	12	U302	36	1	104L	7	8
144	193	120	120	12	U302	36	1	104	7	8
147	193	120	120	12	U302	36	1	104L	7	8
150	193	120	120	12	U302	36	1	104L	7	8

Salmonella Enteritidis

Laboratory/Strain no.	21	22	23	24	25	26	27	28	29	30
Original	15a	21	6a	14b	3	13a	6	8	1	4
36	15a	21	6a	14b	3	13a	6	8	1	4
49	15a	21	6a	14b	3	13a	6	8	1	4
55	19	21	6a	14	3a	13a	6	34	1	4
92	60	21	6	14b	3	22	21a	2	1	1a
106	15a	21	6a	14b	3	13a	6	8	1	4
108	15a	21	6a	14b	3	13a	6	8	1	4
129	15a	21	6a	14b	3	13a	6	8	1	4
142	15	21c	6a	14b	3	13a	6c	8	1b	4b
144	15	21	6a	14b	3a	13a	6	8	1	4
147	15a	21	6a	14b	3	13a	6	8	1	4
150	15a	21	6a	14b	3	13a	6	8	1	4
406	15	21c	6a	14b	ND	13a	ND	8	1	4b

Incorrect result

Annex 8. Reference strains

Reference strains for the MLVA part

	STTR9-Allele	STTR5-Allele	STTR6-Allele	STTR10-Allele	STTR3-Allele
STm-SSI001	6	9	13	10	211
STm-SSI002	7	15	12	12	311
STm-SSI003	8	11	NA	NA	211
STm-SSI004	9	14	NA	NA	211
STm-SSI005	3	12	11	21	311
STm-SSI006	3	16	13	24	311
STm-SSI007	3	19	10	NA	211
STm-SSI008	3	21	11	NA	211
STm-SSI009	2	23	22	13	212
STm-SSI010	2	24	NA	NA	111
STm-SSI011	2	26	7	8	212
STm-SSI012	2	11	13	9	212
STm-SSI013	3	15	14	11	311
STm-SSI014	3	14	15	23	311
STm-SSI015	2	12	24	8	212
STm-SSI016	2	10	25	8	312
STm-SSI017	3	14	29	NA	311
STm-SSI018	2	11	13	4	212
STm-SSI019	2	9	12	5	212
STm-SSI020	3	16	13	29	311
STm-SSI021	4	9	6	8	314
STm-SSI022	2	20	13	11	12
STm-SSI023	2	16	9	14	310
STm-SSI024	4	17	8	6	105
STm-SSI025	2	12	13	6	106
STm-SSI026	3	17	19	16	311
STm-SSI027	5	12	8	10	11
STm-SSI028	5	13	6	7	8
STm-SSI029	3	7	16	31	311
STm-SSI030	2	5	4	13	9
STm-SSI031	3	12	7	NA	511
STm-SSI032	3	17	21	18	311
STm-SSI033	2	13	9	11	112

Annex 9. Guide to BioNumerics database

Guide for setting up your EQA database

There are two possibilities for setting 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 this instruction. The database is packaged in the zip archive called "Listeria EQA db.zip" or 'Salmonella EQA db.zip'. If you have a BioNumerics version prior to 6.0 or wish to set up the database yourself, please use the instruction below.

