TECHNICAL REPORT

Fifth external quality assessment scheme for typing of verocytotoxin-producing *Escherichia coli* (VTEC)

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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 Statens Serum Institut, Denmark.

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Contents

Figures

Tables

Abbreviations

A/EEC	Attaching and effacing *Escherichia coli*
aaiC	Chromosomal gene marker for Enteroaggregative *E. coli*
aggR	Gene encoding the master regulator in Enteroaggregative *E. coli*
BN	BioNumerics software suite
eae	CVD434. *E. coli* attaching and effacing gene probe ehxA
EAEC	Enteroaggregative *E. coli*
EIA	Enzyme immunoassay
eltA	G119. Heat labile enterotoxin (LT). Almost identical to cholera toxin
EQA	External Quality Assessment
ESBL	Extended Spectrum Beta Lactamase
estA	DAS101. Heat stable enterotoxin (porcine variant) STp (STIa)
EU/EEA	European Union/European Economic Area
FWD	Food- and Waterborne Diseases and Zoonoses
GFN	Global Foodborne Infections Network, Food Safety, WHO
HUS	Haemolytic uremic syndrome
ipaH	WR390. Invasion plasmid antigen.
NSF	Non-sorbitol fermenting
PCR	Polymerase chain reaction
PFGE	Pulsed Field Gel Electrophoresis
SF	Sorbitol fermenting
SSI	Statens Serum Institut
STEC	Shiga toxin-producing *E. coli.* STEC is synonymous with VTEC
TESSy	The European Surveillance System
TESSy MSS	EU Molecular Surveillance System, part of the TESSy database
VCA	Vero cell assay
VT1	verocytotoxin 1
VT2	verocytotoxin 2
VTEC	verocytotoxin-producing *E. coli.* VTEC is synonymous with STEC
vtx1	The gene encoding VT1
vtx2	The gene encoding VT2
WHOCC	WHO Collaborating Centre for Reference and Research on Escherichia and Klebsiella

Executive summary

Main findings

- Twenty-nine public health national reference laboratories from 29 EU/EEA countries signed up for the fifth international external quality assessment (EQA) scheme on typing of verocytotoxin-producing *Escherichia coli* (VTEC) organised by ECDC.
- Sixty-nine percent (20 out of 29) of the laboratories participated in the pulsed-field gel electrophoresis (PFGE) part of the EQA, and 60% of the participants were able to produce a PFGE gel of sufficiently high quality to allow for the profiles to be comparable with profiles obtained by other laboratories. The subsequent normalisation and interpretation of the profiles were performed using the specialised software BioNumerics (BN). Seventeen laboratories (87%, 17 out of 20) completed the gel analysis and 65% performed in a fair to good accordance with the guidelines.
- Twelve (41%) laboratories participated in full O:H serotyping for all ten strains, and 83% of the participating laboratories were able to correctly determine the full O:H serotype of all ten strains.
- Correct typing of virulence genes was 98% for *eae, ehxA* and *vtx1* and 94% for *vtx2.*
- Subtyping of vtx was performed correctly by 92% of laboratories on average.
- Correct phenotypic characterisation was high, from 86% for VT production to 100% for ESBL production.

This report presents the results of the fifth round of the external quality assessment scheme for typing of verocytotoxin-producing *E. coli* (VTEC) (further EQA-5) funded by ECDC. The EQA-5 was carried out from January 2014 to May 2014 and included the following methods: pulsed-field gel electrophoresis (PFGE), O:H serotyping, detection of virulence genes (*eae, vtx1, vtx2 and ehxA*), subtyping of the *vtx* genes, phenotypic detection of verocytotoxin/Shiga toxin production (VT/Stx), fermentation of sorbitol, production of beta-glucuronidase, enterohaemolysin, and extended beta-lactamase (ESBL).

A total of 29 laboratories participated in at least one part of the EQA-5. Twenty laboratories (69%) reported PFGE results, 12 laboratories (41%) participated in full O:H serotyping of all strains (26 laboratories submitted O group results for at least one strain and 19 laboratories submitted H-types for at least one strain). Genotypic detection of *eae, vtx1 and vtx2* was performed by 26–28 laboratories (an average of 89-97%), 19 (66%) for *ehxA,* and 22 (75%) participated in subtyping of *vtx* genes. The average participation in phenotypic detection was eight laboratories (28%) for VCA (Vero cell assay), 26 (90%) for fermentation of sorbitol, 15 (52%) for beta-glucuronidase, 14 (48%) for enterohaemolysin and 18 (62%) for ESBL.

Twenty laboratories participated in the PFGE part of the EQA-5, and 12 (60%) were able to produce a PFGE gel of sufficiently high quality to allow comparison with profiles obtained by other laboratories. The subsequent normalisation and interpretation of the profiles were performed using the specialised software suite BioNumerics (BN). Seventeen laboratories completed the gel analysis, and 65% performed in fair to good accordance with the guidelines.

Of 12–19 participants, an average of 69% (range 58–100%) could correctly determine the O:H serotype of the strains (some laboratories only typed a selection of the test strains). The more common serotypes received better typing results: O157:H7 was typed correctly (100%) by all 19 participants, while O145:H34 was associated with a significantly poorer result (58%).

The results for the genotypic detection of virulence genes were generally very good: *eae* (98%), *vtx1* (98%), *vtx2* (94%) and *ehxA* (98%). False positive results were reported five times for *vtx1*, one for *vtx2*. One false negative result was received for *vtx1*, but 15 for *vtx2* (10 of these were failure to detect the *vtx2f* gene). The virulence genes *aggR* and *aaiC* were submitted by 20 and 16 participants, respectively. The *aggR* and *aaiC* positive test strain was correctly determined by all participants; however two laboratories submitted false positive *aggR* results for one or two other strains.

The percentage of correct results for phenotypic detection was 86% for VT, 97% for fermentation of sorbitol, 90% for beta-glucuronidase, and 94% for enterohaemolysin. All participants correctly determined ESBL.

This EQA-5 scheme is the second EQA specifically organised for laboratories participating in the European Food- and Waterborne Diseases and zoonoses network (FWD-Net) that includes molecular typing method. The number of participating laboratories in the EQA-5 is encouraging. The molecular surveillance system relies on the capacity of the FWD-Net laboratories to produce comparable typing results and follow the ECDC guidance for VTEC detection. At the moment, the molecular typing method used for EU-wide surveillance of VTEC is PFGE. The surveillance of VTEC infections also relies on conventional typing/phenotypic strain characteristics in combination with molecular typing. However, the PFGE results of the EQA-5 show that 40% of the laboratories need to improve their performance in order to produce useful typing profiles for an inter-laboratory exchange. However, for the majority of laboratories with identified technical issues, achieving an acceptable quality level is within reach if they optimise procedures, receive trouble-shooting assistance, and additional 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 assessment schemes [1]. External quality assessment (EQA) is a part of quality management systems and evaluates performance of laboratories by an external evaluator 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 objectives of external quality assessment 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
- provision of 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. The 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 3 (VTEC) covers the organisation of an EQA exercise for PFGE, O:H serotyping, virulence gene detection, subtyping of *vtx* genes and common phenotypic traits of VTEC, including ESBL production. The present report presents the results of the second VTEC EQA-exercise of this contract (*E.coli* EQA-5).

1.2 Surveillance of VTEC infections

Verocytotoxin-producing *Escherichia coli* (VTEC) are a group of *Escherichia coli* (E. coli) that are characterised by the ability to produce toxins that are designated verocytotoxins (VT). Human pathogenic VTEC often harbour additional virulence factors that are important in the development of the disease in humans. A large number of serotypes of *E. coli* have been recognised as VT producers. The majority of reported human VTEC infections are sporadic cases. The symptoms associated with VTEC infection in humans vary from mild to bloody diarrhoea, which is often accompanied by abdominal cramps, usually without fever. VTEC infections can result in Haemolytic uremic syndrome (HUS), which is characterised by acute renal failure, anaemia and lowered platelet counts.

In 2012, the overall EU notification rate of VTEC was 1.15 cases per 100 000 population. The total number of confirmed VTEC cases in the EU was 5 671, which represents a decrease of 40% compared with 2011 (N = 9 485) with the large O104:H4 outbreak. However the level of 2012 was still 35% higher than the 2010 level (N=3 656) [3].

Since 2007, ECDC's FWD programme has been responsible for the EU-wide surveillance of VTEC, 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 systems in the EU in order to increase the scientific knowledge regarding aetiology, risk factors and burden of food- and waterborne diseases and zoonoses. 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 were included: *Salmonella*, STEC/VTEC and *L. monocytogenes*, the overall goals of integrating molecular typing in EU level surveillance are to:

- foster rapid detection of dispersed international clusters/outbreaks
- facilitate the detection and investigation of transmission chains and relatedness of strains across Member States and globally
- detect emergence of new evolving pathogenic strains
- 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 States users access to EU-wide molecular typing data for the included pathogens. The pilot project also gives its users the opportunity to perform cluster searches and analyses of EU-level data in order to determine whether isolates characterised by molecular typing at the national level are part of a multinational cluster that may require a cross-border response.

Since 2012 the ECDC FWD programme has supported EQA schemes with a focus on expert support for molecular typing, namely PFGE and multi-locus-variable-number tandem repeat analysis (MLVA) of *Salmonella* PFGE of STEC/VTEC, and *L. monocytogenes*. ECDC has also supported EQA activities for virulence gene detection and serotyping of the selected pathogens. The EQA-5 scheme was targeted at those public health national reference laboratories in EU/EEA countries and EU candidate countries that already conduct molecular surveillance at the national level.

1.3 VTEC characterisation methods

The state-of-the-art characterisation of VTEC includes O:H serotyping in combination with a few selected virulence genes, i.e. the two genes for production of verocytotoxin VT1 (*vtx1*) and VT2 (*vtx2*), and the intimin (*eae*) gene associated with the attaching and effacing lesion of enterocytes – also seen in attaching and effacing of non-VTEC *E. coli* (A/EEC) including enteropathogenic *E. coli* (EPEC). The combination of the toxin genes is clinically relevant in some subtypes of VT2. VT2a in *eae*-positive VTEC and the activatable VT2d subtype in *eae*-negative VTEC seem to be highly associated with the serious sequela HUS [4-6]. VT2c-positive VTEC has also been associated with HUS [5,6]. Other specific subtypes or variants of VT1 and VT2 are primarily associated with milder course of disease without HUS [4-6], and VT2e-positive VTEC strains are probably not pathogenic to humans [7]. Our understanding of the epidemiology of the VT subtypes is therefore important for reducing the risk of VTEC infection and for the surveillance of VTEC.

Some of the existing VT-subtyping methods using a combination of specific polymerase chain reaction (PCR) and restriction fragment length polymorphism (RFLP) are inadequate and may result in misleading conclusions. For example, typing of *vtx2* has been based on the absence of the PstI site as an indicator of the presence of the mucus-activatable *stx2*d subtype [8-11]. However, the PstI site is also absent in six variants of *vtx2a*, in two variants of *vtx2c*, in *stx2f* and in all four variants of subtype *stx2g* [12]. Furthermore, the most commonly detected VTEC serotype – O157:H7 – may be divided into two groups: one with the unusual property of failing to ferment sorbitol within the first 20 hours of incubation (the non-sorbitol fermenters, NSF) and a highly virulent variant of O157 fermenting sorbitol (SF). NSF O157 is most often characterised by failure to produce beta-glucuronidase. Approximately 75% of all VTEC produce enterohaemolysin, a toxin that can cause lysis of erythrocytes. Enterohaemolysin may be detected either phenotypically on sheep blood agar plates or by detection of the *ehxA* gene encoding enterohaemolysin.

VTEC EQA-5 included O:H serotyping, detection and genotyping of virulence genes (*eae, vtx1, vtx2* and *ehxA*), subtyping of ten *vtx* subtype genes by conventional gel-based PCR using the recently published protocol [12], phenotypic detection of VT production through VCA or enzyme immunoassay (EIA), fermentation of sorbitol, production of β-glucuronidase, enterohaemolysin and ESBL.

1.4 Objective of the EQA-5 scheme

1.4.1 Pulsed-field gel electrophoresis (PFGE) typing

The objective of EQA-5 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 results.

1.4.2 Serotyping

The EQA scheme assessed the determinations of somatic 'O' and flagella 'H' antigens for STEC/VTEC strains.

1.4.3 Virulence determination

The EQA scheme covered both genotypic and phenotypic testing of STEC/VTEC strains, taking into account the virulence data currently collected at the EU level (with the possibility to report optional genes). The EQA included the following:

- detection of virulence genes *eae*, *vtx1*, *vtx2* and *ehxA*. Virulence gene testing included detection and typing of intimin (*eae*) gene, verocytotoxin 1 gene (*vtx1)* and verocytotoxin 2 gene (*vtx2)*
- subtyping of *vtx1* and *vtx2* genes
- detection of other virulence genes (*aggR* and *aaiC* were expected by public health national reference laboratories).

1.4.4 Phenotypic tests

Phenotypic assay for the detection of production of verocytotoxin, fermentation of sorbitol, enterohaemolysin, beta-glucuronidase, and ESBL.

2. Study design

2.1 Organisation

The fifth round of the external quality assessment scheme for typing of verocytotoxin-producing *E. coli* (VTEC) (VTEC EQA-5) was funded by ECDC and arranged by Statens Serum Institut (SSI) to be conducted from January 2014 through to May 2014. It included PFGE, O:H serotyping, virulence determination by genotypic methods (detection and typing of virulence genes *eae*, *vtx1*, *vtx2* and *ehxA*, subtyping of *vtx1* and *vtx2*) and by phenotypic detection of VT production, fermentation of sorbitol, production of beta-glucuronidase, enterohaemolysin and ESBL. A recently published protocol by conventional gel-based PCR (14) was tested for subtyping of the ten *vtx* subtype genes.

The EQA-5 (without the PFGE part) was conducted according to ISO/IEC 17043:2010, entitled '*Conformity assessment – General requirements for proficiency testing'* (first edition, February 1st 2010) [13].

Invitations were e-mailed to the ECDC contact points in the FWD-Net (30 countries) on the 4 November 2013. In addition, the ECDC coordinator sent invitations to the EU candidate countries Montenegro, Serbia, the former Yugoslav Republic of Macedonia and Turkey.

Twenty-nine public health national reference laboratories in EU/EEA and EU candidate countries accepted the invitation and these are listed in Annex 1.

The EQA-5 test strains were sent to the participating laboratories in mid-January 2013.

The participants were asked to submit their PFGE results by e-mail and report the rest of the results through an online form (Annex 25) by the 17 March 2014. In addition, 22 laboratories from the international WHO Global Foodborne Infections Network (GFN) were invited to participate.

2.2 Selection of strains

The strains for EQA-5 were selected based on representativeness: all strains should be representative for strains reported from Europe. In addition, strains should remain stable during the preliminary testing period at the laboratory of the EQA provider. The selected types should be easy to type, and they should represent the three different subtypes of *vtx1* and cover as many of the seven different subtypes of *vtx2*. The PFGE profile should be stable and represent the diversity of the occurring VTEC profiles in Europe.

Table 1. Test strains

Method	No. of test strains	Characterisation
PFGE	10	AA1, BB2, CC3, DD4, EE5, FF6, GG7, HH8, II9 and JJ10
O:H serotyping	10*	KK11, LL12, MM13, NN14, OO15, PP16, QQ17, RR18, SS19 and TT20 O78:H2, O111:H-/H8, O26:H1, O103:H2, O55:H7, O121:H19, O157:H7, O91:H14, O16:H15 and O145:H34
Virulence gene determination	10*	*eae*, *vtx1a*, *vtx2a, vtx2b, vtx2c, vtx2d* and *vtx2f, ehxA*, *aggR*, *aaiC*
Phenotypic testing	10*	VCA, sorbitol, β-glucuronidase, enterohaemolysin, ESBL

**Same 10 strains*

Detailed information about the strains is shown in Annex 8.

In addition to the 20 test strains, laboratories participating in EQA-5 for PFGE could request the *Salmonella* Braenderup H9812 reference strain and reference strains for the *vtx* subtyping (Annex 22).

2.3 Carriage of strains

By the end of January, all strains were blinded, packed and shipped (shipping began on the 15 January 2014). Almost all of the participants received their dispatched strains within 1–3 days. One parcel was delayed by customs and was not delivered until after 12 days. The parcels were shipped from SSI Copenhagen, labelled as UN 3373 Biological Substance, Category B.

The participants were e-mailed their specific blinded numbers as an extra control. No participants reported shipment damages or errors in their specific numbers.

On 6 February, instructions on how to submit results were e-mailed to participants. Instructions included a link to a Google Docs submission form (see Annex 25), zipped files for the BN database experiment settings (PFGE part), and guidelines on how to export XML files from BN (Annex 23 and 24).

