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TECHNICAL REPORT

Sixth external quality assessment scheme for typing of verocytotoxin-producing *Escherichia coli*

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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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Abbreviations

AEEC	Attaching and effacing *E. 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*
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 (*estA*$_p$; porcine variant: STp. *estA*$_h$; human variant: STh) (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 uraemic syndrome
ipaH	WR390. Invasion plasmid antigen. This gene is found in several copies chromosomally as well as on plasmids
NSF	Non-sorbitol fermenting
PCR	Polymerase chain reaction
PFGE	Pulsed-Field Gel Electrophoresis
RT PCR	Real Time PCR
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
WGS	Whole genome sequencing

Executive summary

- Twenty-nine public health national reference laboratories from 30 EU/EEA countries signed up for the sixth international external quality assessment (EQA) scheme on typing of VTEC organised by ECDC.
- Seventy-six percent (22 out of 29) of the laboratories participated in the pulsed-field gel electrophoresis (PFGE) part of the EQA, and 73% of the participants were able to produce a PFGE gel of sufficiently high quality to allow for the profiles to be comparable to profiles obtained by other laboratories. The subsequent normalisation and interpretation of the profiles were performed using the specialised software suite BioNumerics (BN). Fifteen laboratories (68%, 15 out of 22) completed the gel analysis and 65% performed in a fair to good accordance with the guidelines.
- Seventeen (59%) laboratories participated in full O:H serotyping for all ten strains, and 78% of the participating laboratories were able to correctly determine the full O:H serotype of all ten strains.
- Correct typing of virulence genes was done by 97% the laboratories for *eae*, 98-99% for *ehxA*, *vtx1* and *vtx2*.
- Subtyping of *vtx1 and vtx2* was performed correctly by 91% of the laboratories on average.
- Correct phenotypic characterisation was done by 100% of the laboratories for VT production through VCA, 99% for ESBL production, 89% haemolysin production, 96% for Beta-glucuronidase production and 98% for sorbitol fermentation.

This report (EQA-6) presents the results of the sixth round of the external quality assessment scheme for typing of verocytotoxin-producing *E. coli* (VTEC) funded by ECDC. The EQA-6 was carried out from January to April 2015 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 BetaBeta-lactamase (ESBL).

A total of 29 laboratories participated in at least one part of the EQA-6. Twenty-two laboratories (76%) reported PFGE results, 17 laboratories (59%) participated in full O:H serotyping of all strains (26 laboratories submitted O group results for at least one strain and 17 laboratories submitted H-types for at least one strain). Genotypic detection of *eae*, *vtx1* and *vtx2* was performed by 24–26 laboratories (an average of 83–90%), 19 (66%) for *ehxA*, and 22 (76%) participated in subtyping of *vtx* genes. Sixteen laboratories who participated in phenotypic detection; 7 (25%) for VCA (Vero cell assay), 24 (83%) for fermentation of sorbitol, 15 (55%) for Beta-glucuronidase, 15 (52%) for enterohaemolysin and 16 (55%) for ESBL.

Twenty-two laboratories participated in the PFGE part of the EQA-6, and 16 (73%) 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 BN. Fifteen laboratories completed the gel analysis, and 87% performed fair to good, in accordance with the guidelines.

Of the 17 participants, an average of 78% (range 59–100%) could correctly determine the O:H serotype of the strains (some laboratories only typed a selection of the ten test strains). The more common serotypes obtained better typing results: O157:H7 serotype was typed correctly (100%) by all 17 participants, while both O41:H26 and O174:H21 were typed correctly by 10 laboratories (59%).

The results for the genotypic detection of virulence genes were generally very good: *eae* (97%), *vtx1* (99%), *vtx2* (98%) and *ehxA* (98%). False positive results were reported once for *vtx1* and *vtx2* and false negative results were submitted four times for *vtx2*. For *vtx* subtyping, eight false negative results were received for *vtx2*; six laboratories failed to detect the *vtx2d* gene. The virulence genes *aggR* and *aaiC* were reported by 19 and 16 participants, respectively. The one *aaiC* positive test strain was correctly determined by two participants while 14 participants failed to detect the gene. Whole genome sequencing (WGS) of this strain revealed a new variant of *aaiC*, not previously described. The primers and probe for either conventional PCR or Real Time PCR (RT PCR) used by the 14 participants with negative *aaiC* results are presumed to be unable to anneal to the new *aaiC* gene variant. One of the test strains harboured the *aggR* gene which was correctly reported by all laboratories.

The percentage for correct results for phenotypic characterisation was 100% for VCA, 99% for ESBL production, 89% haemolysin production, 96% for Beta-glucuronidase production and 98% for sorbitol fermentation.

This EQA-6 scheme is the third EQA specifically organised for laboratories participating in the European Food- and Waterborne Diseases and Zoonoses network (FWD-Net) that includes molecular typing methods. The number of participating laboratories in the EQA-6 is reassuring. The molecular surveillance system relies on the capability of the FWD-Net laboratories to produce comparable typing results and follow the ECDC guidance for VTEC detection. Presently, 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. PFGE results of the EQA-6 showed that 36% of the laboratories need to improve their performance in order to produce useful typing profiles for an inter-laboratory exchange. Compared with EQA-5, this is only a small decrease. Nevertheless, the majority of laboratories with identified technical issues have, within their reach, the possibility to achieve an acceptable quality profile if they optimise their 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 epidemiological surveillance of communicable diseases as in the Decision No 1082/2013/EU [2], and to ensure the reliability and comparability of results in laboratories from all EU/EEA countries.

The main objectives of the EQA 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 and
- identification of needs for training activities

In 2012, a framework service contract on 'Microbiological characterisation services to support surveillance of *Salmonella*, Shiga toxin/verocytotoxin-producing *Escherichia coli* (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 in Denmark won the three lots covering *Salmonella*, 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 third VTEC EQA-exercise of this contract (*E. coli* EQA-6).

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 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. Notably, 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 uraemic syndrome (HUS) which is defined clinically by the triad of haemolytic anaemia, thrombocytopenia, and acute renal failure.

In 2013, the overall EU notification rate of VTEC was 1.59 cases per 100 000 population. The total number of confirmed VTEC cases in the EU was 6 043, which represents a decrease of 36% compared with 2011 (N = 9 485) with the large O104:H4 outbreak. However, the rate in 2013 was 65% higher than the 2010 one (N=3 656) and 6% higher than that in 2012 (N=5 680) [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. 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
- to facilitate the detection and investigation of transmission chains and relatedness of strains across Member States and contribution to global investigations
- to detect emergence of new evolving pathogenic strains
- to support investigations to trace-back the source of an outbreak and to identify new risk factors
- to aid in studying the characteristics of a particular pathogen and its behaviour in a community of hosts.