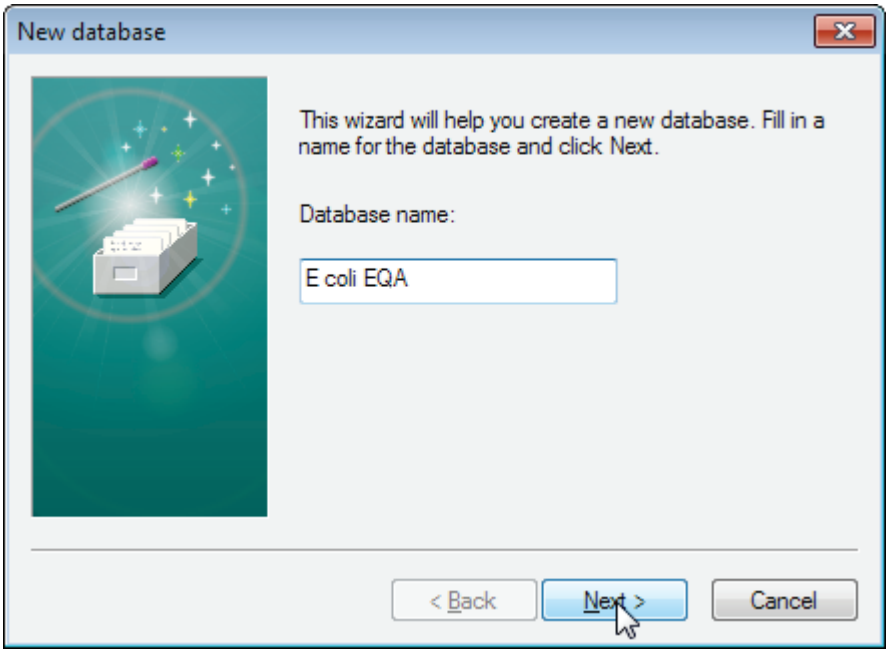
Set up a database from scratch

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

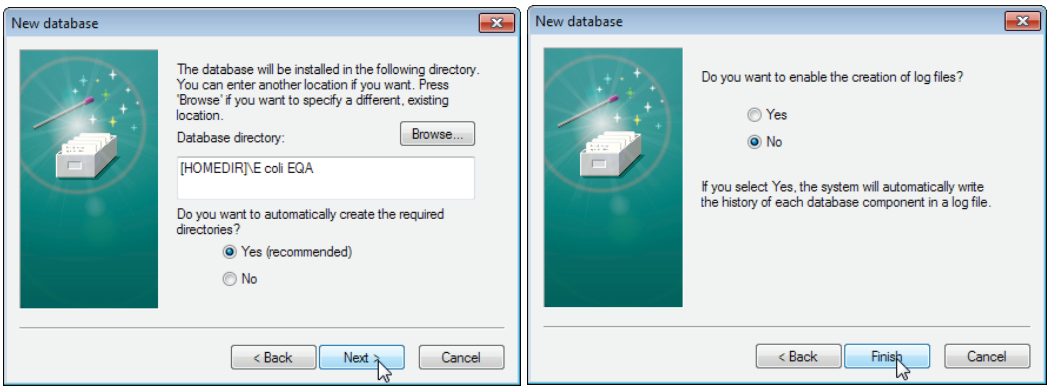
The database is set up by first setting up an empty database followed by an import of an XML file containing experiment settings and field definitions.

Set up the empty database

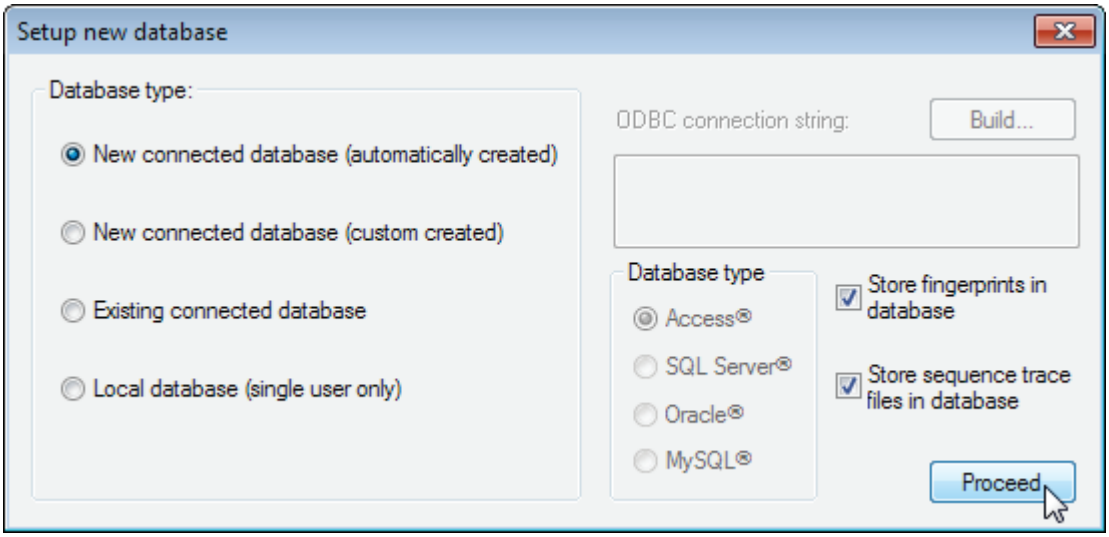
Choose to 'Create a new database'