2.4 Testing

In the PFGE part, ten *E. coli* strains representing different serotypes were tested, and participants could opt to only participate in the laboratory part (by submitting the TIFF file of the PFGE gel) or also take part in the additional analysis of the gel (by submitting normalised profiles with assigned bands). For the laboratory procedures, the participants were instructed to use the laboratory protocol O157 Standard PulseNet PFGE *E. coli* – one-day (24–26 hour) standardised laboratory protocol for molecular subtyping of *Escherichia coli* O157:H7, *Salmonella* serotypes, *Shigella sonnei*, and *Shigella flexneri* by pulsed-field gel electrophoresis (PFGE) [14].

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

In the other parts of EQA-5, ten additional *E. coli* strains were included. All results were submitted online to *Google Docs*. The participants' ability to obtain the correct serotype, both O group and H type, by either serological methods (suggested protocol [15] or molecular typing (no international standard but the applied methods should be submitted together with the results) was tested.

In the genotyping part, the participants' ability to detect the virulence genes *eae*, *vtx1*, *vtx2* and *ehxA* genes and the ability to subtype *vtx1* (*vtx1a)* and *vtx2* (*vtx2a, vtx2b, vtx2d and vtx2f*) were assessed (suggested protocol [16]).

The phenotypic part of the EQA involved the detection of VT production, fermentation of sorbitol, enterohaemolysin, beta-glucuronidase and production of ESBL.

For the detection of virulence, characteristics related to enteroaggregative VT2-producing *E. coli* O104:H4 (EAEC-VTEC), e.g. the chromosomally encoded protein gene (*aaiC)* and enteroaggregative adhesion transcription regulator gene (*aggR*), one strain with these characteristics was included. Additionally, one strain harbouring genes *eltA* (for ETEC) and several harboured *eae* (for EPEC and A/EEC).

Participants were requested to test for additional virulence genes at their own convenience and capacities. This voluntary and additional testing was not a core part of the EQA-5 scheme but meant as a source for sharing information on the capacities found within the network of laboratories. It provided additional information on the test strains, which may be valuable if laboratories wish to set up new tests.

2.5 Data analysis

When the results from the laboratories were received, the PFGE results were added to a dedicated *E.coli* EQA-5 BN database at SSI. For PFGE, the gel quality was evaluated according to a modified version of the ECDC FWD 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-4 'TIFF Quality Grading Guidelines 2013' is 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. The modification of the scheme was necessary in order to get a category that clearly shows when a gel is not comparable to other gels. The BN 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-4 "BioNumerics Gel Analysis Quality Guidelines 2013" is in Annex 6. In general, the large difference between the schemes is the reduction of categories from 4 to 3 (only excellent, fair and poor). After the results from all laboratories were submitted in the online forms, SSI exported a copy of all results to an Excel spreadsheet. Results were then analysed; scores of the serotyping, genotyping, and phenotyping tests were evaluated based on correct results and a percentage score was calculated.

3. Results

3.1 Participation

Laboratories could choose to participate in the full scheme or a selection of the methods. The methods were PFGE, O:H serotyping, virulence determination including genotyping (virulence gene detection and subtyping) and phenotyping (VT, sorbitol, β-glucuronidase, enterohaemolysin, ESBL). Twenty-nine laboratories submitted results – however not all submitted the results that they originally had planned. Twenty laboratories (69%) participated in the PFGE part, 17 (85%) also in the BioNumerics analysis. Twelve (41%) participated in the full O:H serotyping of all 10 strains. An additional seven laboratories submitted O:H data for only a limited number of the EQA strains. The reasons for omitting some of the strains were not always specified, but in some cases, it was based on the obtained O results. Nineteen laboratories (66%) submitted O:H serotype data for strain QQ17 (O157:H7). In addition to the FWD-Net participants, 22 laboratories from the international WHO Global Foodborne Infections Network (GFN) participated (results not included in this report).

The participation rate in O group/H type depends on the laboratories' abilities, including the range of available antisera. Laboratories that only used a limited panel of antisera were encouraged to report the result as 'non-typeable' (NT) for strains that they could not type. For the genotyping part (virulence gene detection and subtyping), some participants only performed the analysis on a selection of the test strains, which was typically based on the serotyping results. This means that the participation rate for a method varies for each strain and these are therefore presented as a range.

The high participation, 15–26 laboratories, was in the O typing, 12–19 laboratories participated in the H typing (Table 3).

In the genotyping part (virulence gene detection and subtyping), 28 laboratories (average 97%) submitted results for *eae* and *vtx* genes, while 19 (66%) laboratories submitted results for *ehxA* genes. Twenty-two laboratories (76%) submitted results for *vtx* subtypes, and 16/20 laboratories (69%/55%) reported results for EAEC (*aggR* and *aaiC)*. In the phenotyping part, 26 laboratories (90%) participated in one or more of the phenotyping methods. Participation is presented in Table 2, details are listed in Table 3.

Table 2. Number of FWD-Net laboratories submitting results for each method

Methods	PFGE		O:H serotyping[1]	Virulence determination[2]	Phenotypic test[3]
	TIFF	XML			
Number of participants	20	17	19	28	26
% of participants	69	85	66	97	90

1 Participation in O grouping was 15–26 (average 71%) laboratories and 12–19 (average 54%) laboratories in H typing

2 Participating in one or more of the virulence gene determination parts (eae, vtx1, vtx2 or ehxA)

3Participating in one or more of the phenotypic test parts (VCA, sorbitol, enterohaemolysin, β-glucuronidase or ESBL)

Note: Twenty-nine laboratories participated in at least one method

Table 3. Detailed participation table

Participants / method	O:H serotype group	O group[1]	H type[2]	Vero cell assay	ESBL	Haemolysin production	β-glucuronidase production	Sorbitol fermentation	*eae* gene	*ehxA* gene	*vtx1* gene	*vtx2* gen	*vtx* subtypes	*aggR*	*aaiC*
n_{max}	19	26	19	8	18	14	15	26	26	19	28	28	22	20	16
Average	66%	90%	66%	28%	62%	48%	52%	90%	90%	66%	97%	97%	76%	69%	55%

nmax: highest number of participants

1 Participation in O grouping was 15–26 laboratories

2 Participation in H typing was 12–19 laboratories

Participation in the detection of virulence gene *eae* was 90%, *ehxA* was 66%, detection of *vtx1* and *vtx2* was 97%, and subtyping was 76% on average.

Participation in the phenotypic detection was 28–90% (8–26 labs). The lowest participation was for the VT assay: only eight participants (28%) delivered results for 10 strains. Participation in the sorbitol fermentation was 26 laboratories (90% on average). The test for enterohaemolysin production was performed by 14 laboratories (48% on average). The test for production of β-glucuronidase was performed by fifteen laboratories (52%). Eighteen laboratories (62%) submitted results for the production of ESBLs.

3.2 Pulsed-field gel electrophoresis (PFGE)

Twenty laboratories participated in the PFGE, sending TIFF files (raw gel images). Seventeen of these laboratories also analysed their gels in BN and submitted data as XML files.

3.2.1 Gel quality

All laboratories were able to produce profiles that were recognisable as the profile for the relevant EQA strain. The gels were graded according to the modified TIFF Quality Grading Guidelines, where seven parameters are used in 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. In general, acceptable quality (fair – score of 2) should be achieved for each parameter. A score of 1 in just one category resulted in a non-acceptable gel, making inter–laboratory comparison impossible.

A wide variation in quality was seen between laboratories (Table 4). For three parameters participants obtained a high average score, 3.6 and above, i.e. between good and excellent (Table 4). The three parameters were Cell Suspension, Lanes and Restriction. Participants obtained an average score of 3.3 in DNA degradation and 3.0 in Gel Background and Image Acquisition and Running conditions. For the last parameter Bands participants had an average score below 3 (2.4), i.e. between fair and good.

Table 4. Results of PFGE gel quality for 20 participating laboratories

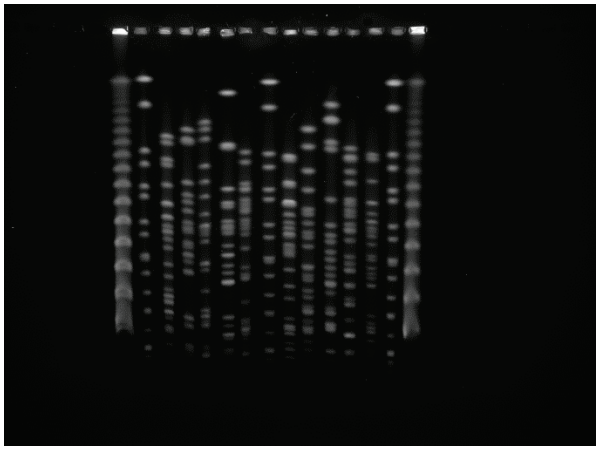
Parameters	1 – poor	2 – fair	3 – good	4 – excellent	Average
Image acquisition and running conditions	5%	40%	10%	45%	3.0
Cell suspension	0%	5%	10%	85%	3.8
Bands	30%	30%	10%	30%	2.4
Lanes	0%	10%	25%	65%	3.6
Restriction	5%	0%	5%	90%	3.8
Gel background	0%	30%	45%	25%	3.0
DNA degradation	5%	25%	10%	60%	3.3

The average scores between 1 and 4 and percentages of laboratories in the seven TIFF Quality Grading Guideline parameters. Also shown is the average score, based on all laboratories.

The laboratories obtained very diverse scores for the parameter Image Acquisition and Running Conditions (Table 4). Ninety-five percent of participants were graded fair [2], good [3] or excellent [4] in this parameter resulting in 5% of participants having a critical score [1]. In the parameter Bands, 70% of laboratories were graded a score of 2 or above (Table 4). Thirty percent of participants obtained the score 1 in the parameter Bands, making further analysis of the gel impossible. Eight (40%) laboratories produced gels that were graded 1 (poor) in at least one of the seven parameters. Profiles from gels with poor quality in just one parameter are impossible to compare with profiles produced on other gels. All the participants Gel Quality scores are listed in Annex 7.

The gel in Figure 1 was graded 1 (poor) in the parameter Bands. The low score was caused by fuzzy bands. The gel was also graded 2 in four other parameters.

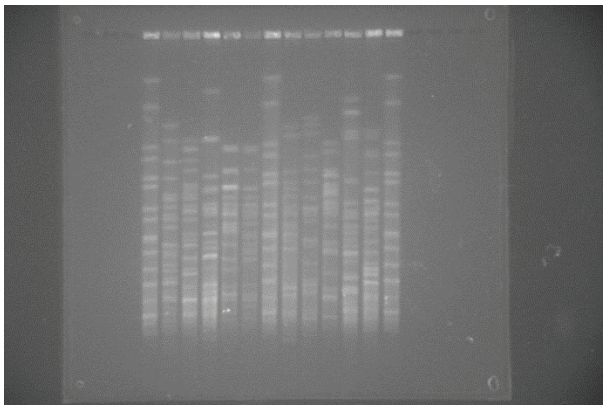
Figure 1. A gel graded 1 in parameter Bands, and a score of 2 in the parameters Image acquisition and running conditions, Lanes, Gel background and DNA degradation.



Note: The most critical score was in the parameter Bands.

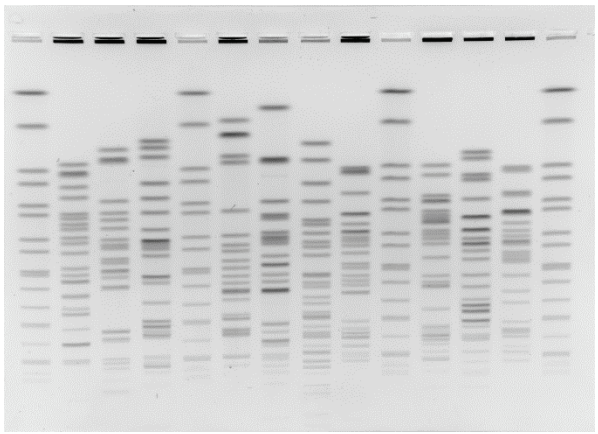
Figure 2 depicts a gel with low scores in the parameters Bands and DNA degradation. Scores in Image acquisition and running conditions were also low because of incorrect running conditions and the reference strains are therefore not 1–1.5 cm from the bottom of the gel.

Figure 2. A gel graded 1 in Bands and DNA degradation.



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

Figure 3. A gel with high scores in all 7 parameters.



3.2.2 Gel analysis with BioNumerics

Seventeen laboratories analysed their gel in BioNumerics and were able to produce XML files according to the protocol attached to the invitation letter (Annex 23 and 24). The participants' ability to perform gel analysis was graded according to the modified BioNumerics Gel Analysis Quality Guidelines developed at SSI, including five parameters for the grading (Annex 5).

Table 5. Results of the BN analysis for 17 laboratories

Parameters	1 – poor	2 – fair	3 – excellent	Average
Position of the gel	6%	18%	76%	2.7
Strips	0%	29%	71%	2.7
Curves	0%	41%	59%	2.6
Normalisation	0%	6%	94%	2.9
Band assignment	29%	35%	35%	2.1

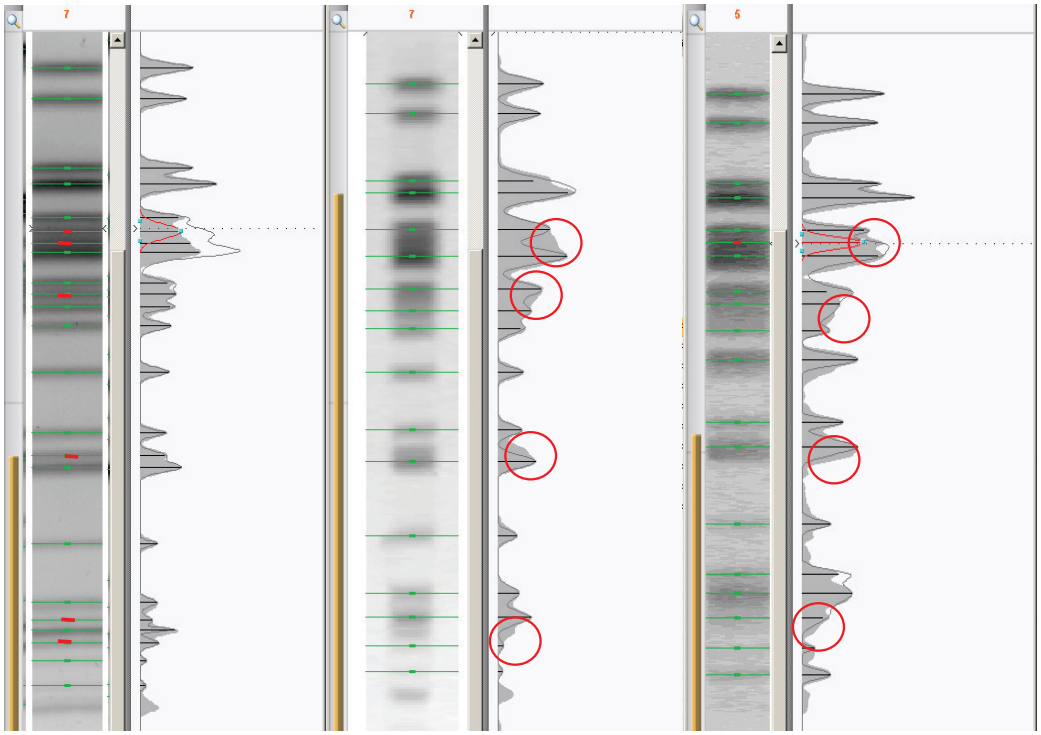
The average scores between 1 and 3 and percentages of laboratories in the five BioNumerics gel analysis Quality Grading Guideline parameters. Also shown is the average score, based on all laboratories.

For three parameters, Position of the Gel, Strips and Normalisation, participants obtained a very high average score, of 2.7 or above (Table 5). The participants were graded a bit lower with an average of 2.6 in the parameter Curves. The average score for participants in Band assignment was 2.1 – corresponding to 29% of the laboratories (five) who were unable to make Band assignment that could be used for inter-laboratory comparison. Additionally, one laboratory included the wells in the frame position.

An optimal Band assignment in BN is crucial, and this is very dependent on the overall quality of the gel and the score of the parameter Band from the TIFF quality grading guidelines (Annex 5). Very fuzzy and/ or thick bands make correct Band assignment an impossible task. In Figure 4, the comparison of three gels from two participants and the reference strain illustrates differences in band quality.

In Figure 4 the left lane is the *E.coli* strain 2 (BB2) run by the EQA provider, the second lane is from a gel with the score 2 in the parameter Bands and lane 3 is from a gel with the score 1 in the parameter Bands. However, both participants scored 1 in the Band assignment in the BN analysis.