The molecular typing surveillance gives Member State users access to EU-wide molecular typing data for the included pathogens. It also gives the opportunity to perform cluster searches and analyses of EU-level data in order to determine whether isolates characterised by molecular typing at the nation al 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 *Salmonella*, STEC/VTEC and *L. monocytogenes*. ECDC has also supported EQA activities for virulence gene detection and serotyping of the selected pathogens. The EQA-6 scheme was targeted at 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 non-VTEC *E. coli* (AEEC) 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 a 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 are inadequate and may result in misleading conclusions. For example, the typing of *vtx2* have been based on the absence of the PstI site as an indicator of the presence of the mucus-activatable *vtx2d* subtype [8-11]. However, the PstI site is also absent in six variants of *vtx2a*, in two variants of *vtx2c*, in *vtx2f* and in all four variants of subtype *vtx2g* [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 often characterised by failure to produce Beta-glucuronidase. Furthermore, approximately 75% of all VTEC strains produce enterohaemolysin, a toxin that can cause lysis of erythrocytes. Enterohaemolysin can either be detected phenotypically on sheep blood agar plates, or by detection of the *ehxA* gene.

VTEC EQA-6 included O:H serotyping, detection and genotyping of virulence genes (*eae*, *vtx*1, *vtx*2 and *ehx*A), 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, fermentation of sorbitol, and the production of Beta-glucuronidase, enterohaemolysin and ESBL.

1.4 Objective of the EQA-6 scheme

1.4.1 Pulsed-field gel electrophoresis typing

The objective of EQA-6 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 flagellar '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* andvtx2 genes
- detection of other virulence genes (*aggR* and *aaiC* were expected by public health national reference laboratories).

1.4.4 Phenotypic tests

Phenotypic assay was used for the detection of production of verocytotoxin (VT), fermentation of sorbitol, enterohaemolysin, Beta-glucuronidase, and ESBL (Extended Spectrum Beta Lactamase) production.

2. Study design

2.1 Organisation

The VTEC EQA-6 was funded by ECDC and arranged by SSI to be conducted from January 2015 through May 2015. The EQA scheme included PFGE, O:H serotyping, virulence determination by genotypic methods (detection and typing of virulence genes *eae*, *vtx1*, *vtx2*, *ehxA*, *aggR*, *aaiC* and subtyping of *vtx1* and *vtx2* using PCR) and phenotypic testing (detection of VT production, fermentation of sorbitol, production of Beta-glucuronidase, enterohaemolysin and ESBL). A recently published protocol for conventional gel-based PCR [13] was tested for subtyping of the *vtx* subtype genes.

The EQA-6 was conducted according to ISO/IEC 17043:2010, entitled 'Conformity assessment – General requirements for proficiency testing' (first edition, February 1st 2010) [14].

Invitations were e-mailed to ECDC contact points in the FWD-Net (30 countries) on 4 of November 2014. In addition, the ECDC coordinator sent invitations to the EU candidate countries Montenegro, Serbia, the Former Yugoslav Republic of Macedonia and Turkey.

Thirty public health national reference laboratories in EU/EEA and EU candidate countries accepted the invitation and are listed in Annex 1. Unfortunately, one participant had to be excluded as they were unable to obtain an import permit.

The EQA-6 test strains were sent to the participating laboratories in February 2015.

The participants were asked to submit their PFGE results by e-mail and report the rest of the results through an online form (by the 13 of April 2015). In addition, 23 laboratories from the international WHO Global Foodborne Infections Network (GFN) were invited to participate.

2.2 Selection of strains

The strains for EQA-6, were selected based on their 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 O26:H11, O41:H26, O63:H6, O104:H7, O111:H-/H8, O121:H19, O157:H7, O157:H7, O166:H15 and O174:21
Virulence gene determination	10*	*eae*, *vtx1a*, *vtx1c*, *vtx1d*, *vtx2a, vtx2b, vtx2c, vtx2d,vtx2f, ehxA*, *aggR* and *aaiC*
Phenotypic testing	10*	VCA, sorbitol, Beta-glucuronidase, enterohaemolysin and ESBL

**Same 10 strains*

Detailed information about the strains is shown in Annex 6. In addition to the 20 test strains, laboratories participating in EQA-6 for PFGE could request the *Salmonella* Braenderup H9812 reference strain and reference strains for the *vtx* subtyping (Annex 20).

2.3 Carriage of strains

All strains were numbered AA1-TT20, packed and shipped (shipping began on the 5th of February 2015). Almost all of the participants received their dispatched strains within 1–5 days. One parcel was delayed by customs and delivered after eleven days. Another participant was unable to obtain an import permit in order to get material through customs. In agreement with local regulations, the strains were therefore returned to SSI. As such, and because of time constraint to reach the deadline it was decided that the participant should not engage in EQA-6

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 26 February 2015, instructions on how to submit results were e-mailed to participants. Instructions included a link to a Google Docs submission form, zipped files for the BN database experiment settings (PFGE part), and guidelines on how to export XML files from BN (Annex 21 and 22).

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) [13].

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-6, 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, vtx1c* and *vtx1d)* and *vtx2* (*vtx2a, vtx2b, vyx2c, vtx2d and vtx2f*) were assessed (suggested protocol [16]).

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

Characteristics related to the enteroaggregative VT2-producing *E. coli* O104:H4 (EAEC-VTEC), e.g. the chromosomally encoded protein gene (*aaiC)* and enteroaggregative adherence transcription regulator gene (*aggR*) were included.

2.5 Data analysis

Once the results from the laboratories were received, the PFGE results were added to a dedicated *E.coli* EQA-6 BN database at SSI. In the case of PFGE gel quality, the gel was evaluated according to a modified version of the ECDC Food and Waterborne Disease MolSurv Pilot (SOPs 1.0 - Annex 5) PulseNet US protocol PFGE Image Quality Assessment (TIFF Quality Grading Guidelines 2014 - Annex 3), by scoring the gel according to seven parameters (scores in the range 1–4, 4 being the top score). The score of 1 - 'Poor' – is a category which clearly shows that the gel is not usable for inter-laboratory comparison. The BN analysis was evaluated according to BioNumerics Gel Analysis Quality Guidelines 2015 (Annex 4). The BN analysis was graded with respect to five parameters (scores in the range 1–3, 3 being the top score). After the results from all laboratories were submitted online, 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 either, the full scheme or in 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, Beta-glucuronidase, enterohaemolysin, ESBL). Twenty-nine laboratories submitted results – however not all submitted the results that they had originally planned. Twenty-two laboratories (75%) participated in the PFGE part, and 15 (68%) also participated in the BioNumerics analysis. Seventeen (59%) participated in the full O:H serotyping of all 10 strains. Of the seventeen laboratories, three submitted O:H data for only a limited number of the EQA strains. The reasons for omitting some strains were often not stated by the participants. Seventeen laboratories submitted correct O:H serotype data for strain RR18 (O157:H7). In addition to the FWD-Net participants, 23 laboratories from the international WHO GFNparticipated (results not included in this report).

In general, the participation rate in O group/H type depends on the abilities of the laboratories and the antisera available. 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 particular method varies for each strain, however all ND or NT were calculated as an error. (See Annex (7-19) for detailed submission data).

There was a higher participation for O typing, 26 laboratories, compared with the seventeen laboratories which participated in the H typing (Table 3).