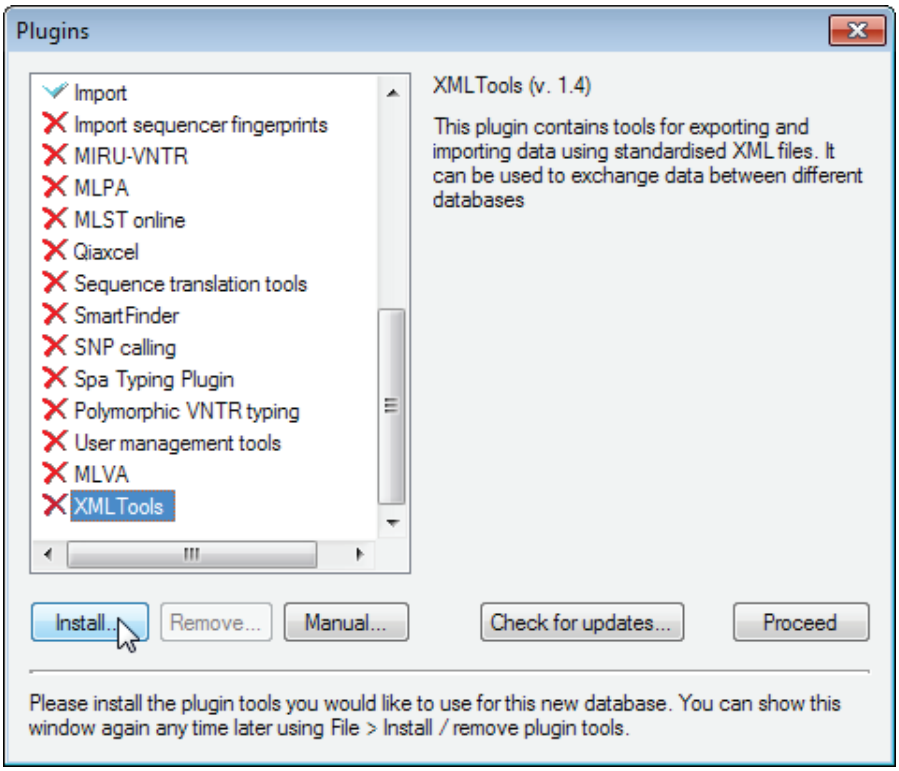
Enter a database name, 'Salmonella EQA' or 'Listeria EQA'



Use default values.



Choose a new connected database of Access type.