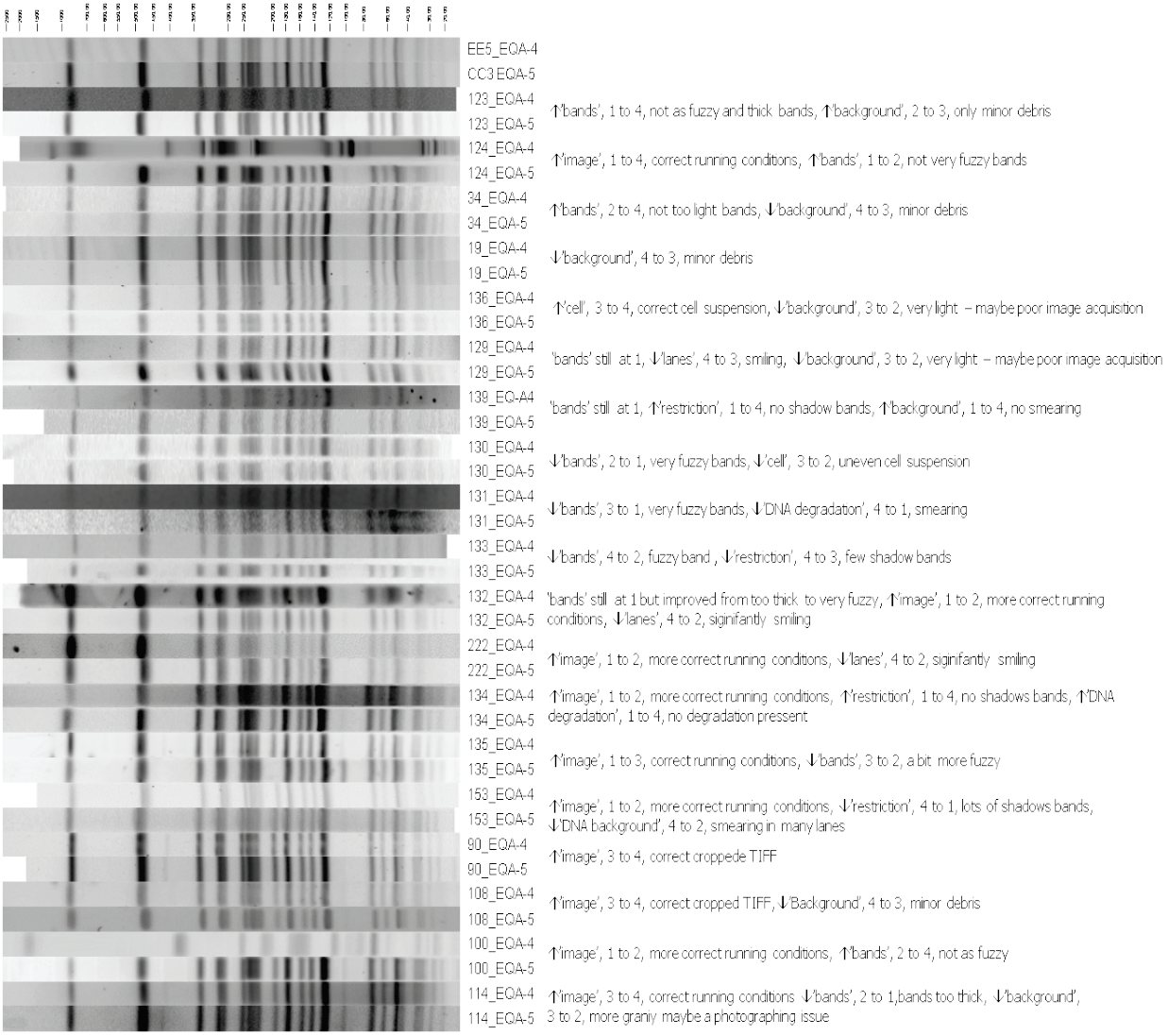
Figure 4. Comparison of Band assignment from two participants in BN analysis



The comparisons of the profiles could have been improved, despite the poor quality of the bands, by using the densitometric curves during the band assignment. Marked with the red circles are the areas where additional bands should be added based on the densitometric curve – although the quality of the gel is poor.

To investigate the progress of the laboratories performance in the PFGE EQA, two strains from EQA-4 were included in EQA-5. Strain EE5 and GG7 from EQA-4 are numbered AA1 and CC3 in the EQA-5. Figure 5 show an example of the evaluation of the laboratories that participated in both EQAs (19 participants). The figure shows a comparison of strain EE5 (EQA-4) and strain CC3 (EQA-5) for each of the participants. In general, many of the participants in the VTEC EQA have improved their performance – and only limited comments are included in the Figure 5.

Figure 5. Improvement of gel quality from EQA-4 to EQA-5



Comparison of strain EE5 (EQA-4) and strain CC3 (EQA-5) for each of the 19 participants, are represented by arbitrary numbers. ↑(improved) from score x to y.

3.3 Serotyping

Ten (38%) out of the 26 participating laboratories could correctly perform O grouping for all 10 test strains. Overall an average of 80% of the strains were correctly, O grouped (Table 6). Results were lowest (50%) for serotype O166 (SS19) and highest for serotype O157 (QQ17), which was correctly typed by all laboratories. The highest correct scores were obtained for serotypes O26, O103, O111, and O157, which is included in the minimum requirements of ECDC [17].

H typing was correctly performed 53% (10/19) for all 10 strains. Nineteen participants submitted H typing results, which represents only 73% of the number of participants performing O grouping. Results were lowest (58%) for the TT20 (H34) and highest (100%) for QQ17 (H74). The majority of incorrect H-types were due to reporting a strain as not typeable. Correct scores above 68% were obtained for the H types H2, H-/H8, H7, H11 and H19.

Table 6. Average scores for the O:H serotyping

Strain/method		O:H Serotype	O group	Type of incorrect antigens	H type	Type of incorrect antigens
n_{max}		19	26		19	
KK11	O78:H2	68% (13)	69% (18)	NT/ND (7), O119 (1)	68% (13)	NT/ND (6)
LL12	O111:H-/H8	68% (13)	96% (25)	NT/ND (1)	68% (13)	NT/ND (6)
MM13	O26:H11	68% (13)	96% (25)	NT/ND (1)	68% (13)	NT/ND (6)
NN14	O103:H2	68% (13)	88% (23)	NT/ND (2), O128 (1)	68% (13)	NT/ND (6)
OO15	O55:H7	68% (13)	77% (20)	NT/ND (6)	74% (14)	NT/ND (4), H5 (1)
PP16	O121:H19	68% (13)	73% (19)	NT/ND (6), O124 (1)	68% (13)	NT/ND (6)
QQ17	O157:H7	100% (19)	100% (26)		100% (19)	
RR18	O91:H14	63% (12)	65% (17)	NT/ND (7), O32 (1), O114 (1)	63% (12)	NT/ND (7)
SS19	O166:H15	63% (11)	50% (13)	NT/ND (11), O55 (1), O110 (1)	63% (12)	NT/ND (7)
TT20	O145:H34	58% (11)	85% (22)	NT/ND (3), O103 (1)	58% (11)	NT/ND (6), H28(1), H7 (1)
Average		**69%**	**80%**		**70%**	

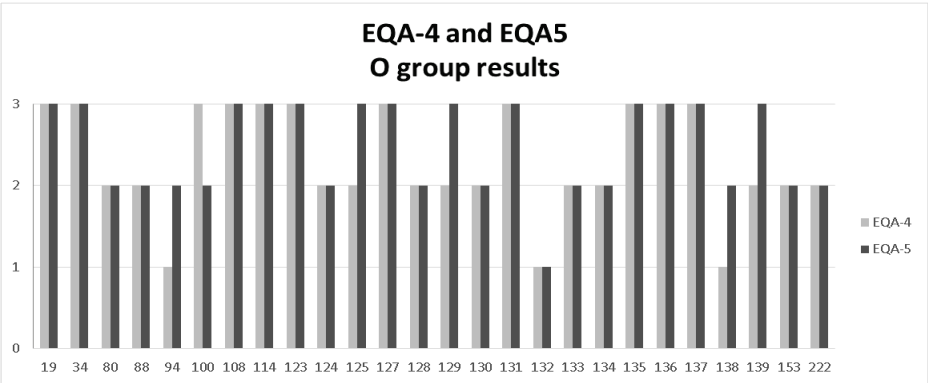
n_{max} = number of participants. Percentages are calculated based on the results submitted by all participants (see Annexes 9 and 10).

An average of 69% (58–100%) of laboratories could correctly identify O:H serotype in the 10 test strains. Correct O:H serotyping ranged from 100% for serotypes O157:H7 to 58% for serotype O145:H34 (Table 6). Nine participants (47%) could identify the correct O:H serotype for all 10 test strains.

This leads to the conclusion that it is more difficult for laboratories to serotype a strain correctly if the serotype are less common.

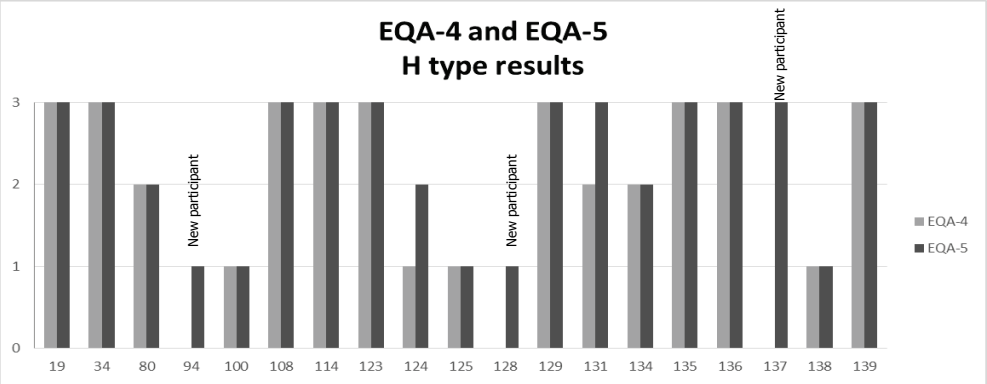
To show the exact progress of the laboratories' performance, three strains from EQA-4 were included in EQA-5. Strain GG7 (O111:H-), II9 (O157:H7) and MM13(O166:H15) from EQA-4 are numbered LL12, QQ17 and SS19 respectively in the EQA-5. Figure 6 and Figure 7 show the performance based only on the three isolates.

Figure 6. Comparing EQA-4 and EQA-5 O group results



The participating laboratories are represented by arbitrary numbers. Bars represent the number of correctly assigned O groups of the three strains (GG7, II9 and MM13 from EQA-4 and LL12, QQ17, and SS19 from EQA-5).

Figure 7. Comparing EQA-4 and EQA-5 H type results



The participating laboratories are represented by arbitrary numbers. Bars represent the number of correctly assigned H types of the three strains GG7, II9 and MM13 from EQA-4 and LL12, QQ17, and SS19 from EQA-5.

3.4 Virulence determination

3.4.1 Detection of virulence genes *eae*, *vtx1*, *vtx2* and *ehxA*

Genotypic detection of virulence genes *eae*, *vtx1, vtx2* and *ehxA* was performed by 19–28 laboratories for all the 10 test strains, with high average scores (94–98% correct) (Table 7). With regard to the detection of *eae*, a perfect score was obtained for six strains. Four strains (of 10) were incorrectly identified by one laboratory, with false negative results. Detection of *vtx*1 genes had a high average correct score of 98%; however, detection of *vtx*2 genes had an average correct score of 94%. Ten laboratories missed the presence of *vtx2* in the strain TT20 that has the *vtx2f* gene. Five false positive *vtx1* genes were submitted, one in strain KK11 (O78:H2) and two in RR18 (O91:H14) and in TT20 (O145:H34). Three of them were submitted by one laboratory, which also detected three false negative *vtx2* gene. Additionally, one participant reported false negative *vtx1* results in the strain OO15 (O55:H7). One of the participants detected false negative vtx2 gene in strains QQ17 (O157:H7) and TT20 (O145:H34) as well as false positive *vtx2* gene in strain NN14 (O103:H2). The remaining false negative results were submitted by different participants. In total, *vtx1* and *vtx2* were misidentified 22 times: *vtx*1 (one false negative, five false positives), *vtx2* (15 false negatives, one false positive).

Table 7. Average scores for virulence determination

Strain/method	*eae* gene	*vtx*1 gene	*vtx*2 gene	*ehx*A gene
N	26	28	28	19
KK11	100%	96%	100%	100%
LL12	100%	100%	100%	100%
MM13	96%	100%	100%	100%
NN14	100%	100%	96%	100%
OO15	96%	96%	100%	100%
PP16	96%	100%	93%	95%
QQ17	100%	100%	100%	95%
RR18	100%	93%	93%	95%
SS19	100%	100%	96%	100%
TT20	96%	93%	64%	100%
Average	**98%**	**98%**	**94%**	**98%**

n = number of participants. Percentages are calculated based on the results submitted by all participants (see Annexes 16, 17, 18 and 19).

An average score of 98% was reported for the detection of the *ehx*A. Three false negative results were obtained by one laboratory for strain PP16 (O121:H19), QQ17 (O157:H7) and RR18 (O91:H14). Then complete results are presented in Annexes 16, 17, 18 and 19.

3.4.2 Subtyping of *vtx1* and *vtx2*

The number of laboratories participating in subtyping of *vtx* genes were 22 (79% of the participants participated in the *vtx* detection). The average subtyping results of vtx genes were calculated based on the number of participants, including laboratories, which reported false negatives for *vtx1* or *vtx2*. The results indicate that the participants followed our suggestion of performing the subtyping on all test strains despite the results of the detection of *vtx1* and *vtx2*. Three laboratories have correctly subtyped strains despite the negative results in the *vtx* detection, and only one participant submitted a negative in both the *vtx* detection and subtyping – if this is because of the *vtx* detection results or if the subtyping was really performed is not clear. In EQA-5 only strains with *vtx1a* were included and *vtx1* was correctly subtyped by an average of 98% of the participants. The range was 90% (*vtx1a*) in strain OO15 (O55:H7), but 100% for *vtx1a* in strain (O103:H2). One laboratory failed to discriminate between *vtx1a* and *vtx1c*, despite the fact that the combination of two *vtx1* genes in the same strain rarely occurs. In addition, only one false negative *vtx1* was reported for strain OO15 (O55:H7). *Vtx2* was correctly typed by an average of 94% of the participants. The range was from 73% for *vtx2d* in strain SS19 (O166:H15) to 95% for *vtx2b* in strain RR18 (O91:H14*).* False positive and negative results are included in Table 8. Strain SS19 (*vtx2d*, O166:H15) was the strain with the lowest percentage of correct results (73%) and the highest false positive results was mainly *vtx2c +vtx2d*. The complete results are presented in Annex 20.

Table 8. Subtyping results for vtx1 and vtx2, including false positive and false negative results

Strain/method	Original	*vtx1* subtyping			*vtx2* subtyping			*vtx* subtyping
n		**22**			**22**			**22**
		Found *vtx1* gene	False positive	False negative	Found *vtx2* gene	False positive	False negative	Correct
KK11		100% (22)			100% (22)			100%
LL12	*vtx1a*	95% (21)	1 *vtx1a* + *vtx1c*		100% (22)			95%
MM13	*vtx1a*	95% (21)	1 *vtx1a* + *vtx1c*		100% (22)			95%
NN14	*vtx1a*	100% (22)			100% (22)			100%
OO15	*vtx1a*	91% (20)	1 *vtx1a* + *vtx1c*	1	100% (22)			91%
PP16	*vtx2a*	100% (22)			91% (20)	1 *vtx2b*	1	91%
QQ17	*vtx2a* and *vtx2c*	100% (22)			91% (20)	1 *vtx2c* + *vtx2d* 1 *vtx2d*	1 (vtx2c)	91%
RR18	*vtx2b*	100% (22)			95% (21)	1 *vtx2b* + *vtx2g*		95%
SS19	*vtx2d*	100% (22)			73% (16)	*5 vtx2c* + *vtx2d* 1 *vtx2a* + *vtx2c*		73%
TT20	*vtx2f*	100% (22)			91% (20)	1 *vtx2d* and *vtx2f*	1	91%
Average		**98%**			**94%**			**92%**

n = number of participants. Percentages are calculated based on the results submitted by the participants listed in Annex 20.

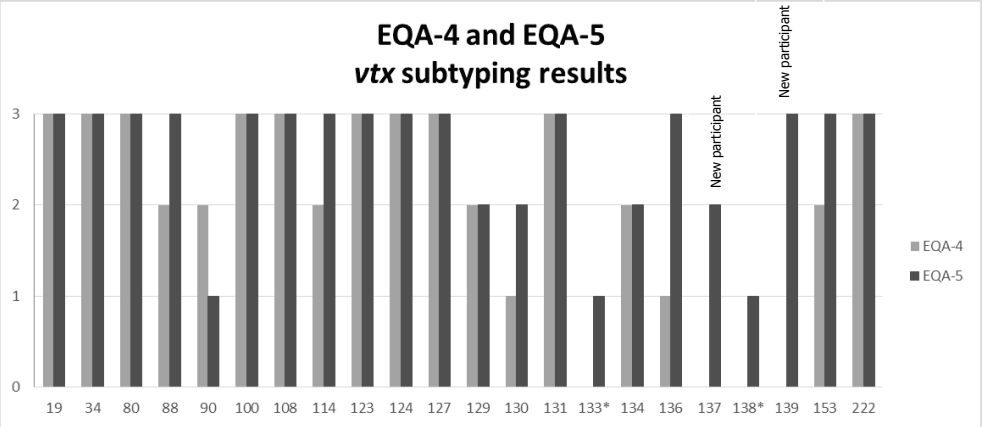
Sensitivity and specificity of the subtyping of the one vtx1 and five vtx2 subtypes are presented in Table 9. Sensitivity was 1.00 for vtx1a, vtx2b and vtx2f, and between 0.77 and 0.98 for vtx2a, vtx2c, vtx2d. Specificity was 0.98 to 1.00 for all subtypes.