In the genotyping part (virulence gene detection and subtyping), 28 laboratories (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, 16/19 laboratories reported results for EAEC (*aaiC* (55%) and *aggR* (66%). 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	22	15	17	26	26
% of participants	76	68*	59	90	90

† Twenty-nine laboratories participated in at least one method
1 Participation in O grouping or H typing
2 Participation in one or more of the virulence gene determination parts (eae, vtx1, vtx2 or ehxA)
3Participation in one or more of the phenotypic test parts (VCA, sorbitol, enterohaemolysin, Beta-glucuronidase or ESBL)
**out of the 22 participants in the TIFF*

Table 3. Detailed participation table

Participants/ method	O:H serotype group	O group	H type	Vero cell assay	ESBL	Haemolysin production	Beta-glucuronidase production	Sorbitol fermentation	*eae* gene	*ehxA* gene	*vtx1* gene	*vtx2* gene	*vtx* subtypes	*aggR*	*aaiC*
nmax	17	26	17	7	16	15	16	24	24	19	26	26	22	19	16
Average	59%	90%	59%	25%	55%	52%	55%	83%	83%	66%	90%	90%	76%	66%	55%

n_{max}: highest number of participants

Participation in the detection of virulence gene *eae* was 83%, *ehxA* was 66%, detection of *vtx1* and *vtx2* was 90%, and subtyping was 76% on average.

Participation in the phenotypic detection was 25–83% (7–24 laboratories). The lowest participation was for the VCA assay: only seven participants (25%) delivered results for the 10 strains. Participation in the sorbitol fermentation was 24 laboratories (83%). The test for enterohaemolysin production was performed by 15 laboratories (52%). The test for production of Beta-glucuronidase was performed by 16 laboratories (55%). Finally, 16 laboratories (55%) submitted results for the production of ESBLs.

3.2 Pulsed-field gel electrophoresis

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

3.2.1 Gel quality

All laboratories were able to produce profiles that were recognisable as the correct 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). An acceptable quality should be obtained for each parameter since a low quality score in just one category can have a significant impact on the ability to further analyse the image and compare with other profiles. In general, acceptable quality ('Fair' – score of 2) should be achieved for each parameter. A score of 1 ('Poor') 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 four of the parameters, the participants obtained a high average score, 3.5 and above, i.e. between 'Good' and 'Excellent' (Table 4). The four parameters were 'Cell Suspension', 'Lanes', 'Gel background' and 'DNA degradation'. The participants obtained an average score of 3.3 in the two categories 'Image Acquisition and Running conditions', and 'Restriction'. For the last parameter 'Bands', participants had an average score below 3 (2.6), i.e. between 'Fair' and 'Good'.

Table 4. Results of PFGE gel quality for 22 participating laboratories

Parameters	1 – Poor	2 – Fair	3 – Good	4 – Excellent	Average
Image acquisition and running conditions	0%	18%	36%	45%	3.3
Cell suspension	0%	9%	23%	68%	3.6
Bands	23%	23%	23%	32%	2.6
Lanes	0%	14%	5%	82%	3.7
Restriction	5%	27%	5%	64%	3.3
Gel background	0%	14%	9%	77%	3.6
DNA degradation	0%	14%	18%	68%	3.5

The 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 quite diverse scores for the parameter 'Image Acquisition and Running Conditions' (Table 4). All participants were graded 'Fair' (2), 'Good' (3) or 'Excellent' (4) in this parameter and 81% scored 'Good' or 'Excellent'. In the parameter 'Bands', 78% of the laboratories were graded a score of 2 ('Fair') or 3 ('Good') (Table 4). Twenty-three percent of participants obtained the score 1 – 'Poor', in the parameter 'Bands', making analysis of the gel unsatisfactory.

Six (27%) 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 cannot satisfactorily be compared with profiles produced on other gels. All the participant's Gel Quality scores are listed in Annex 5.

Poor band quality is the most common reason for getting a score of poor and thereby a gel that cannot reliably be compared with other gels. Figure 1a shows the full image, and 1b a portion of the gel illustrating the fuzziness. There are several possible causes for fuzzy bands and the gel illustrates three of the problems. The gel uses narrow plugs, something that always reduces the sharpness of the bands compared to wide plugs. There is much less tolerance for errors with narrow plugs. The pixel dimensions of the file is 822x614 px (493k). In itself, this is noth too low, but the lane area of the gel only uses 186k pixels, meaning that only 38% of the actual pixels are used. This is a very low resolution for a gel with the numbers of bands you get in an *E. coli Xba*I PFGE. The final reason for the fuzzy bands is that the image is slightly out of focus, something that would not have been detrimental in itself, but it adds to the fuzziness.

Figure 1. A gel graded 1 in parameter 'Bands', but high scores in the other parameter.

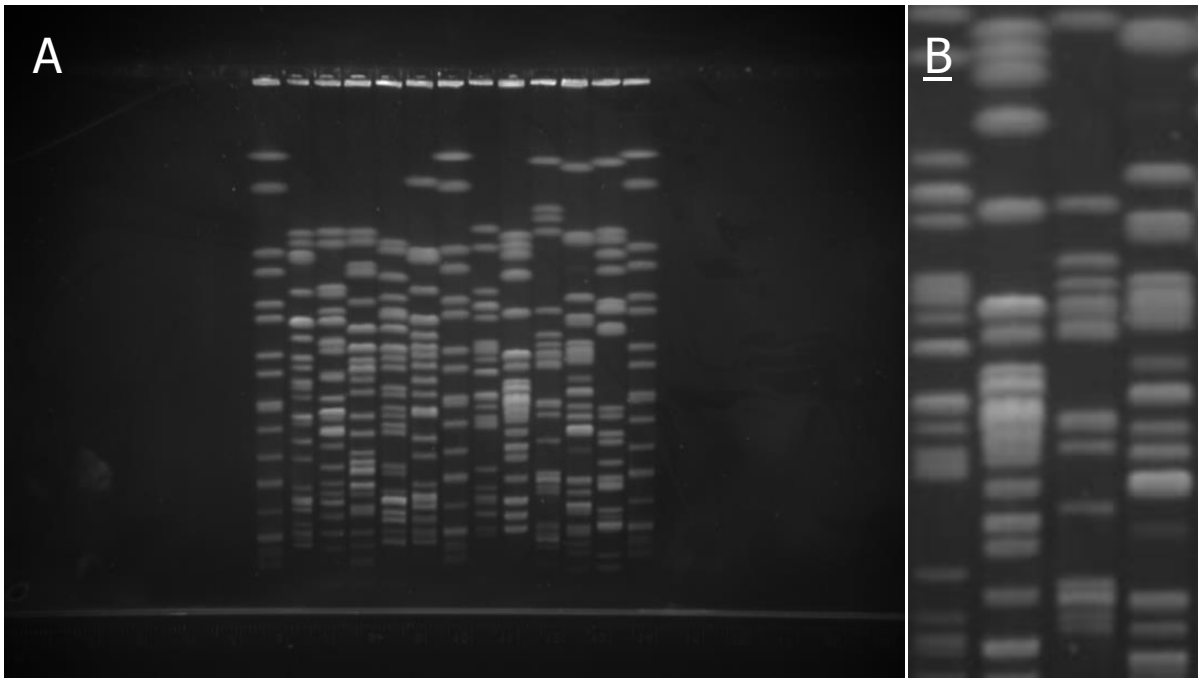
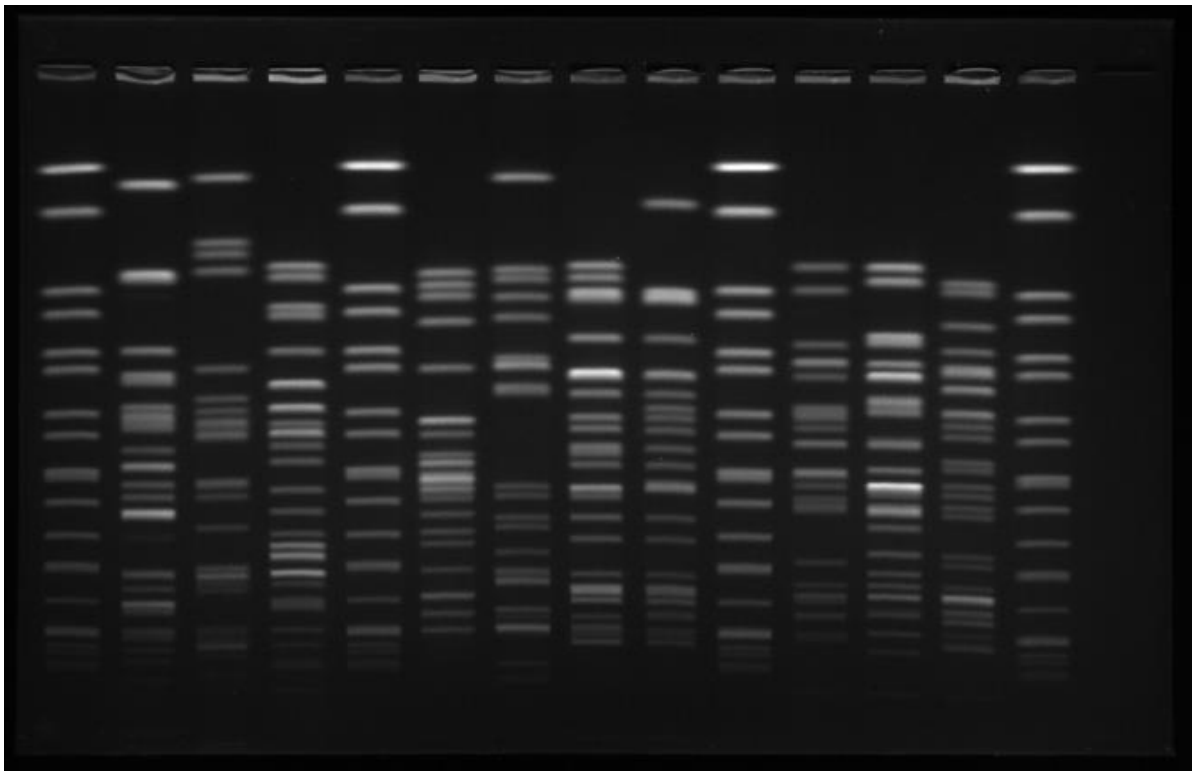