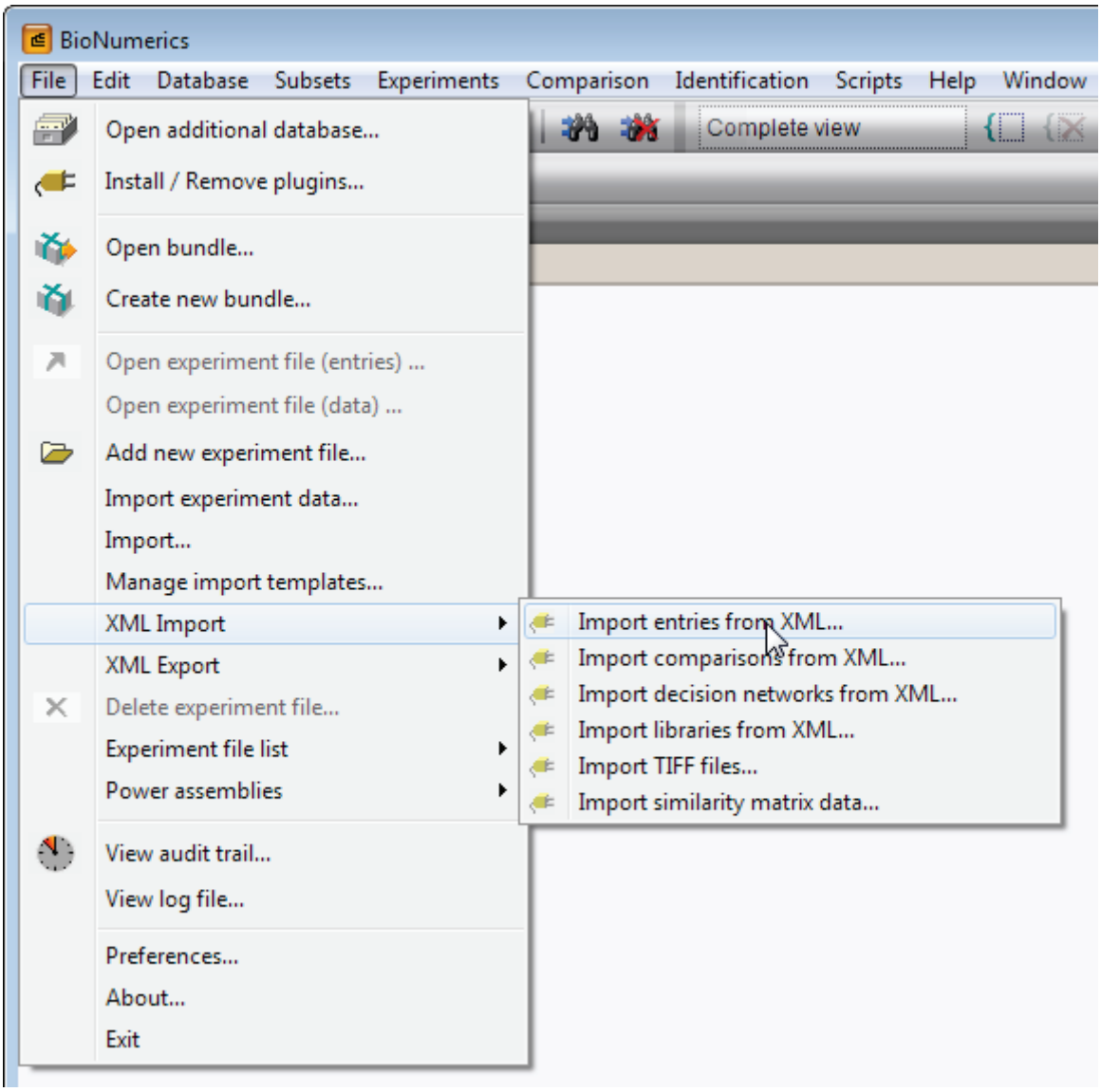


When choosing plugins, add the XML Tools plugin by selecting the plugin in the list and press 'Install'