Table 9. Sensitivity and specificity of vtx subtyping results

	vtx1a	*Vtx2a*	*vtx2b*	*vtx2c*	*vtx2d*	*vtx2f*
Sensitivity	1.00	0.98	1.00	0.77	0.88	1.00
Specificity	0.99	0.98	1.00	0.99	0.99	0.99

To show the exact progress of the laboratory's performances, three strains from EQA-4 were included in EQA-5. Strain GG7(*vtx1a*), II9(*vtx2a* and *vtx2c*) and MM13(*vtx2d*) from EQA-4 are numbered LL12, QQ17 and SS19 respectively in the EQA-5. Figure 8 show the performance based only on the three isolates.

Figure 8. Comparing EQA-4 and EQA-5 vtx subtyping performance



*The participating laboratories are represented by arbitrary numbers. Bars represent the number of correctly assigned vtx subtypes of the three strains GG7, II9 and MM13 from EQA-4 and LL12, QQ17, and SS19 from EQA-5. * indicates the laboratory participated in EQA-4, but none of the three strains were correctly subtyped.*

3.4.3 Detection of other virulence genes (*aggR, aaiC, aatA and eltA*)

Results for relevant additional virulence genes (non-VTEC genes) are presented in Tables 10 and 11.

Table 10 presents the virulence genes considered part of the standard repertoire of virulence genes in EU public health national reference laboratories; additional genes presented in Table 11 are not considered part of this repertoire.

Twenty laboratories detected *aggR* correctly in strain KK11 (O78:H2). Sixteen laboratories also detected the *aaiC gene*, however two laboratories wrongly detected *aggR* in additional strains. ETEC-related genes *elt*A in strain SS19 (O166:H15) were correctly determined by 13 laboratories; Six laboratories correctly detected the gene *aatA* for EAggEC in strain KK11 (O78:H2).

Table 10. Detection of additional virulence genes, including false positive

Strain no.	False positive results (n)	*aggR*	*aaiC*	*aatA*	*eltA*
KK11		20	16	6	
LL12	*aggR* (1)				
MM13					
NN14					
MM15					
PP16					
QQ17	*aggR* (1)				
RR18					
SS19					13
TT20	*aggR* (1)				

n = number of participants. Percentages are calculated based on the results submitted by the participants listed in Annex 21.

Note: These genes are considered part of the standard repertoire of virulence genes in EU public health national reference laboratories.

No false negative results were submitted for any of the genes

Designations for accepted heat-labile enterotoxin were: elt (LT1), eltA (ltcA), eltI

A high number of false positive results for astA in the EQA-4 together with the observation of WHOCC recorded both positive and negative results of astA while testing the strains for stability of the EQA-5 – therefore astA is not included in this table but in table 11 "Additional virulence genes in the fifth EQA test strains".

Other additional virulence genes detected by the participating laboratories are shown in Table 11 and are only included for future reference. The EQA provider did not test these genes.

Table 11. Additional virulence genes in the fifth EQA test strains

Strain no.	Positive gene results (n)	Suggested negative gene results
KK11	*pet* (1), *pic* (2), *iha* (1), *cdtVA* (1), *fyuA* (1), *aer* (1), *bfpA** (1)	*elt, estA, estA$_{p/h}$, invE, cdtVB, saa, bfp, toxB, ipaH, bfpB, bfpA, aap, daaC*
LL12	*eae-θ* (1), *rfbO111* (1), *fliCH8* (1), *lpfAO113* (1), *iha* (1), *efa1* (1), *lacY* (1), *ehaA* (1), *aer* (1), *hlyA* (1)	*iha, elt, estA, estA$_{p/h}$, invE, cdtVA, cdtVB, saa, bfp, toxB, ipaH, bfpB, bfpA, aap, daaC*
MM13	*eae-β* (1), *wbuAO26* (1), *flicH11* (1), *ipfAO113* (1), *Iha (2)*, *efa1* (1), *lacY* (1), *ehaA* (1), *fyuA* (1), *aer* (1)	*elt, estA, estA$_{p/h}$, invE, cdtVA, cdtVB, saa, bfp, toxB, ipaH, bfpB, bfpA, aap, daaC*
NN14	*eae-ε* (1), *wzxO103* (1), *efa1* (1), *bla TEM* (1), *lacY* (1), *ehaA* (1), *hlyA* (1)	*iha, elt, estA, estA$_{p/h}$, invE, cdtVA, cdtVB, saa, bfp, toxB, ipaH, bfpB, bfpA, aap, daaC*
MM15	*eae-γ* (1), *flicH7* (2), *ipfAO157* (1), *OI-141/lpfAO157-OI141/ipfAO154* (1), *efa1* (1), *lacY*(1), *etp* (1), *ehaA* (1)	*iha, elt, estA, estA$_{p/h}$, invE, cdtVA, cdtVB, saa, bfp, toxB, ipaH, bfpB, bfpA, aap, daaC*
PP16	*lacY*(1), *espP* (1), *toxB ** (1), *ehaA* (1), *hlyA* (1)	*iha, elt, estA, estA$_{p/h}$, invE, cdtVA, cdtVB, saa, bfp, toxB, ipaH, bfpB, bfpA, aap, daaC*
QQ17	*eae-γ* (1), *rfbO157* (1), *fliCH7* (2), *lpfAO157/OI-141, ipfAO157/OI-154* (1), *iha* (2), *toxB* (2), *efa1* (1), *lacY*(1), *espP* (1), *etp* (1), *katP* (1), *ehaA* (1), *astA* (1)	*elt, estA, estA$_{p/h}$, invE, cdtVA, cdtVB, saa, bfp, ipaH, bfpB, bfpA, aap, daaC*
RR18	*wbsDO91* (1), *subAB* (3), *ipfAO113* (1), *iha* (2), *saa* (5), *efa1* (1), *estA* (1), lacY (1), *espP* (1), *ehaA* (1), *hlyA* (1)	*elt, estA, estA$_{p/h}$, invE, cdtVA, cdtVB, bfp, toxB, ipaH, bfpB, bfpA, aap, daaC*
SS19	*Bla TEM-1* (2), *bla CTX-M-15* (2), *astA* (4), *lacY* (1), *qnrS1* (1), *hlyA* (1)	*iha, elt, estA, estA$_{p/h}$, invE, cdtVA, cdtVB, saa, bfp, toxB, bfpB ,ipaH, bfpA, aap, daaC*
TT20	eae-ι(1), *wzxO145* (1), *efa1* (1), *lacY* (1), *cdt* (1), *ibeA* (1)	*iha, estA, estA$_{p/h}$, invE, cdtVA, cdtVB, saa, bfp, toxB, ipaH, bfpB, bfpA, aap, daaC*

Note: *These genes are not considered part of the standard repertoire of virulence genes in EU public health national reference laboratories.*

**Reported both positive and negative*

3.4.4 Phenotypic test

Participation in phenotypic detection ranged from 28% (VCA/EIA) to 90% (sorbitol fermentation). Correct results of 100% were reported for the production of ESBL, this test was introduced in the EQA-4 (Table 12).

Table 12. Average scores of the phenotypic tests

Strain/method	VCA/EIA	ESBL production	Haemolysin production	beta-glucuronidase production	Sorbitol fermentation
n	8	18	14	15	26
KK11	88%	100%	100%	93%	100%
LL12	100%	100%	86%	80%	96%
MM13	100%	100%	100%	93%	100%
NN14	100%	100%	93%	73%	96%
OO15	100%	100%	100%	93%	88%
PP16	100%	100%	86%	93%	100%
QQ17	100%	100%	86%	100%	100%
RR18	38%	100%	93%	93%	96%
SS19	56%	100%	100%	93%	92%
TT20	75%	100%	100%	87%	96%
Average	**86%**	**100%**	**94%**	**90%**	**97%**

n =Number of participants. The percentages are calculated based on the results of the participants presented in Annexes 11, 12, 13, 14 and 15.

Average correct results were 86% for VCA/EA and 94% for enterohaemolysin production, 90% for beta-glucuronidase production, 97% for fermentation of sorbitol and 100% for ESBL (Table 12).

Most of the errors in the detection of beta-glucuronidase production were submitted by a single laboratory, which submitted all 10 strains negative. We suspect that the laboratory disregarded our suggestion of testing all strains with each analysis – and not only test (in this case) the O157 strain. In the detection of enterohaemolysin production also one laboratory submitted only negative results for all 10 strains, which might also be a submission error – the additional errors were also submitted by an additional laboratory. One laboratory submitted four out of the nine errors in sorbitol fermentation.

Detailed results for all phenotypic tests can be found in Annexes 11 (VCA/EIA), 12 (ESBL), 13 (enterohaemolysin), 14 (beta-glucuronidase), and 15 (sorbitol).

4. Conclusions

Twenty-nine laboratories signed up for the EQA-5 on VTEC typing funded by ECDC. For the second time, the EQA also included PFGE, and 20 laboratories participated in the PFGE exercise. Twelve (60%) of the laboratories were able to produce a PFGE gel of sufficiently high quality to allow comparison with profiles obtained by other laboratories. This is an increase from 45% in EQA-4. In the critical parameter Image acquisition and running conditions, only 5% of the participants scored poor (1) compared with 40% in the EQA-4. The production of distinct bands is another important gel quality assessment parameter; however, six out of the eight laboratories scoring poor (1) in any of the gel quality parameters were caused by too fuzzy/thick bands - a level equal to EQA-4. The BN software suite was used for the normalisation and interpretation of profiles. Seventeen (85%) laboratories analysed the resulting gels and 71% of these laboratories performed in good accordance with the guidelines.

Nineteen of the laboratories (66%) participated in the full O:H serotyping, and 69% of the serotyping results were correct. The correct results of O grouping and H grouping were reported for an average of 80% and 70% of the test strains, respectively. However not all laboratories have the full scheme of all O groups and H types.

Participation rate was 90% for *eae*, 97% for both *vtx1* and *vtx2,* and 66% for *ehxA.* Subtyping for *vtx* was performed by 76% of the participants, with an average of 92% of correct results. Gene detection of *eae, vtx1*, *vtx2* and *ehxA* was 94–98% correct. The score of the vtx2 detection (94%) was mainly caused by one strain (TT20) that was *vtx2f* positive.

Phenotypic characterisation generally showed very good results: 86% correct results for VT and 94% enterohaemolysin production, 97% for fermentation of sorbitol, 90% for beta-glucuronidase production, and 100% for detection of ESBL production. Phenotypic characterisation was not performed as often as genotypic characterisation: detection of VT production (28% of the participants), enterohaemolysin production (48%), beta-glucuronidase production (52%), sorbitol (90%), and ESBL (62%).

Overall, the EQA-5 showed that there is an increasing number of laboratories that perform O:H serotyping at a very high level. Virulence genes (*eae*, *vtx1*, *vtx2*, *ehxA*, *aggR* and *aaiC)* were correctly detected and *vtx* genes were generally subtyped correctly. A few laboratories need to improve the quality of both genotypic and phenotypic tests. If this relatively small number of laboratories are excluded from the overall results, the performance level is very high.

For the few laboratories with poor PFGE results, additional trouble shooting and training activities should be considered.

5. Discussion

The WHO Collaborating Centre for Reference and Research on *Escherichia* and *Klebsiella* (WHOCC), Unit of Foodborne Infections at the SSI in Copenhagen, Denmark, has played a leading role in establishing a worldwide international network of quality evaluation and assessment for the typing of *E. coli* since 2002.

5.1 Pulsed-field gel electrophoresis (PFGE)

Twenty laboratories participated in the PFGE part of the EQA-5. 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 modified TIFF quality grading guidelines, which evaluate seven parameters. Scores were given between 1 and 4 (poor, fair, good and excellent). The majority (60%) of laboratories were able to produce gels with sufficiently high quality (above a score of 1) in all seven parameters.

The main issue in this VTEC EQA-5 was in the parameter Bands. Seventy percent were graded fair and above, while 30% gels scored 1 in this parameter. In general, major improvements could be made when capturing the image and producing a TIFF image. 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 because of blurs. This together with overloading plugs with DNA are major contributors to the low score in the category Bands.

Only three laboratories each obtained a score of 1 in parameter: Image acquisition and running conditions, Lanes or DNA degradation. In the gel graded 1 in the category Image Acquisition and Running Conditions the top two bands of the *Salmonella* Braenderup reference strain were merged into one thick band – resulting in an unacceptable Normalization. It is important to use running conditions as described for the relevant organism as these varie significantly between species. It is also important to have equipment that is running properly as well as making sure that the running temperature is as described in protocol. The modification of the grading scheme was a necessity to ensure that the score 1 (poor) is only obtained for the parameter Image Acquisition and Running Conditions when the band spacing of the standard is incorrect and highly affects the analysis. Nevertheless, in this EQA, 60% all of the gels have obtained at least the score 2 in all parameters and are therefore suitable for inter-laboratory comparison and increase from 45% in EQA-4. Other common deviations from protocol is seen in image acquisition, where some 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. The other parameters are 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.

Eighty-five percent (17 out of 20) of the laboratories that performed PFGE did the subsequent gel analysis, i.e. the normalisation and band assignment that produces the actual PFGE profiles for comparison. This analysis requires specialised software, usually the BN software suite. Some laboratories might not have access to this software or have limited experience working with PFGE analysis in BN. However, to be able to perform national surveillance as well as submit profiles to the EU-wide molecular surveillance system (TESSy MSS database), it is necessary to have the capacity to analyse and interpret PFGE gels. Eleven of the 17 (65%) laboratories, that submitted the BN analysis, achieved fair to excellent (2–3) scores, compared with EQA-4 an increase from 50% to 65% (30%).

5.2 Serotyping

Participation in O:H serotyping in EQA-5 was roughly the same in the EQA-3 and EQA-4 (>50% in average). An average of 80% of the 15–26 participating laboratories could correctly perform O grouping of the 10 test strains, which is the same as the EQA-4.

Participation in the EQA-5 (26/29) was however a bit lower compared with EQA-4 (26/28) in O group typing, but somewhat higher (66% versus 64%) in H typing. However, both analyses are biased by the reporting of non-typeable (NT) results.

This EQA-5 had 29 EU/EEA participants, which is one more than the last EQA-4. Correct O:H serotyping ranged from 100% correct typing of one of the O157:H7 strain to 58% correct typing of serotype O145:H34. Nevertheless correct results for O:H serotyping in the EQA-5 were lower (69%) than in the EQA-5 (80%). This is however, not influenced by the H typing but by the errors in the O typing.

However, the general trend (in both EQAs) is that the more common serotypes are identified more reliably. No systematic typing errors were observed. Only eight O types were mistypes and three H groups. The remainder of incorrect typing was submitted as NT or not done (ND).

The comparisons (Figure 6 and 7) of the strains that were included in both EQA-4 and EQA-5 shows that 10 of 26 the laboratories had all three strains correctly O grouped in both EQA-4 and EQA-5. Only one laboratory decreased their performance. In the H typing (Figure 7) 9 out of 19 the laboratories had all three strains correctly H typed in both EQAs.

In addition, 17 of 26 (65%) laboratories were able to correctly determine the O group of the top 6 strains (O157, O26, O103, O111, O121, and O145) that were included in EQA-5, which are a part of the suggested minimal requirement for the typing of VTEC in the EU-level by ECDC (17, not published). Fifty-three percent of these laboratories (9 out of 17) were also able to correctly determine the O:H serotype in the same top 6 strains.

In addition to O grouping, H typing is crucial in the detection of outbreaks, for epidemiological surveillance, for taxonomic differentiation *of E. coli*, and for detecting pathogenic serotypes within the species. It therefore remains a main challenge to enable more of the PHNRLs to perform complete and reliable O:H serotyping – and H typing in particular. As the number of PHNRLs performing H typing has not increased significantly over the first ECDC funded EQA schemes (13-16 for all H types, and 16-18 for H7 on average), data submitted to TESSy and in relation to outbreaks will be reliable for only a little over half of EU PHNRLs.

5.3 Virulence determination

5.3.1 Genotypic tests

Genotypic detection of virulence genes *eae*, *vtx1, vtx2* and *ehxA* was performed by 19–28 laboratories for all the 10 test strains; results were 94–98% correct. The participation rate varied substantially between the different tests in the fifth EQA, being highest for the genotypic detection of the *vtx* genes (97%) and lowest for the detection of *ehxA* (66%). In general, the percentage of correct results was very high (94–98%). The incorrect results for the *eae* gene originated from errors by four different laboratories. The average correct score has improved from 96% in EQA-4 and EQA-3 to 98% in EQA-5. Compared with the EQA-3 and EQA-4 , the average correct score of *ehxA* was slightly decreased from 99 to 98%, only three false negative results submitted by one laboratory was submitted.