Figure 2 displays a gel which scored 'Excellent' in all seven parameters. The running time is good, with not too much free space at the bottom of the gel. There is even cell suspension between lanes, the bands are clear and distinct all the way to the bottom, lanes are straight, there are no shadow bands and there is no background debris or smearing.

Figure 2. Gel with high scores in all seven parameters



3.2.2 Gel analysis with BioNumerics

Fifteen laboratories analysed their gel in BioNumerics and were able to produce XML files according to the protocol attached to the invitation letter (Annex 21 and 22). The participants' ability to perform gel analysis was graded according to the modified BioNumerics Gel Analysis Quality Guidelines developed at SSI. These guidelines use a five parameter scheme for the grading (Annex 3).

Table 5. Results of the BN analysis for 15 laboratories

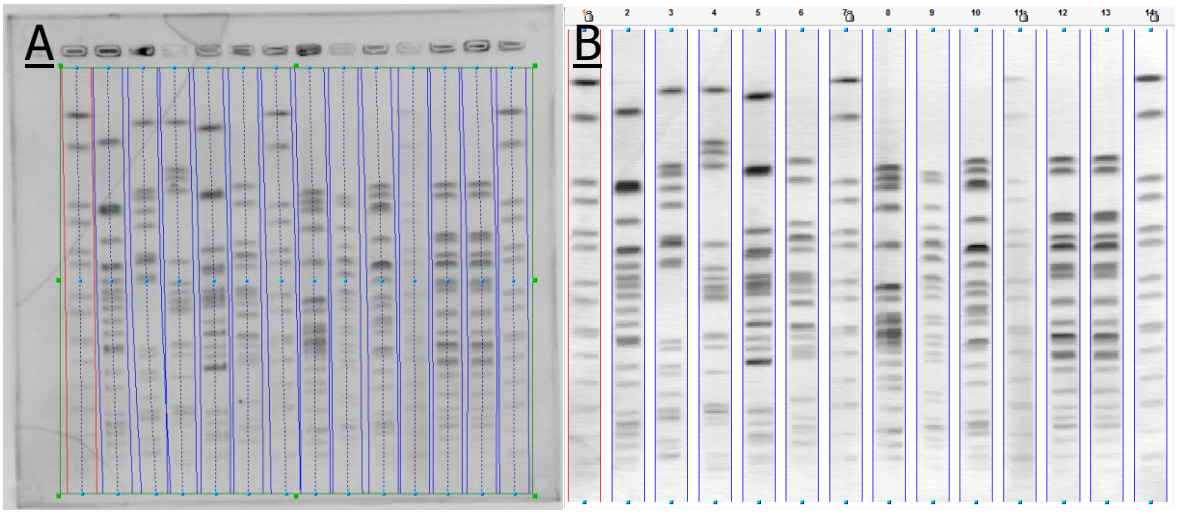
Parameters	1 – Poor	2 – Fair	3 – Excellent	Average
Position of the gel	7%	33%	60%	2.5
Strips	0%	40%	60%	2.6
Curves	0%	40%	60%	2.6
Normalisation	7%	27%	67%	2.6
Band assignment	0%	67%	33%	2.3

The 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, 'Strips', 'Curves' and 'Normalisation', participants obtained a very high average score, of 2.6 (Table 5). In the parameter 'Position of the Gel' the participants were graded slightly lower with an average of 2.5. The average score for 'Band assignment' was 2.3, the lowest of the five parameters, but not a single participant scored 1 ('Poor'). This can be compared with the last EQA when 29% of laboratories (five) were unable to make 'Band assignment' which could be used for inter-laboratory comparison. One participant scored a 1 ('Poor) by including the wells in the analysis and one by failing to include the reference lanes in the submitted analysis.