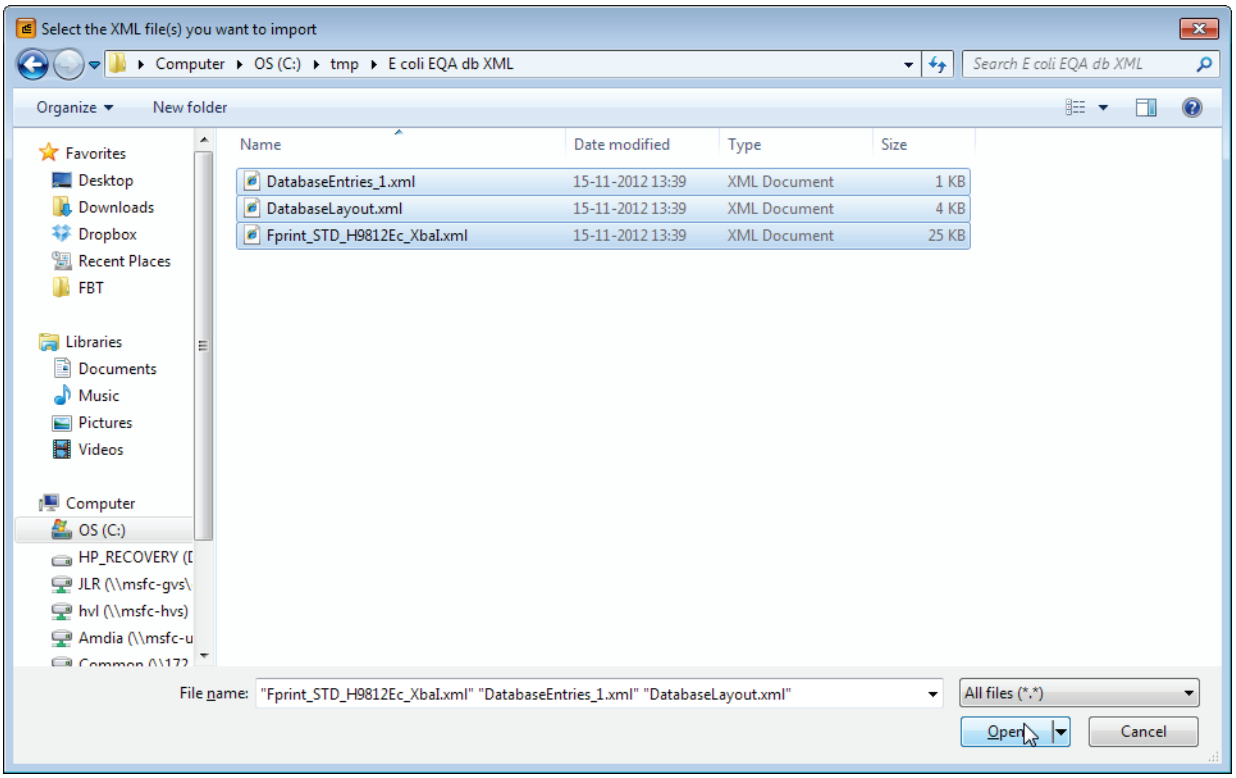
Proceed to the next window. The database is now set up and ready to import the database definitions.

Importing the XML structure

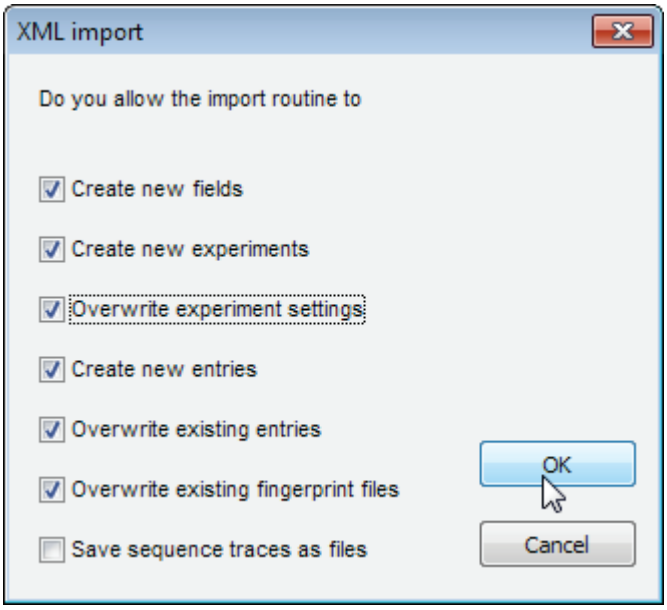
Unzip the contents of the supplied file 'Listeria EQA db XML.zip' or 'Salmonella EQA db XML.zip'



Select the 'Import entries from XML' menu item



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



Mark the box 'Overwrite experiment settings' And click OK

Restart the database.