Detection of *vtx1* and *vtx2* genes was achieved with a high percentage of correct results (98%/ 94%), *vtx1* very similar to the EQA-4/EQA-3 (98%). The percentage of correct results of *vtx2* was a bit lower in this EQA than previous EQA-4/EQA-3 (99%/ 98%). However, the majority of false negative results originated from testing the strain TT20 (*vtx2f*). Ten laboratories did not detect the strain positive for *vtx2,* which suggest that they did not include primers that detected *vtx2* in their analysis. Resent cases of HUS caused by strains harbouring *vtx2f* have been discussed, however not published, but the importance of awareness of *vtx2f* has been described by Friesema et al., 2014 [18]. Maybe detection of *vtx2f* needs to be included in the expected repertoire of VTEC in Europe in the future.

The comparison (Figure 8) of the strains that were included in both EQA-4 and EQA-5 shows that 10 of 22 (45 %) the laboratories had all three strains correctly subtyped in both EQAs. Only one laboratory decreased their performance.

In the EQA-3, the major problem in subtyping the *vtx* genes was to distinguish between *vtx2a*, *vtx2b*, *vtx2c* and *vtx2d*. A revised protocol for subtyping of *vtx* genes was published in September 2012 [16]. Previously recorded problems with significant sensitivity to different PCR cycler equipment and use of different DNA polymerases seem to have been resolved in the majority of participating laboratories, mainly by adherence to the revised protocol. The new protocol was used by 97% of the participants, and correct results ranged from 91–100% for subtyping *vtx1* and from 73–95% for subtyping *vtx2*. Correct subtyping of both *vtx1* and *vtx2* was obtained at an average of 92%. A small improvement compared with EQA-3 (90%). Laboratories received advice on how to test and calibrate their PCR cyclers (increasing the annealing temperature up to 67°C or higher) in order to improve their performance.

5.3.2 Phenotypic tests

The participation in the phenotypic detection was between 28 and 90% on average (8–26 laboratories). The lowest participation was for VCA/EIA, where only eight participants (28%) delivered results for 10 strains. A decrease from the ten participants delivering results in the third EQA and fourth EQA.

In general, the most important phenotypic test is the sorbitol fermentation, which is used to screen for the highly virulent SF O157:H7 clone. It is therefore encouraging that the fermentation of sorbitol was performed more often (90% compared to 78%) in the third EQA, however a bit lower that in the EQA-4 (93%). The average results (97% correct) have increased from EQA-4 (95%). The second highest participation was for ESBL (62%) almost the same as EQA-3 (61%) followed by beta-glucuronidase activity 52% (decreased from 58% EQA-4), enterohaemolysin (48%, compared with 49% in the EQA-4) and VCA/EAI production (28%) compared with 40% in the EQA-3 and 38% EQA-4.

Both in the detection of beta-glucuronidase production and enterohaemolysin production all strains were submitted by a single laboratory as 'negative', which suggests that only one or a few strains were tested by the analysis and the 'negative' results were selected because 'not done' was not an option. ESBL was always correctly detected (correct results by all 18 participating laboratories) however, as the EQA provider we encourage the participants to participate in as much as the can.

In summary, the performance level for phenotypic characterisation was very high but some laboratories need to ensure their analysis.

5.3 General remarks

The inconsistency in the number of performed tests per strain and per laboratory was notable in all VTEC EQAs so far. Laboratories never explained why a specific test was not performed on all 10-test strains. This was particularly evident for O grouping where laboratories submitted multiple instances of 'NT'. A similar situation was encountered for H typing. However we observed ten negative results submitted for both beta-glucuronidase and enterohaemolysin production by two different laboratories. These inconsistencies reduce comparability between the tests and the laboratories and complicate the analyses.

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 the application of controlled laboratory procedures. Therefore, laboratories can optimise performance by strictly adhering to the protocol which details, for example, temperatures, times, and the number of repeated washing steps. Deviations from the protocol should be avoided unless thoroughly evaluated. Certain elements cannot be modified, especially the electrophoresis conditions including temperature and switch times. It should be noted that although many steps are similar for different organisms, important species-specific differences have to be taken into account.

Several laboratories probably produced a high quality gel, but failed to document this due to sub-optimal staining, poor destaining and imprecise image capturing. It is highly recommended that laboratory personnel invest the time and effort to improve their familiarity with image acquisition equipment and ensure proper maintenance of imaging and electrophoresis equipment.

A number of avoidable errors were made. Many errors could have been avoided if laboratory personnel had carefully read the instructions on how to produce and submit TIFF and XML files of the PFGE results. However, the number of participants in the BN analysis increased from 12 (EQA-4) to 17 (approximately 42%).

Again, in this EQA only half of the laboratories participated in O:H serotyping. Serotyping is essential for the characterisation of *E. coli* and VTEC. We still suggest a survey among non-participating laboratories could explore the underlying reasons.

Regarding both genotypic and phenotypic tests, it is evident from the results and discussion that only a small number of laboratories encountered difficulties. If these laboratories are excluded from the overall results, the level of performance is very high. Additional trouble shooting and training activities should be considered for laboratories with poor performance. Still some laboratories has difficulties in creating and sending TIFF and XML files of the PFGE results, however laboratories seem to proofreading the results both before submission and during the grace period of the 24 hours.

6.2 ECDC and FWD-Net

The PFGE part of the VTEC EQA-5 had a 69% participation rate; 85% of the participating laboratories performed the BN gel analysis. Sixty percent of the gels produced were of sufficiently high quality for inter-laboratory comparison, and 65% of the BN analyses were at an acceptable level, compared with the results of EQA-4 for VTEC the gel quality and the BN analysis performance increased by 33% and 30%, respectively. However, there is still a need to improve laboratory procedures, gel analysis, and interpretation with BN software. Training to get familiar with the electrophoresis equipment and image acquisition equipment.

The relatively low levels of participation in full O:H typing needs to be explored and the reasons for the low participation rates have to be addressed. Similarly, the phenotypic tests for VT production and beta-glucuronidase production are only performed by a small number of laboratories and may be excluded in the next EQA.

6.3 The EQA provider

The modification of grading the quality of PFGE gels was necessary because the methodology did not automatically result in a score, which clearly indicates whether a gel is suitable for interlaboratory comparisons: occasionally, a fair overall score would be given to a gel that could not be compared with other gels. A score of one in any of the parameters is given when the gel cannot be used for comparisons with gels from other laboratories.

The EQA provider consider making an guide on optimising the image acquisition and recommends that the laboratories use our expertise in troubleshoot.

In this EQA the laboratories O group detection renders a 'Non typeable' or 'Not done', were considered as a negative result and scored accordingly.

The separation of the deadlines gave more time to finish the evaluation reports which is important 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. 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.

In addition, it should be mentioned that implementing the BN7.1 was not without trouble. The XML export files are encrypted and we are not able to help when field names or experiment types are incorrect. This however could be avoided if the participant onlyused the distributed BN databases. Next year standardised comments will be added to the evaluation report.

7. References

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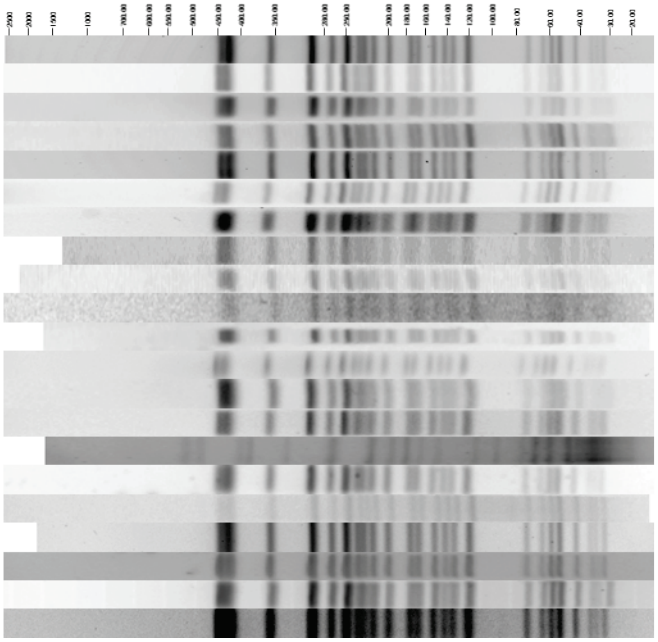
Annex 1. List of participants

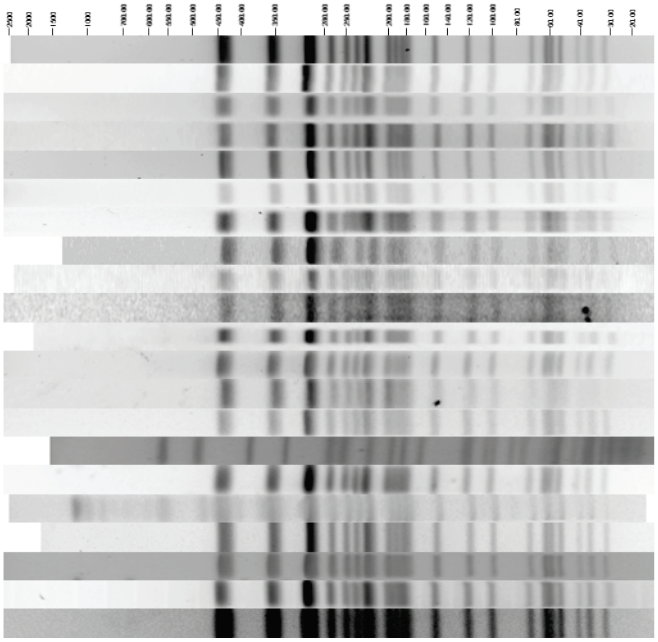
Country	Institute/organisation	Laboratory
Austria	Austrian Agency for Health and Food Safety, Institute for Medical Microbiology and Hygiene	National Reference Center for *Escherichia coli*
Belgium	Vrije Universiteit Brussel	Microbiology
Bulgaria	National Center of Infectious and Parasitic Diseases	NRL for enteric pathogens
Cyprus	Medical and Public Health Services, Nicosia General Hospital	Microbiology department
Czech Republic	National Institute of Public Health	NRL for *E. coli* and *shigella*
Denmark	Statens Serum Institut	Unit of Foodborne Infections
Estonia	Health board	Central Laboratory of Communicable Diseases
Finland	Institute of Health and Welfare	Bacteriology unit
France	Institut Pasteur	Centre national de référence des escherichia coli, des *shigella* et *Salmonella*
Germany	Robert Koch Institute, Bereich Wernigerode	National reference centre for *Salmonella* and other bacterial enterics
Greece	National School of Public Health, microbiology department	National reference centre for *Salmonella, Shigella- VTEC*
Hungary	National Center for Epidemiology, országos epidemiológiai központ	National reference laboratory
Iceland	Landspitali University Hospital	Department of clinical microbiology
Ireland	Cherry Orchard Hospital	Public health laboratory
Italy	Istituto superiore di sanità, Dipartimento di Sanità Pubblica Veterinaria e Sicurezza Alimentare	Foodborne Zoonoses Unit
Latvia	Riga East Clinical University Hospital	Latvian Centre of Infectious Deseases
Lithuania	National public health surveillance laboratory	Molecular testing subderpartment
Luxembourg	Laboratoire national de sante	Surveillance Epidemiologique
Netherlands	National Institute for Public Health and the Environment, Centre for Infectious Disease Control/IDS	BSR
Norway	Norwegian Institute of Public Health, Division for Infectious Disease Control	Reference Laboratory for Enteropathogenic Bacteria
Poland	National Public Health Institute, National Institute of Hygiene	Laboratory of Enteric Rods
Portugal	Instituto nacional de saúde dr. Ricardo Jorge	LNR de Salmonella e E.coli
Romania	Cantacuzino National Institute of Research-Development for Microbiology & Immunology	Molecular epidemiology laboratory
Serbia	Military Medical Academy, Institute of Epidemiology	Laboratory for molecular genetics
Slovenia	National Institute of Public Health, Department of Medical Microbiology	Laboratory of Bacteriology
Spain	National Center of Microbiology, Institute of Health Carlos III	Unidad de Enterobacterias
Sweden	Smittskydsinstitutet, Diagnostik & Vaccin	Livsmedel & Vatten
Turkey	Public Health Institution of Turkey, Microbiology Reference Laboratory Department	National reference laboratory for enteric pathogens
United Kingdom	Public Health England, Microbiology Services	Gastrointestinal Bacteria Reference Unit (GBRU)

Annex 2. Examples of PFGE profiles

Figure A2-1. Sample profile from participants



Strain 6



Strain 8

Annex 3. TIFF quality grading 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 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).

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

Annex 4. TIFF quality grading guidelines 2013[2]

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])

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

Annex 5. BioNumerics (BN) gel analysis quality guidelines

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 (BN) 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 too low Too much space framed at the bottom of the gel Too much space framed on the sides of the gel (Guidelines recommend to frame just beneath the wells)		Frame includes wells Gel not with light bands on dark background
Strips	All lanes correctly defined	A single lane is not correctly defined	Lanes defined too narrowly (users should include the whole gel lane)	Lanes not defined correctly: too wide/not following the actual gel lanes
Curves	1/3 or more of the lane is used for averaging curve thickness	Curves defined either as very narrow strip or encompassing almost the whole lane (Average thickness 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 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 with regard to the quality of the gel	Some double bands are assigned wrong	Some shadow bands are assigned (Guidelines requires control of band assignment after using auto search)	The positions are correct, but double bands assigned at the exact same positions Band assignment not correct (Commonly caused by thickness of the bands/overexposure) Only used auto search to find bands, no manual corrections (Guidelines requires control of band assignment after using auto search)

Annex 7. Scores of the PFGE results

Gel quality

Parameters\laboratory	19	34	90	100	108	114	123	124	129	130	131	132	133	134	135	136	138	139	153	222
Image and Running Conditions	4	4	4	2	4	4	4	4	3	2	2	2	2	2	3	4	1	2	2	2
Cell Suspension	4	4	4	4	4	4	4	4	3	2	4	4	4	4	4	4	3	4	4	4
Bands	4	4	4	4	2	1	4	2	1	1	1	1	2	4	2	3	3	1	2	2
Lanes	4	4	4	4	4	4	4	3	3	4	4	2	3	4	3	4	4	4	3	2
Restriction	4	4	4	4	4	4	4	4	4	4	4	4	3	4	4	4	4	4	1	4
Gel Background	3	3	4	4	3	2	3	3	2	3	3	2	2	4	3	2	3	2	4	4
DNA Degradation	4	3	4	4	4	4	4	2	4	2	1	2	4	4	4	3	2	4	2	4

Scored according to Annex 3 (TIFF quality grading guidelines)

BN analysis

Parameters\laboratory	19	34	90	100	108	123	124	129	130	132	133	134	135	136	139	153	222
Position of Gel	3	3	3	3	3	3	3	3	3	1	2	3	3	3	2	2	3
Strips	3	3	3	3	3	3	2	2	2	3	2	3	3	2	3	3	3
Curves	3	3	3	3	3	3	3	3	2	2	2	3	2	2	3	2	2
Normalization	3	3	3	3	3	3	3	3	2	3	3	3	3	3	3	3	3
Band Assignment	2	3	3	2	3	3	1	1	1	2	1	2	3	3	1	2	2

Scores according to Annex 5 (BN gel analysis quality guidelines)

Annex 8. Original data (serotyping, genotyping and phenotyping)

Strains/method	O group	H type	Vero cell assay	ESBL production	Haemolysin production	Beta-glucuronidase	Sorbitol fermentation	*eae* gene	*ehxA* gene	*vtx1* gene	*vtx2* gene	*vtx* Subtypes			Additional virulence genes	Pathogenic group
KK11	O78[a)]	H2	-	-	-	+	+	-	-	-	-				*aggR, aaiC , aatA*	EAggEC
LL12	O111	H-/H8	+	-	+	+	+	+	+	+	-	*vtx1a*				STEC/VTEC
MM13	O26	H11	+	-	-	+	+	+	-	+	-	*vtx1a*				STEC/VTEC
NN14	O103	H2	+	-	+	+	+	+	+	+	-	*vtx1a*				STEC/VTEC
OO15	O55	H7	+	-	-	+	-	+	-	+	-	*vtx1a*				STEC/VTEC
PP16	O121 [a)]	H19	+	-	+	+	+	+	+	-	+		*vtx2a*			STEC/VTEC
QQ17	O157	H7	+	-	+	-	-	+	+	-	+		*vtx2a*	*vtx2c*		STEC/VTEC
RR18	O91	H14	+	-	+	+	+	-	+	-	+		*vtx2b*			STEC/VTEC
SS19	O166	H15	+	+	-	+	+	-	-	-	+		*vtx2d*		*eltA*	STEC/VTEC-ETEC
TT20	O145	H34	+	-	-	+	-	+	-	-	+		*vtx2f*			STEC/VTEC

+ = Positive,- = Negative, alfa = positive for alfahaemolysin, but entero/alfahaemolysin results were accepted for all strains. Intermediate result noted in the VCA was accepted as a positive result. Other additional virulence genes are described in Table 10.
a) Lactose negative