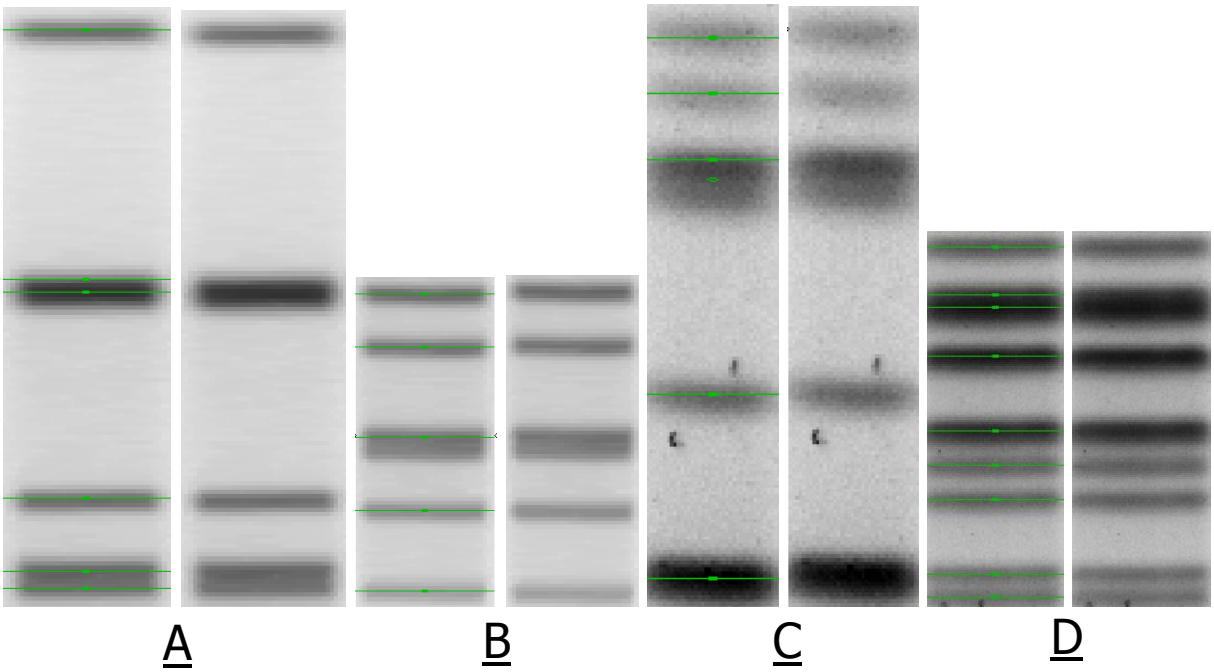
An optimal Band assignment in BN is crucial, and this is dependent on the overall quality of the gel and in particular the score of the parameter 'Bands' from the TIFF quality grading guidelines (Annex 3). Very fuzzy and/ or thick bands make correct Band assignment an impossible task. However, the Strips and curves placement also have some impact on the analysis of the gel and it is an easy part to improve. Figure 3A shows the strips assignment where too much space is included on the sides. The strip definition should not be wider but rather slightly narrower than the actual bands themselves. The strip in lane 11 is also clearly not following the actual lane. Figure 3B show that the curves have been defined to encompass the whole lane, where it should be approximately1/3 or slightly wider. The erroneous strip definition is mostly a visual problem impacting the quality of a comparison, whereas the curves error results in poorer band finding and poorer performance of curve-based cluster algorithms.

Figure 3. A gel with erroneous definitions in the 'Strips' and 'Curves' categories.



The rule is to assign double bands when you can see whitespace between the bands, and otherwise assign them as single bands. The examples are shown in pairs with the bands assigned to the left and the raw gel to the right. In 4a and 4d there are thick bands assigned as doublets even though it is not possible to split them visually. These darker bands do most likely represent two or more bands overlaid on each other, but using the intensity of the bands as a means of assigning doublets is not an approach that can yield consistent results. In 4b there is a clear strip of white space between the bands, but only one band has been assigned. Figure 4c shows a case where a band has been marked as uncertain even though there is some white spaces. The approach with uncertain bands is something that makes analysis in a larger database more complicated and is discouraged.

Figure 4. Band assignment errors



3.3 Serotyping

Eight (31%) out of the 26 laboratories submitting serotyping data could correctly perform O grouping for all 10 test strains. Overall, an average of 78% of the strains were correctly O grouped (Table 6). The lowest (46%) results were seen for serotype O174 (TT20) and the highest for serotype O157 (QQ17 and RR18), which was correctly typed by all laboratories. The highest correct percentage scores were obtained for serotypes O26, O104, O111, and O157; all are included in the minimum requirements of ECDC [17].

H typing was correctly performed for 53% (9/17) all 10 strains. Seventeen participants submitted H typing results, which represents 65% of the number of participants performing O group typing. Results were lowest (65%) for the LL12 (H26) and highest (100%) only for RR18 (H7). The majority of incorrect H-types were due to reporting a strain as not typeable (NT). Correct scores above 75% were obtained for the H types H6, H-/H8, H7, H11, H15 and H21.

Table 6. Average scores for the O:H serotyping

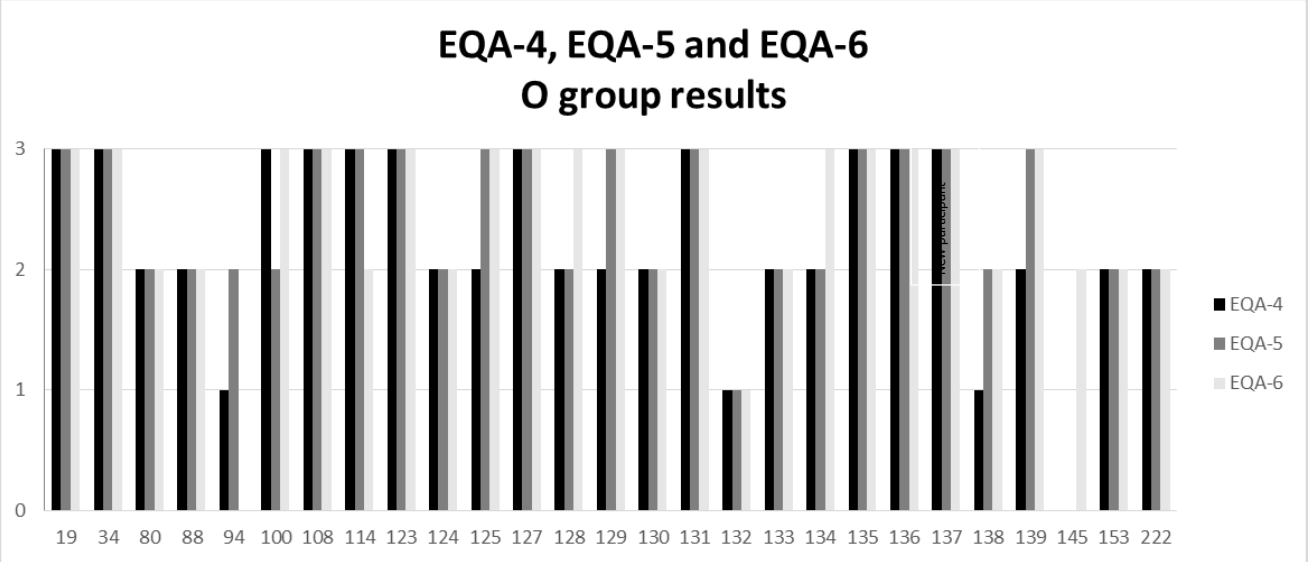
Strain/method		O:H Serotype	O group	Type of incorrect antigens	H type	Type of incorrect antigens
nmax		17	26		17	
KK11	O26:H11	76% (13)	96% (25)	ND (1)	76% (13)	NT/ND (3), H21 (1)
LL12	O41:H26	59% (10)	50% (13)	NT/ND (8), O115 (1), O121 (2), O126 (1), NON-O157 (1)	65% (11)	NT/ND (4), H5 (1), H27 (1)
MM13	O63:H6	71% (12)	58% (15)	NT/ND (8), O125 (1), NON-O157 (1), Rough (1)	76% (13)	NT/ND (4)
NN14	O104:H7	88% (15)	85% (22)	ND (2), O109 (1), O157 (1)	94% (16)	NT (1)
OO15	O111:H-/H8	88% (15)	96% (25)	ND (1)	88% (15)	NT/ND (2)
PP16	O121:H19	76% (13)	88% (23)	NT/ND (3)	76% (13)	NT/ND (3), H4 (1)
QQ17	O157:H7	88% (15)	100% (26)		88% (15)	NT (1), H- (1)
RR18	O157:H7	100% (17)	100% (26)		100% (17)	
SS19	O166:H15	71% (12)	58% (15)	NT/ND (8), O23 (1), O86 (1), NON-O157 (1)	76% (13)	NT/ND (4)
TT20	O174:H21	59% (10)	46% (12)	NT/ND (11), O104 (1), NON-O157 (1), Rough (1)	76% (13)	NT/ND (3), H11 (1)
Average		**78%**	**78%**		**82%**	