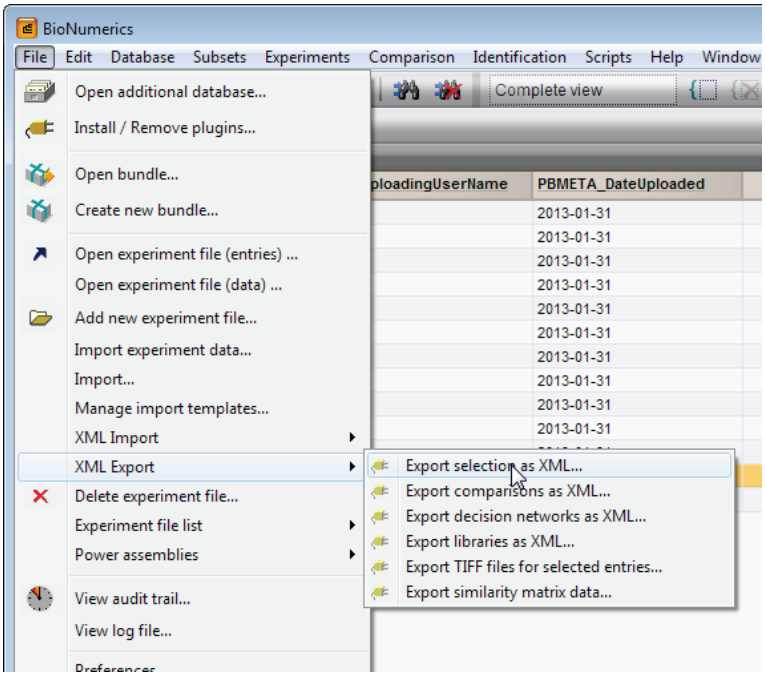
Annex 10. Guide to XML export

Exporting XML data from your database

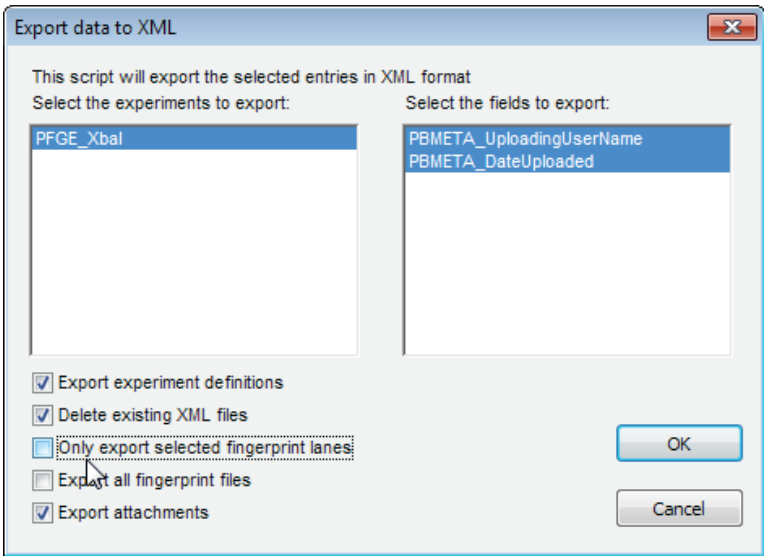
After analysing you data, select all isolates that you would like to export