Gene abbreviations			
eae	CVD434. *E. coli* attaching and effacing gene probe	*eltI*	G119. Heat-labile enterotoxin (LT). Almost identical to cholera toxin
ehxA	CVD419. Plasmid-encoded O157-enterohaemolysin	*aatA*	PCR fragment. The gene encodes the dispersin (aap) transporter protein, which is a good plasmid marker for enteroaggregative *E. coli*
vtx1	NTP705. Verotoxin1; almost identical with Shiga toxin	*aaiC*	Chromosomal gene marker for enteroaggregative *E. coli*
vtx2	DEP28. Verotoxin2; variants exist. Approximately 60% homology to *vtx1*	*aggR*	Gene encoding the master regulator in enteroaggregative *E. coli*

Annex 9. O group serotyping results

Strain/lab	Ori.	19	34	80	88	94	100	108	114	123	124	125	127	128	129	130	131	132	133	134	135	136	137	138	139	153	222
KK11	O78	O78	O78	O78	O78	O78	O78	O78	O78	O78	NT	O78	O78	NT	O78	NT	O78	ND	NT	O78	O78	O78	O78	NT	O78	NT	O119
LL12	O111	O111	O111	O111	O111	O111	O111	O111	O111	O111	O111	O111	O111	O111	O111	O111	O111	ND	O111	O111	O111	O111	O111	O111	O111	O111	O111
MM13	O26	O26	O26	O26	O26	O26	O26	O26	O26	O26	O26	O26	O26	O26	O26	O26	O26	ND	O26	O26	O26	O26	O26	O26	O26	O26	O26
NN14	O103	O103	O103	O103	NT	O128	O103	O103	O103	O103	O103	O103	O103	O103	O103	O103	O103	ND	O103	O103	O103	O103	O103	O103	O103	O103	O103
OO15	O55	O55	O55	O55	NT	O55	NT	O55	O55	O55	O55	O55	O55	NT	O55	O55	O55	ND	NT	O55	O55	O55	O55	O55	O55	NT	O55
PP16	O121	O121	O121	O121	NT	NT	O121	O121	O121	O121	O121	NT	O121	O121	O121	NT	O121	ND	NT	O121	O121	O121	O124	O121	O121	O121	O121
QQ17	O157	O157	O157	O157	O157	O157	O157	O157	O157	O157	O157	O157	O157	O157	O157	O157	O157	O157	O157	O157	O157	O157	O157	O157	O157	O157	O157
RR18	O91	O91	O91	O91	O32	O114	O91	O91	O91	O91	NT	NT	O91	NT	O91	O91	O91	ND	NT	O91	O91	O91	O91	NT	O91	NT	O91
SS19	O166	O166	O166	NT	O110	NT	NT	O166	O166	O166	NT	O166	O166	NT	O166	NT	O166	ND	NT	NT	O166	O166	O166	NT	O166	O55	NT
TT20	O145	O145	O145	O145	O145	NT	O145	O145	O145	O145	O145	O145	O145	NT	O145	O145	O145	ND	O145	O145	O145	O145	O145	O103	O145	O145	O145

Incorrect result

Stains included from the EOA-4

Annex 10. H type serotyping results

Strain/lab	Original	19	34	80	94	100	108	114	123	124	125	128	129	131	134	135	136	137	138	139
KK11	H2	H2	H2	H2	NT	ND	H2	H2	H2	NT	NT	ND	H2	H2	H2	H2	H2	H2	ND	H2
LL12	H-/H8	H-	H-	H8	NT	ND	H8	H-	H8	H-	NT	ND	H8	H8	NT	H8	H8	H8	ND	H8
MM13	H11	H11	H11	H11	NT	ND	H11	H11	H11	NT	NT	ND	H11	H11	H11	H11	H11	H11	ND	H11
NN14	H2	H2	H2	H2	NT	ND	H2	H2	H2	NT	NT	ND	H2	H2	H2	H2	H2	H2	ND	H2
OO15	H7	H7	H7	H7	NT	ND	H7	H5	H7	H7	NT	ND	H7	H7	H7	H7	H7	H7	H7	H7
PP16	H19	H19	H19	H19	NT	ND	H19	H19	H19	NT	NT	ND	H19	H19	H19	H19	H19	H19	ND	H19
QQ17	H7	H7	H7	H7	H7	H7	H7	H7	H7	H7	H7	H7	H7	H7	H7	H7	H7	H7	H7	H7
RR18	H14	H14	H14	NT	NT	ND	H14	H14	H14	NT	NT	ND	H14	H14	H14	H14	H14	H14	ND	H14
SS19	H15	H15	H15	NT	NT	ND	H15	H15	H15	NT	NT	ND	H15	H15	H15	H15	H15	H15	ND	H15
TT20	H34	H34	H34	NT	NT	ND	H34	H28	H34	NT	NT	ND	H34	H34	H34	H34	H34	H34	H7	H34

H- was accepted as a correct result.

Incorrect result

Stains included from the EOA-4

Annex 11. VCA results

Strain/labortory	Original	19	94	114	126	127	128	131	222
KK11	-	-	-	-	-	-	+	-	-
LL12	+	+	+	+	+	+	+	+	+
MM13	+	+	+	+	+	+	+	+	+
NN14	+	+	+	+	+	+	+	+	+
OO15	+	+	+	+	+	+	+	+	+
PP16	+	+	+	+	+	+	+	+	+
QQ17	+	+	+	+	+	+	+	+	+
RR18	+	+	-	+	-	-	-	+	-
SS19	+	+	-	+	+	Intermediate	-	+	+
TT20	+	+	-	+	+	+	-	+	+

Intermediate result noted in the Vero cell assay is accepted as a positive result.

+= Positive, - = Negative

Incorrect result

Annex 12. ESBL production results

Strain/labortory	Original	19	34	80	94	100	114	123	124	125	126	128	130	131	132	132	133	136	153
KK11	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
LL12	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
MM13	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
NN14	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
OO15	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
PP16	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
QQ17	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
RR18	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
SS19	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
TT20	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

+ = Positive, -. = Negative

Neg=negative

Annex 13. Enterohaemolysin production results

Strain/labortory	Original	19	34	94	100	114	123	125	126	127	128	129	131	136	153
KK11	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
LL12	+	+	+	+	+	+	+	-	+	+	-	+	+	+	+
MM13	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
NN14	+	+	+	Alfa	+	+	+	-	+	Alfa	+	+	+	+	+
OO15	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
PP16	+	+	+	Alfa	+	+	+	-	+	+	-	+	+	+	+
QQ17	+	+	+	+	+	+	+	-	+	+	-	+	+	+	+
RR18	+	+	+	Alfa	+	+	+	-	+	+	+	+	+	+	+
SS19	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
TT20	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

Alfa: positive results for alfahaemolysin, but entero/alfahaemolysin results are accepted for all strains.

+= Positive, -= Negative

Incorrect result

Annex 14. beta-glucuronidase production results

Strain/labortory	Original	19	34	80	94	100	114	123	124	127	128	129	130	131	136	153
KK11	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+
LL12	+	+	-	+	-	+	+	+	+	+	+	+	+	+	+	-
MM13	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+
NN14	+	+	-	+	-	+	+	+	+	+	+	+	+	-	+	-
OO15	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+
PP16	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+
QQ17	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
RR18	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+
SS19	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+
TT20	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	-

+= Positive, -= Negative

Incorrect result

Annex 15. Sorbitol fermentation results

Strain/labortory	Original	19	34	80	88	94	100	108	114	123	124	125	127	128	129	130	131	132	133	134	135	136	137	138	139	153	222
KK11	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
LL12	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+
MM13	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
NN14	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+
OO15	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	+	-	-	-	-	-	-
PP16	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
QQ17	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
RR18	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+
SS19	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	+	+
TT20	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

+= Positive, -= Negative

Incorrect result

Annex 16. *eae* gene detection results

Strain/labortory	Original	19	34	80	88	90	94	100	108	114	123	124	127	128	129	130	131	132	133	135	136	137	138	139	145	153	222
KK11	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
LL12	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
MM13	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+
NN14	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
OO15	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+
PP16	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+
QQ17	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
RR18	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
SS19	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
TT20	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+

+= Positive, -= Negative

Incorrect result

Annex 17. *ehx*A gene detection results

Strain/labortory	Original	19	34	80	90	94	100	108	114	123	124	127	129	131	133	134	136	139	153	222
KK11	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
LL12	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
MM13	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
NN14	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
OO15	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
PP16	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+
QQ17	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+
RR18	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+
SS19	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
TT20	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

+= Positive, -= Negative

Incorrect result

Annex 18. *vtx*1 gene detection results

Strain/labortory	Original	19	34	80	88	90	94	100	108	114	123	124	125	127	128	129	130	131	132	133	134	135	136	137	138	139	145	153	222
KK11	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
LL12	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
MM13	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
NN14	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
OO15	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+
PP16	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
QQ17	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
RR18	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-
SS19	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
TT20	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-

+= Positive, -= Negative

Incorrect result

Annex 19. *vtx*2 gene detection results

Strain/lab.	Ori.	19	34	80	88	90	94	100	108	114	123	124	125	127	128	129	130	131	132	133	134	135	136	137	138	139	145	153	222
KK11	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
LL12	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
MM13	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
NN14	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
OO15	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
PP16	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	-	+	+	+	+	+	+	+	+	+
QQ17	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
RR18	+	+	+	+	+	+	-	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
SS19	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
TT20	+	+	+	+	+	+	-	+	+	+	+	+	-	+	-	-	+	+	-	+	-	-	+	+	-	-	-	+	+

Incorrect result

Annex 20. *vtx* subtyping results

Strain/labortory	Original	19	34	80	88	90	100	108	114	123	124	127
KK11	-	-	-	-	-	-	-	-	-	-	-	-
LL12	*vtx1a*	*vtx1a*	*vtx1a*	*vtx1a*	*vtx1a*	*vtx1a*	*vtx1a*	*vtx1a*	*vtx1a*	*vtx1a*	*vtx1a*	*vtx1a*
MM13	*vtx1a*	*vtx1a*	*vtx1a*	*vtx1a*	*vtx1a*	*vtx1a*	*vtx1a*	*vtx1a*	*vtx1a*	*vtx1a*	*vtx1a*	*vtx1a*
NN14	*vtx1a*	*vtx1a*	*vtx1a*	*vtx1a*	*vtx1a*	*vtx1a*	*vtx1a*	*vtx1a*	*vtx1a*	*vtx1a*	*vtx1a*	*vtx1a*
OO15	*vtx1a*	*vtx1a*	*vtx1a*	*vtx1a*	*vtx1a*	*vtx1a*	*vtx1a*	*vtx1a*	*vtx1a*	*vtx1a*	*vtx1a*	*vtx1a*
PP16	*vtx2a*	*vtx2a*	*vtx2a*	*vtx2a*	*vtx2a*	*vtx2a*	*vtx2a*	*vtx2a*	*vtx2a*	*vtx2a*	*vtx2a*	*vtx2a*
QQ17	*vtx2a+ vtx2c*	*vtx2a+ vtx2c*	*vtx2a+ vtx2c*	*vtx2a+ vtx2c*	*vtx2a+ vtx2c*	*vtx2d*	*vtx2a+ vtx2c*	*vtx2a+ vtx2c*	*vtx2a+ vtx2c*	*vtx2a+ vtx2c*	*vtx2a+ vtx2c*	*vtx2a+ vtx2c*
RR18	*vtx2b*	*vtx2b*	*vtx2b*	*vtx2b*	*vtx2b*	*vtx2b*	*vtx2b*	*vtx2b*	*vtx2b*	*vtx2b*	*vtx2b*	*vtx2b*
SS19	*vtx2d*	*vtx2d*	*vtx2d*	*vtx2d*	*vtx2d*	*vtx2c + vtx2d*	*vtx2d*	*vtx2d*	*vtx2d*	*vtx2d*	*vtx2d*	*vtx2d*
TT20	*vtx2f*	*vtx2f*	*vtx2f*	*vtx2f*	*vtx2f*	*vtx2f*	*vtx2f*	*vtx2f*	*vtx2f*	*vtx2f*	*vtx2f*	*vtx2f*

Strain/labortory	Original	129	130	131	133	134	136	137	138	139	153	222
KK11	-	-	-	-	-	-	-	-	-	-	-	-
LL12	*vtx1a*	*vtx1a*	*vtx1a*	*vtx1a*	*vtx1a + vtx1c*	*vtx1a*	*vtx1a*	*vtx1a*	*vtx1a*	*vtx1a*	*vtx1a*	*vtx1a*
MM13	*vtx1a*	*vtx1a*	*vtx1a*	*vtx1a*	*vtx1a + vtx1c*	*vtx1a*	*vtx1a*	*vtx1a*	*vtx1a*	*vtx1a*	*vtx1a*	*vtx1a*
NN14	*vtx1a*	*vtx1a*	*vtx1a*	*vtx1a*	*vtx1a*	*vtx1a*	*vtx1a*	*vtx1a*	*vtx1a*	*vtx1a*	*vtx1a*	*vtx1a*
OO15	*vtx1a*	*vtx1a*	*vtx1a*	*vtx1a*	*vtx1a + vtx1c*	*vtx1a*	*vtx1a*	*vtx1a*	*vtx1a*	*vtx1a*		*vtx1a*
PP16	*vtx2a*	*vtx2a*	*vtx2a + vtx2b*	*vtx2a*		*vtx2a*	*vtx2a*	*vtx2a*	*vtx2a*	*vtx2a*	*vtx2a*	*vtx2a*
QQ17	*vtx2a+ vtx2c*	*vtx2a*	*vtx2a+ vtx2c*	*vtx2a+ vtx2c*	*vtx2a+ vtx2c*	*vtx2a+ vtx2c*	*vtx2a+ vtx2c*	*vtx2a+ vtx2c*	*vtx2c + vtx2d*	*vtx2a+ vtx2c*	*vtx2a+ vtx2c*	*vtx2a+ vtx2c*
RR18	*vtx2b*	*vtx2b*	*vtx2b*	*vtx2b*	*vtx2b*	*vtx2b*	*vtx2b*	*vtx2b + vtx2g*	*vtx2b*	*vtx2b*	*vtx2b*	*vtx2b*
SS19	*vtx2d*	*vtx2d*	*vtx2c + vtx2d*	*vtx2d*	*vtx2c + vtx2d*	*vtx2c + vtx2d*	*vtx2d*	*vtx2c + vtx2d*	*vtx2a + vtx2c*	*vtx2d*	*vtx2d*	*vtx2d*
TT20	*vtx2f*	*vtx2f*	*vtx2d + vtx2f*	*vtx2f*	*vtx2f*	*vtx2f*	*vtx2f*	*vtx2f*		*vtx2f*	*vtx2f*	*vtx2f*

Incorrect result

Stains included from the EOA-4

Annex 21. Virulence genes *aggR* and *aaiC*

aggR

Strain/labortory	Original	19	34	80	88	90	94	100	108	114	123	124	129	130	131	133	134	136	137	153	222
KK11	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
LL12	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
MM13	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
NN14	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
OO15	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
PP16	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
QQ17	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
RR18	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
SS19	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
TT20	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

+= Positive, -= Negative

Incorrect result

aaiC

Strain/labortory	Original	19	34	80	90	94	100	114	123	124	127	130	131	133	134	136	222
KK11	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
LL12	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
MM13	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
NN14	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
OO15	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
PP16	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
QQ17	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
RR18	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
SS19	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
TT20	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

+= Positive, -= Negative

Incorrect result

Annex 22. Reference strains of *vtx* subtypes

SSI collection D number	Strain	Control for toxin subtype	Toxin variant designation	GenBank accession no.	Results	Serotype	Additional virulence genes
D2653	EDL933	VT1a	VT1a-O157-EDL933	M19473	*vtx1a + vtx2a*	O157:H7	*eae, ehxA, astA*
D3602	DG131/3	VT1c	VT1c-O174-DG131-3	Z36901	*vtx1c + vtx2b*	O174:H8	
D3522	MHI813	VT1d	VT1d-O8-MHI813	AY170851	*vtx1d*	O8:K85ab:Hrough	*eae*
D3428	EH250	VT2b	VT2b-O118-EH250	AF043627	*vtx2b*	O118:H12	*astA*
D3648	S1191	VT2e	VT2e-O139-S1191	M21534	*vtx2e*	O139:K12:H1	
D3546	T4/97	VT2f	VT2f-O128-T4-97	AJ010730	*vtx2f*	O128ac:[H2]	*eae, bfpA, astA*
D3509	7v	VT2g	VT2g-O2-7v	AY286000	*vtx2g*	O2:H25	*ehxA, astA, estAp*
D3431	F35790	VT2c	VT2c-O157-310/ VT2c-O157-Y350-1	ND	*vtx2c*	O157:H7	*eae, ehxA, astA*
D4134	1112R15035	VT2d	ND	ND	*vtx2d*	O166:H15	

Replacement strains added to the EQA-5 shipment of vtx *subtypes*

D2435[a]	94C	VT2a	VT2a-O48-94C	Z37725	*vtx1a + vtx2a*	O48:H21	*ehxA, saa*
D2587[b]	031	VT2c	VT2c-O174-031	L11079	*vtx2b + vtx2c*	O174:H21	
D3435[c]	C165-02	VT2d	VT2d-O73-C165-02	DQ059012	*vtx2d*	O73:H18	*astA*

Removed from the EQA-5 shipment for the following reasons:

[a] *Strain was isolated from a patient with HUS and could therefore not be distributed under specification UN 3373*

[b] *Was replaced by D3431 encoding VT2c in order to minimise possible cross-contamination of genes vtx2b and vtx2c*

[c] *Strain was isolated from a patient with bloody diarrhoea and could therefore not be distributed under specification UN 3373. May result in both fragments at 179 bp and 280 bp*

ND: Not done

Annex 23. Guide to BN 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, use the instruction on setting up a database from scratch (B).