nmax = number of participants. Percentages are calculated based on the results submitted by all participants (see Annexes 7 and 8).

An average of 78% (59–100%) of laboratories could correctly identify O:H serotype in the 10 test strains. Eight participants (47%) could identify the correct O:H serotype for all 10 test strains. Correct O:H serotyping ranged from 100% for serotypes O157:H7 to 59% for serotype O174:H21 and O41:H26 (Table 6). It is possibly more difficult for laboratories to correctly serotype a strain if its serotype is less common.

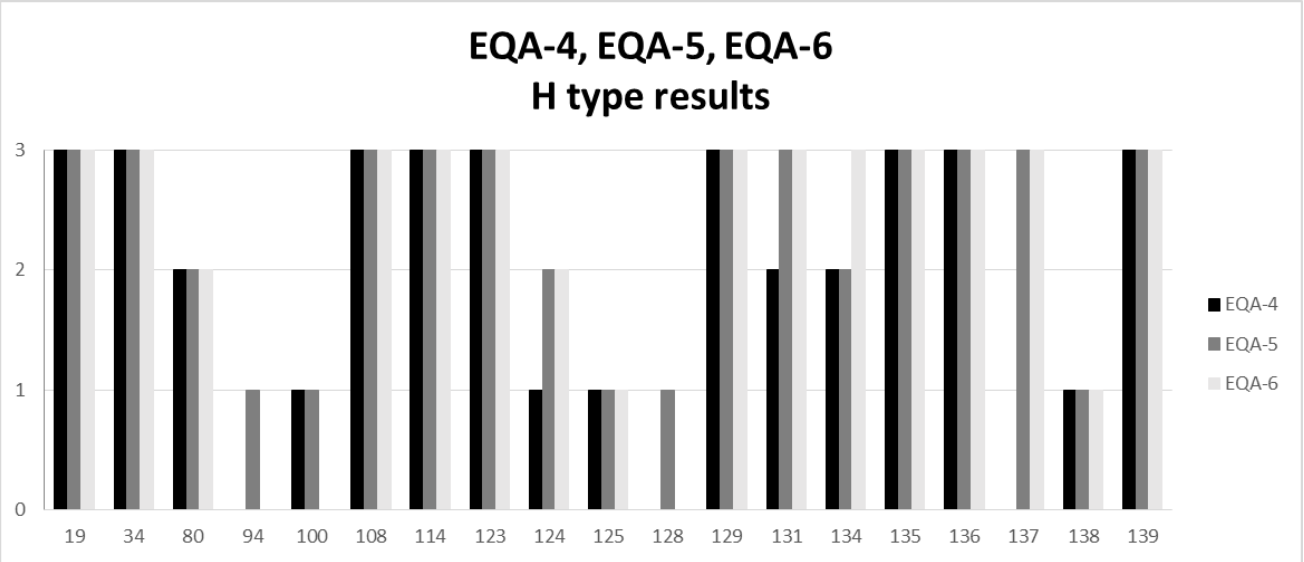
To examine the exact progress of the laboratories' performance, three strains from EQA-4 and EQA-5 were included in EQA-6. Strain GG7 (O111:H-), II9 (O157:H7) and MM13(O166:H15) from EQA-4 are numbered OO15, QQ17 and SS19 respectively in the EQA-6. Figure 6 and Figure 7 show the performance based only on these three isolates.

Figure 5. Comparing EQA-4, EQA-5 and EQA-6 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 OO15, QQ17, and SS19 from EQA-6).

Figure 6. Comparing EQA-4, EQA-5 and EQA-6 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 OO15, QQ17, and SS19 from EQA-6.

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 19–26 laboratories for all the 10 test strains, with high average scores (97–98% correct) (Table 7). With regard to the detection of *eae*, a perfect overall score was obtained for all strains by 22 out of 24 participants. Seven strains (of 10) were incorrectly identified by one laboratory, including both false negative and positive results. Detection of both *vtx*1 and *vtx*2 genes had high average correct scores of 98-99%. Four laboratories missed the presence of *vtx2* in the strain MM13 (O63:H6) that has the *vtx2f* gene, and one participant reported a false positive in strain LL12 (O41:H26). One false positive *vtx1* genes was reported; one in strain KK11 (O26:H11) and one false negative *vtx1* result in strain LL12 (O41:H26). None of the participants reported incorrect results for both *vtx1* and *vtx2,* and in total, *vtx1* and *vtx2* were misidentified seven times: *vtx*1 (one false negative and one false positive), *vtx2* (four false negatives and one false positive).

Table 7. Average scores for virulence determination

Strain/method	*eae* gene	*vtx*1 gene	*vtx*2 gene	*ehx*A gene
N*	24	26	26	19
KK11	100%	96%	100%	100%
LL12	96%	96%	96%	89%
MM13	96%	100%	85%	100%
NN14	92%	100%	100%	100%
OO15	100%	100%	100%	100%
PP16	96%	100%	100%	95%
QQ17	96%	100%	100%	100%
RR18	100%	100%	100%	100%
SS19	96%	100%	100%	100%
TT20	96%	100%	100%	100%
Average	**97%**	**99%**	**98%**	**98%**

**N = number of participants. Percentages are calculated based on the results submitted by all participants (see Annexes 14–17).*

An average score of 98% was reported for the detection of the *ehxA* gene. One false negative result was reported by one laboratory for strain PP16 (O121:H19) and one false positive result was reported for strain LL12 (O41:H26) (see Annex 17).

3.4.2 Subtyping of *vtx1* and *vtx2*

The number of laboratories participating in subtyping of *vtx* genes was 22 (85% of the *vtx* detection participants). 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*. One laboratory correctly subtyped all strains despite the negative results in the *vtx2* detection for strain MM13 (O63:H6), and only one participant submitted a negative in both the *vtx2* detection and subtyping for strain MM13 (O63:H6). However, it is not clear if the subtyping was performed, as that particular laboratory also reported a negative subtyping result for strains KK11 (O26:H11) and PP16 (O121:19). In EQA-6, strains harbouring *vtx1a, vtx1c* and *vtx1d* were included and *vtx1* was correctly subtyped by an average of 100% of the participants. *vtx2* was correctly typed by an average of 91% of the participants. The individual range for *vtx2* genes was from 64% for *vtx2b + vtx2d* in strain TT20 (O174:H21), to 100% for *vtx2a* in strain RR18 (O157:H7). The *vtx2c* gene was correctly reported by all participants in strain QQ17 (O157:H7) except for one. False positive and negative results are included in Table 8. Strain TT20 (*vtx2b and vtx2d*, O174:H21) was the strain with the lowest percentage of correct results (64%) and highest false negative results, mainly due to *vtx2b.* The complete results are presented in Annex 18.