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		

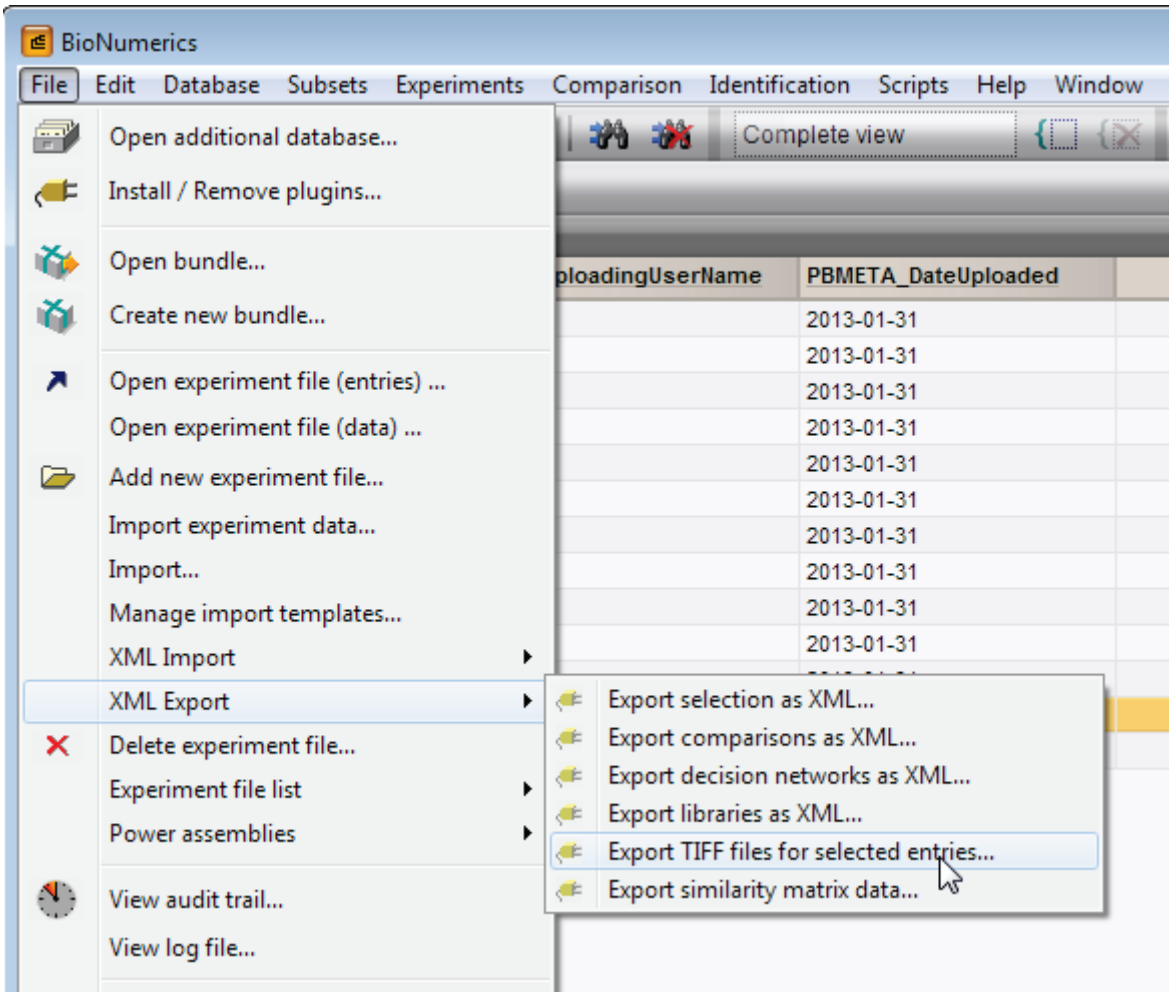
Export selection as XML



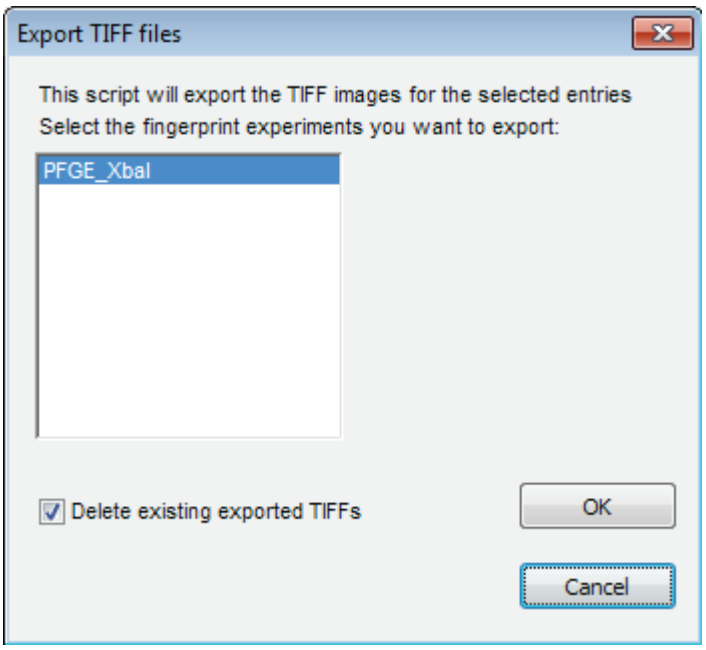
De-select the check box "Only export selected fingerprint lanes"



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'