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 BN and change the home directory to where you placed your database.

Setting up a database from scratch

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

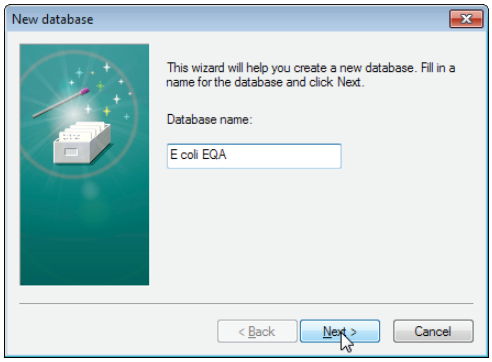
The screen shots are from version 6 of BioNumerics so things probably look slightly different in your version.

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

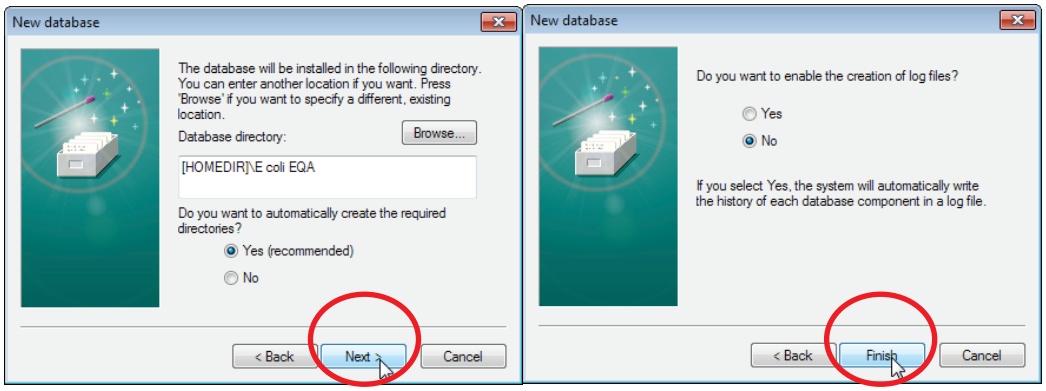
Setting up the empty database

1. Choose 'Create a new database'.

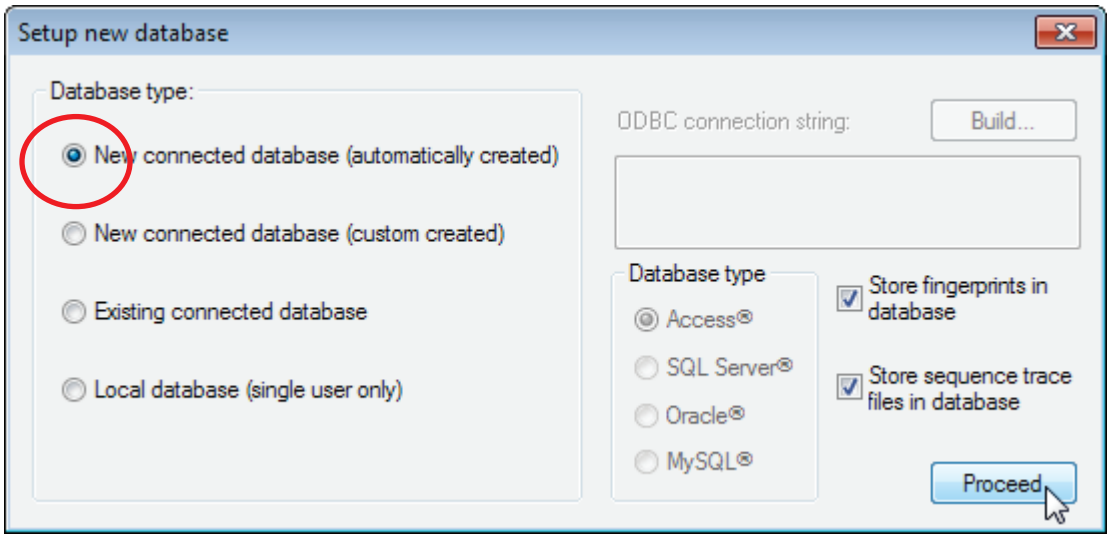
2. Enter a database name. Remember to Enter a database name, "Salmonella "Listeria " or "E coli EQA"



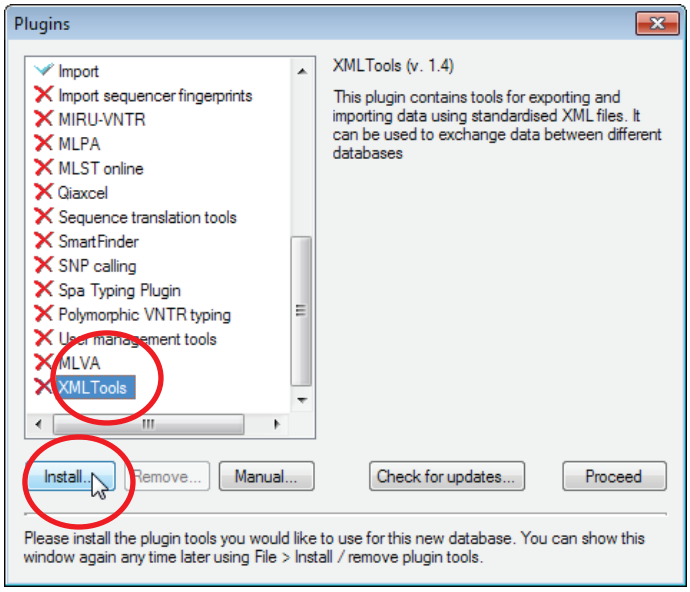
3. Use default values.



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



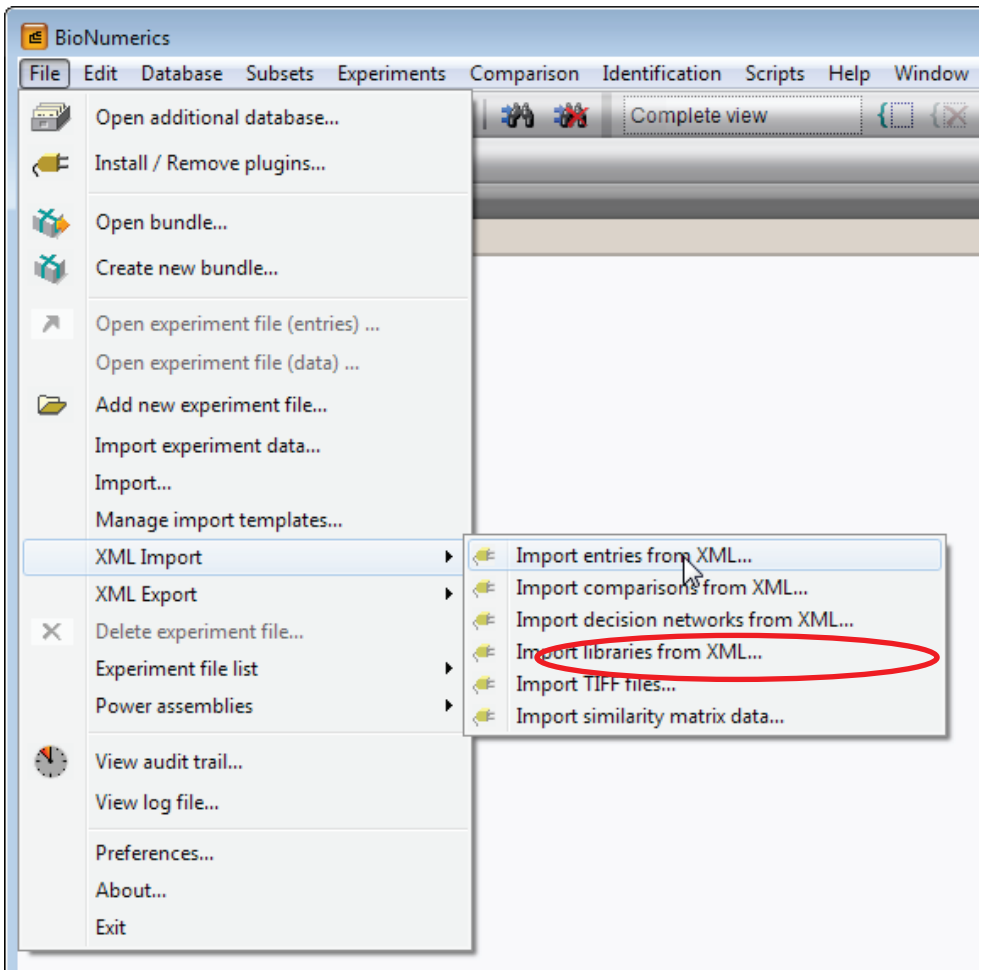
5. When choosing plugins, add the XML Tools plugin by selecting the plugin from the list and pressing 'Install…'



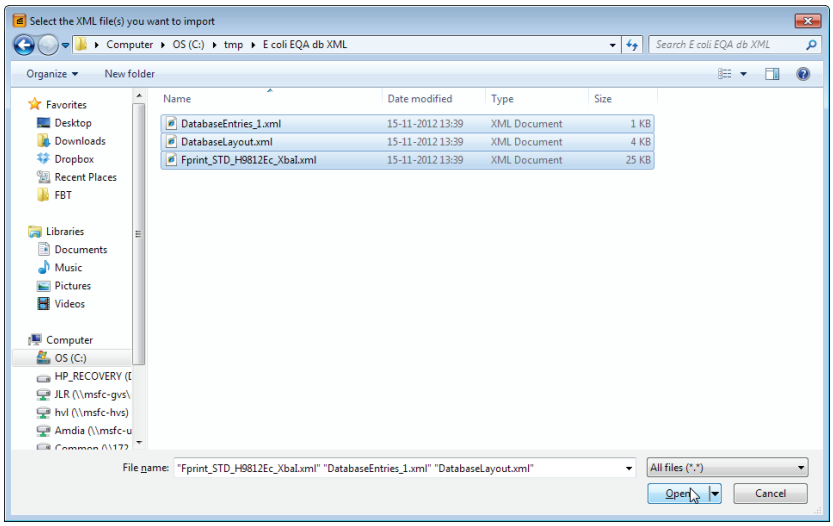
6. Proceed to the next window. The database is now set up and ready to import the database definitions.

Importing the XML structure

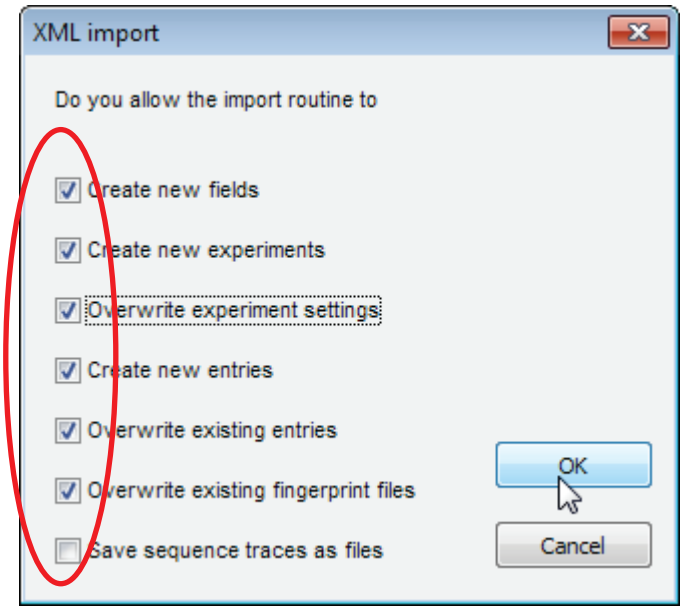
7. Unzip the contents of the supplied file 'Listeria EQA db XML.zip' or 'Salmonella EQA db XML.zip'. into the folder where you would like to place the files.
8. Select 'Import entries from XML' in the menu.



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.

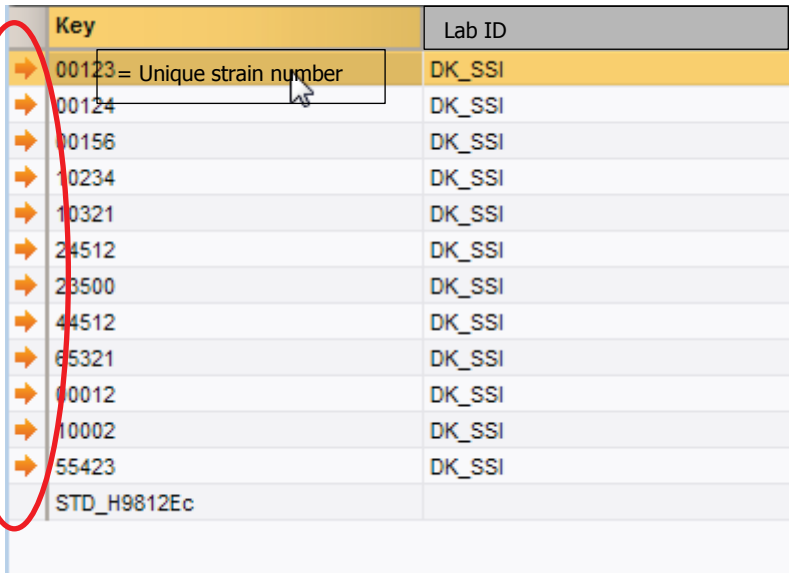
Annex 24. Guide to XML export

After analyzing you data you export all your results in XML format. The procedure looks slightly different in BioNumerics version 6 (A) and 7 (B). If you have an older software version the instruction for version 6 is quite similar.

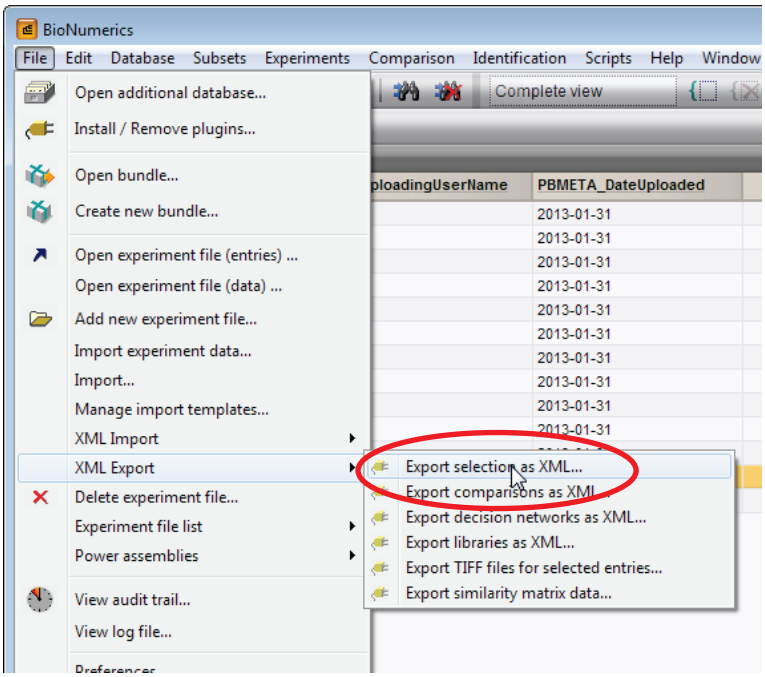
A. Exporting XML data from your database BN version 6

In BioNumerics version 6 and earlier you need to export tiff files separately from the analysed data. Follow all steps of this guide.