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**
		Correct	False positive	False negative	Correct	False positive	False negative	Correct
KK11	*vtx2a*	100% (22)			95% (21)		1	95% (21)
LL12	*vtx1d*	100% (22)			95% (21)	1 (*vtx2d*)		95% (21)
MM13	*vtx2f*	100% (22)			95% (21)		1	95% (21)
NN14	*vtx1c*	100% (22)			100% (22)			100% (22)
OO15	*vtx1a*	100% (22)			100% (22)			100% (22)
PP16	*vtx2a*	100% (22)			91% (20)	1 (*vtx2f*)	1	91% (20)
QQ17	*vtx2a + vtx2c*	100% (22)			95% (21)		1	95% (21)
RR18	*vtx1a + vtx2a*	100% (22)			100% (22)			100% (22)
SS19	*vtx2d*	100% (22)			77% (17)	5 (*vtx2c*)		77% (17)
TT20	*vtx2b + vtx2d*	100% (22)			64% (14)	1 (*vtx2a*), 1 (*vtx2c*)	6 (*vtx2d*) 2 (*vtx2b*)	64% (14)
Average		**100%**			**91%**			**91%**

**N = number of participants. Percentages are calculated based on the results submitted by the participants listed in Annex 18.*

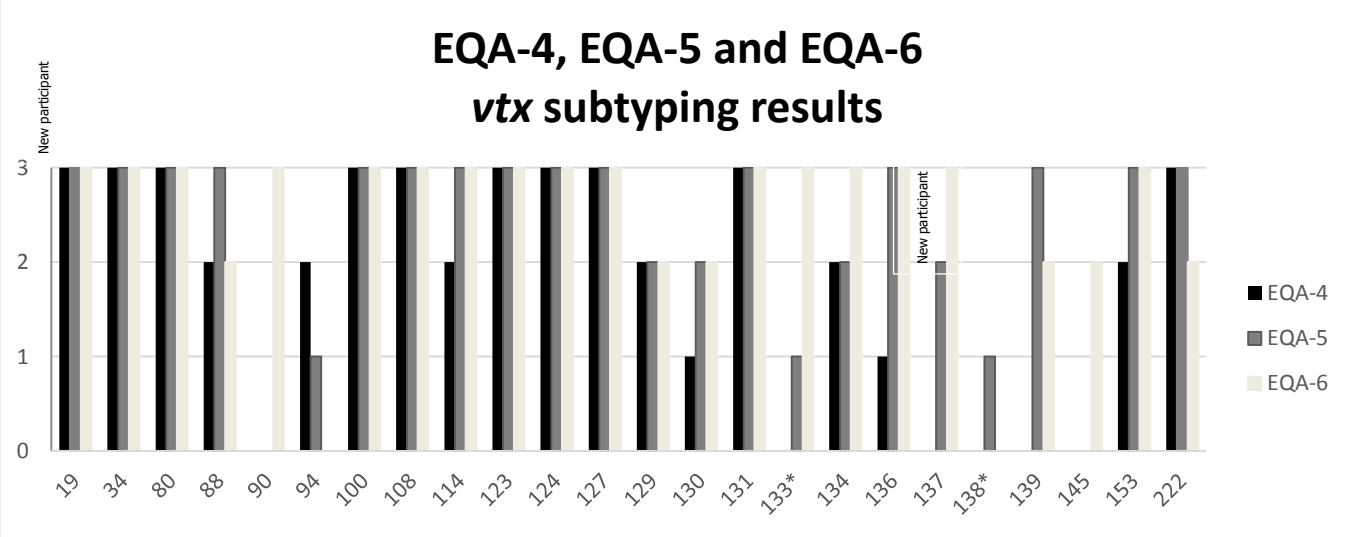
Sensitivity and specificity results of the subtyping of the one *vtx1* and five *vtx2* subtypes are presented in Table 9. Sensitivity was 1.00 for *vtx1a*, *vtx1c*, *vtx1d, vtx2c* and *vtx2f*, and between 0.79 and 0.99 for *vtx2a*, *vtx2b and vtx2d*. Specificity was 0.98 to 1.00 for eight subtypes.

Table 9. Sensitivity and specificity of vtx subtyping results

	vtx1a	*vtx1c*	*vtx1d*	*vtx2a*	*vtx2b*	*vtx2c*	*vtx2d*	*vtx2f*
Sensitivity	1.00	1.00	1.00	0.99	0.91	1.00	0.79	1.00
Specificity	1.00	1.00	1.00	0.99	1.00	0.98	1.00	1.00

To show the exact progress of the laboratory's performances, three strains from EQA-4 and EQA-5 were included in EQA-6. Strain GG7 (*vtx1a*), II9 (*vtx2a* and *vtx2c*) and MM13 (*vtx2d*) from EQA-4 are numbered OO15, QQ17 and SS19 respectively in the EQA-6. Figure 8 shows the performance based only on these three isolates.

Figure 7. Comparing EQA-4, EQA-5 and EQA-6 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 OO15, QQ17, and SS19 from EQA-6.
** 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 and aaiC*)

Nineteen laboratories correctly submitted an *aggR* negative result. However, one laboratory abstained from analysing six out of 10 strains. Sixteen laboratories also detected the *aaiC gene*, however only two laboratories correctly detected the *aaiC* gene in strain NN14 (O104:H7). This strain was *aaiC* positive by dot blot hybridisation in the laboratory of the EQA provider, and by conventional PCR in one of the participating laboratories using primers described for Real Time PCR (RT PCR) by the EU Reference Laboratory for *E. coli*. Our analysis of the procedures in use for *aaiC* detection by the participating laboratories, revealed usage of three different sets of primers and one probe for RT PCR [19, 20, 21]. One of the participants (no. 80) had repeated their conventional PCR (Boisen *et al.* 2008) on the NN14 strain and confirmed their intial *aaiC* negative result. This laboratory provided the EQA provider with their copy of the NN14 strain for re-testing, where the *aaiC* negative result by PCR and positive result by dot blot were confirmed. Therefore, WGS of the NN14 strain was performed, revealing a new variant of the *aaiC* gene. This new variant has three nucleotide differences in the target sequence of the forward primer and five nucleotide differences in the target sequence of the reverse primer (Boisen *et al.* 2008). This published reverse primer has an additional A nucleotide at position 7 compared twith the consensus sequence (nt 323 – 342) (see Annex 23). Both forward and reverse primers, as described by Boisen *et al.* 2012, have five nucleotide differences compared with the target sequences in the consensus sequence. Compared with Acc. No. KF678353, the reverse primer holds one mismatch at position 10 in the target sequence. The forward primer, described by the EU RL_Method_05_Rev 1, has one mismatch only in the target sequence of the *aaiC* variant of NN14 and one in the Acc. No. KF678353. The reverse primer has three and the probe has seven mismatches in their target sequences in the new *aaiC* variant identified in NN14. The alignment of the three known *aaiC* genes and the new variant, including the position of primers and number of mismatches, is shown in Annex 23. In conclusion, it is suspected that both primer sets by Boisen *et al.* 2008 and 2012 and the RT PCR probe by the EU RL_Method_05_Rev 1 are unable to anneal properly under the conditions described. However, the primers by the RL_Method_05_Rev 1 will anneal, and the product can be detected by conventional PCR (shown by participant no. 153) but not RT PCR. Further examination of these primers is underway in the laboratory of the EQA provider. The *aaiC* gene is considered part of the standard repertoire of virulence genes in EU public health national reference laboratories.