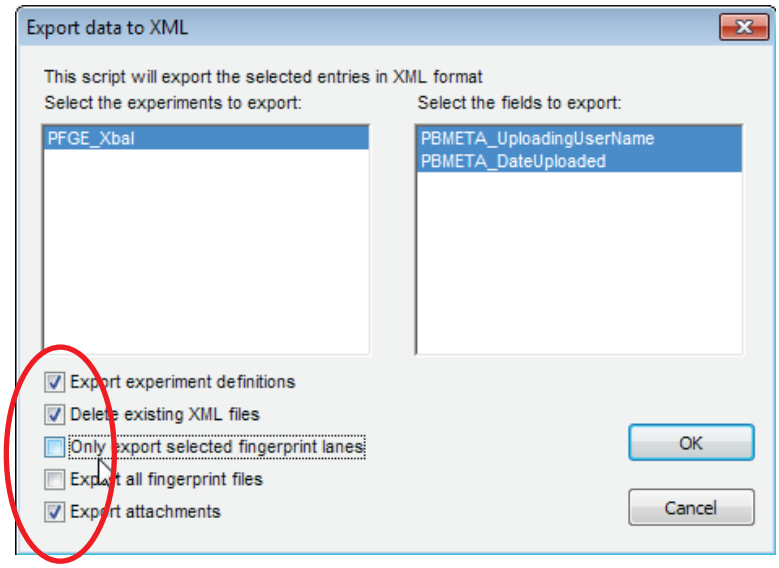
1. After analysing your data, select all the isolates that you would like to export



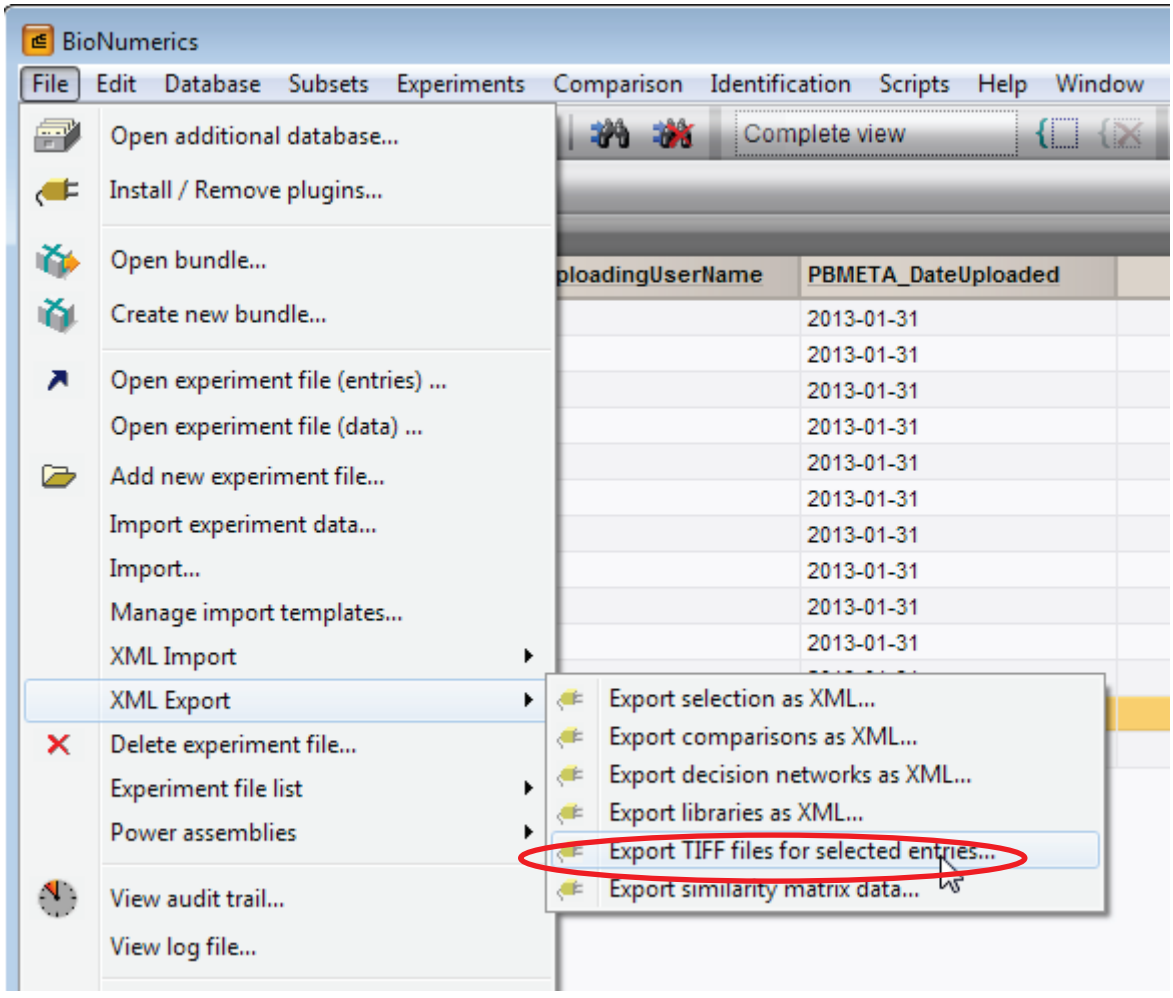
2. Export selection as XML.



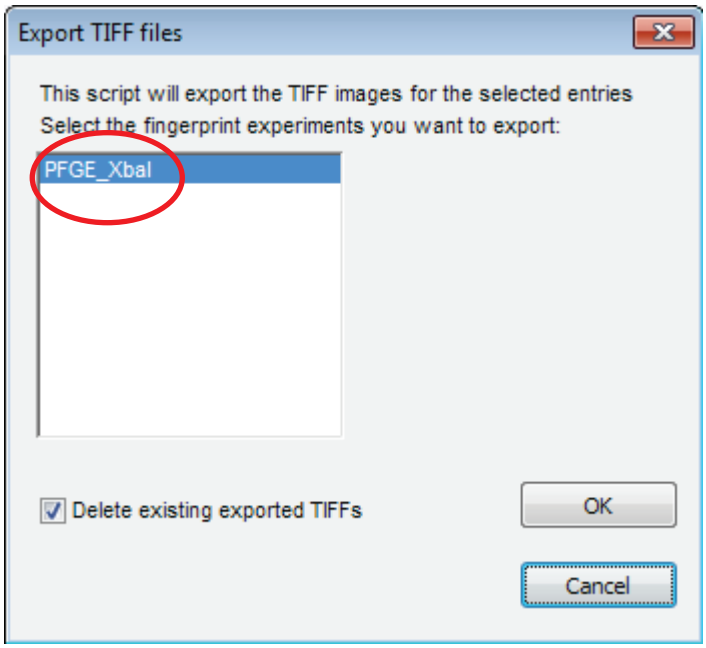
3. De-select the check box 'Only export selected fingerprint lanes'.



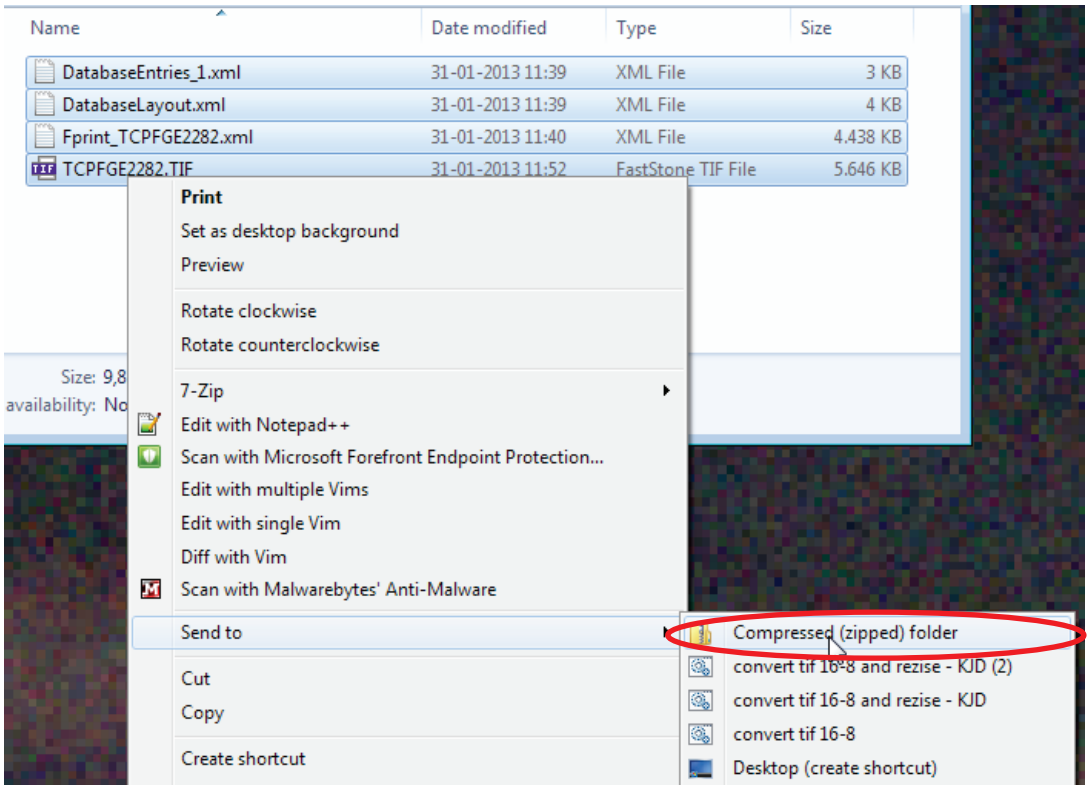
4. Now export the TIFF file(s).



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



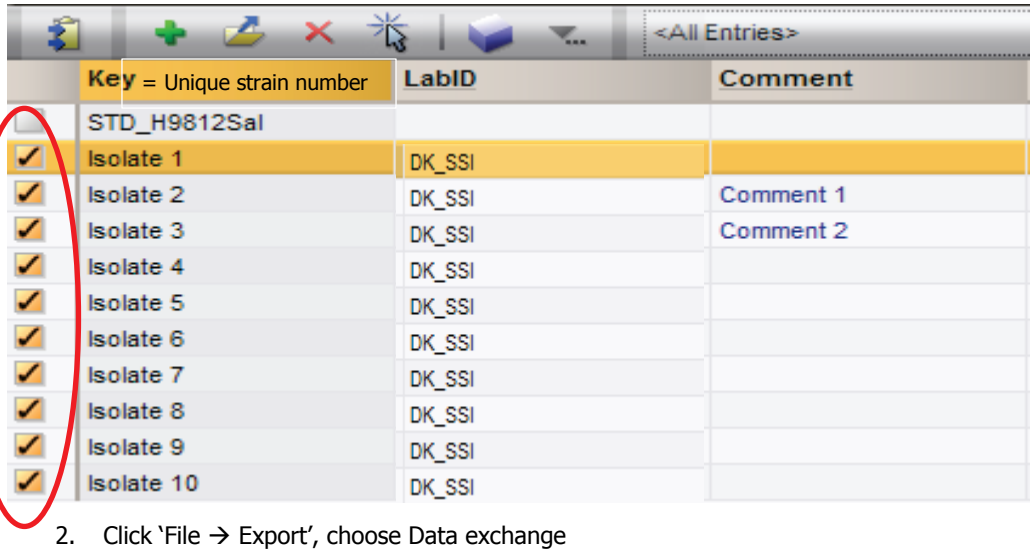
6. Now locate the EXPORT directory in your database directory. Remember to check that the TIFF file is included
7. Send all XML and TIFF files located there via mail.
8. Please compress them into a zip archive. One way of creating the zip archive is to mark all the XML and TIFF files, right click on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



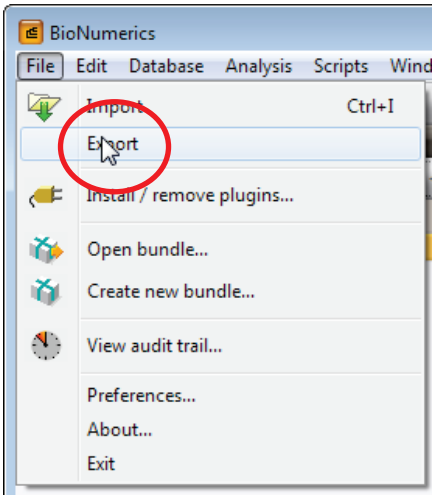
B. Exporting XML data from your database BN version 7

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

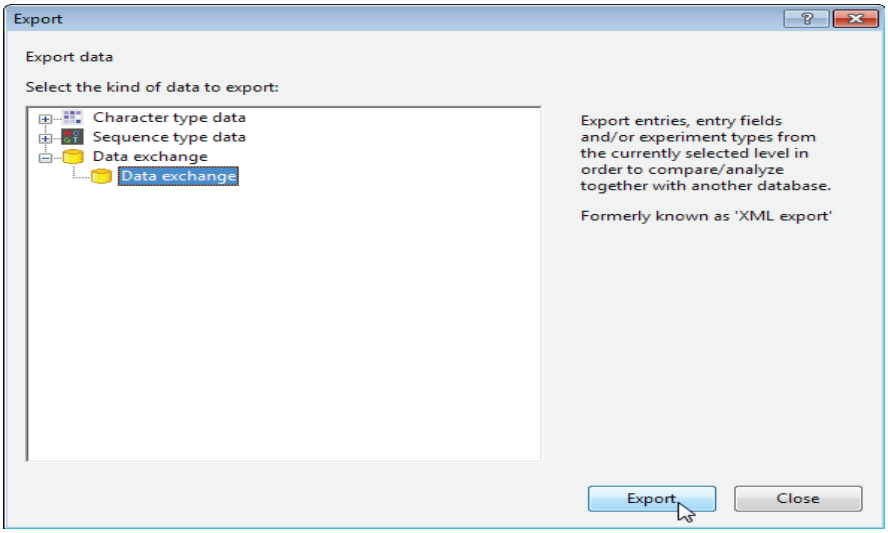
1. Select all isolates that you would like to export



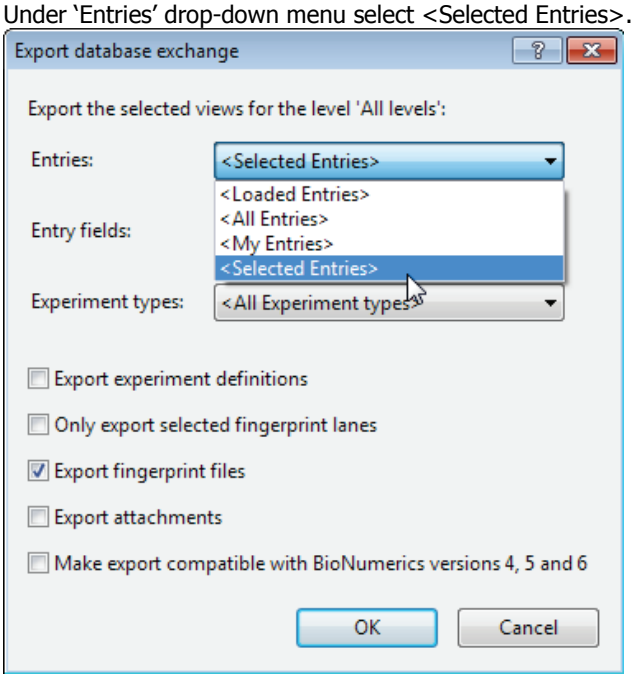
2. Click 'File → Export', choose Data exchange



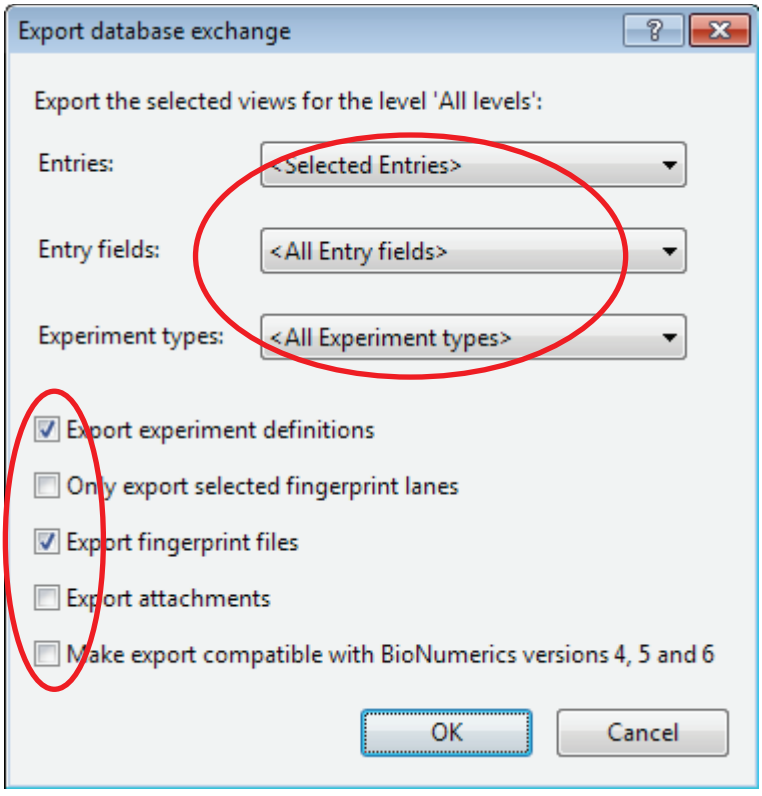
3. and click 'Export'



4. Under 'Entries' drop-down menu select <Selected Entries>.



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

Annex 25. Online submission

Online submission form available from:

Form 1: Submission of results in the EQA-5 VTEC 2013-2014 PFGE, Serotyping, Phenotyping

https://docs.google.com/forms/d/1mlsMToDg3t1BEZ3c7lzHMC75N46Xi3CPDVpahQjHRaI/viewform

Form 2: Submission of results in the EQA-5 VTEC 2013-2014 Genotyping and Subtyping

https://docs.google.com/forms/d/1uy0ABS5gUtB_06vPqQ187lHNiARKdMSupZcgEKijaM8/viewform