It is possible that the lower participation rate of *aaiC* detection can be explained by the correct negative *aggR* result in all 10 test strains. The complete results are presented in Annex 19.

3.4.4 Phenotypic test

Participation in phenotypic detection ranged from 25% (VCA) to 83% (sorbitol fermentation). Correct results of 100% were reported for the VCA test (Table 12).

Table 10. Average scores of the phenotypic tests

Strain/method	VCA	ESBL production	Haemolysin production	Beta-glucuronidase production	Sorbitol fermentation
N*	7	16	15	16	24
KK11	100%	100%	80%	100%	100%
LL12	100%	94%	100%	100%	100%
MM13	100%	100%	100%	100%	96%
NN14	100%	100%	100%	100%	92%
OO15	100%	100%	73%	69%	100%
PP16	100%	100%	73%	100%	100%
QQ17	100%	100%	80%	94%	96%
RR18	100%	100%	80%	94%	100%
SS19	100%	100%	100%	100%	96%
TT20	100%	100%	100%	100%	100%
Average	**100%**	**99%**	**89%**	**96%**	**98%**

**N =Number of participants. The percentages are calculated based on the results of the participants presented in Annexes 9, 10, 11, 12 and 13.*

Average correct results were 100% for VCA, 89% for enterohaemolysin production, 96% for β-glucuronidase production, 98% for sorbitol fermentation and 99% for ESBL production (Table 12).

Most of the errors in the detection of enterohaemolysin were false negatives reported by three participants. Likewise, with β-glucuronidase production, five laboratories obtained false negative results in strain OO15 (O111:H-/H8). In the detection of sorbitol fermentation, two laboratories submitted false negative results for strain OO15 (O111:H-/H8) and one participant reported a false negative for strain SS19 (O166:H15). Overall, the errors reported by participants in the phenotypical tests could either be associated to a few laboratories or individual strains, mainly strain OO15 (O111:H-/H8).

Detailed results for all phenotypic tests can be found in Annexes 9 (VCA), 10 (ESBL), 11 (enterohaemolysin), 12 (β-glucuronidase), and 13 (sorbitol).

4. Conclusions

Twenty-nine laboratories signed up for the EQA-6 on VTEC typing, funded by ECDC. For the third time, the EQA also included PFGE, and 22 laboratories participated in the PFGE exercise. Sixteen (73%) 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, and 60% in EQA-5. In the critical parameters 'image acquisition' and 'running conditions', none of the participants scored poor (1) compared with 40% in the EQA-4 and 5% in EQA-5. The production of distinct bands is another important gel quality assessment parameter; however, five out of the six laboratories scoring poor (1) in any of the gel quality parameters were caused by too fuzzy/thick bands - a level equal to EQA-5 and EQA-4. The BN software suite was used for the normalisation and interpretation of profiles. Fifteen (68%) laboratories analysed the resulting gels and 86% of these laboratories performed in good accordance with the guidelines.

Seventeen of the laboratories (61%) participated in the full O:H serotyping, and 78% of the serotyping results were correct. The correct O and H grouping were reported for an average of 78% and 82% of the test strains, respectively. Notably, not all laboratories have the full capacity to determine all O groups and H types.

Participation rate was 86% for *eae*, 93% for both *vtx1* and *vtx2,* and 68% for *ehxA.* Subtyping for *vtx* was performed by 79% of the participants, with an average of 90% of correct results. Gene detection of *eae, vtx1*, *vtx2* and *ehxA* was 97–98% correct. The low score of the *vtx2* detection was mainly caused by one strain, TT20 (59%) that was *vtx2b* and *vtx2d* positive.

The phenotypic characterisation was generally very good; with 100% correct results for VCA, 89% for enterohaemolysin production, 98% for fermentation of sorbitol, 96% for Beta-glucuronidase production, and 99% for detection of ESBL production. Except for sorbitol fermentation, phenotypic characterisation was not performed as frequently as genotypic characterisation: detection of VCA production (25%), enterohaemolysin production (54%), Beta-glucuronidase production (57%) and ESBL production (57%).

Overall, the EQA-6 showed that there was a slight decrease in the number of laboratories performing O:H serotyping at an average of 78% correct reporting. The virulence genes (*eae*, *vtx1*, *vtx2*, *ehxA*, *aggR* and *aaiC)* detection were on average nearly perfect except for the detection of the *aaiC* gene in strain NN14 (O104:H7), where a new variant was identified. Additionally, the *vtx* genes were overall subtyped correctly with the exception of strain TT20.

For the few laboratories with poor PFGE results, additional trouble shooting and training activities are advised.

5. Discussion

The WHO Collaborating Centre for Reference and Research on *Escherichia* and *Klebsiella*, and the Unit of Foodborne Infections at the SSI in Copenhagen, Denmark, have 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

Twenty-two laboratories participated in the PFGE part of the EQA-6. 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 (73%) 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-6 was in the parameter 'Bands'. Seventy-seven percent were graded fair and above, while 23% 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 parameter 'Bands'. In this EQA, five of the six laboratories obtained a score of 1 in parameter 'Bands' caused by fuzzy bands, which can be caused by either using narrow plugs, not using the correct dimensions of the TIFF file or not focusing during image capturing.

Nevertheless, in the EQA, 73% all of the gels have obtained at least the score 2 in all parameters and are therefore suitable for inter-laboratory comparison and an increase from 45% in EQA-4 and 60% in EQA-5. It is critical and can have a 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.

Sixth-eighty percent (15 out of 22) 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. Thirteen of the 15 (87%) laboratories, that submitted the BN analysis, achieved fair to excellent (2–3) scores, compared with EQA-4 (50%) and EQA-5(65%).

5.2 Serotyping

Participation in O:H serotyping in EQA-6 was roughly the same in the EQA-3, EQA-4 and EQA-5 (>50% on average). Participation in O group typing was the same in EQA-6 (26/29) as EQA-5, however, H typing was lower (17/29) this year compared with EQA-5 (19/29). An average of 78% of the 26 participating laboratories could correctly perform O grouping on the 10 test strains, which is comparable to EQA-4 and EQA-5. Although H type participation was lower in EQA-6 compared with EQA-5, average correct H typing was higher (82%). Note, both analyses are biased by the reporting of non-typeable (NT) results.

EQA-6 had 29 EU/EEA participants, the same number as EQA-5. Correct O:H serotyping ranged from 100% correct typing of one of the O157:H7 strains to 59% correct typing of serotype O174:H21 and O41:H26. The average percentage correct O:H serotyping in this EQA-6 was higher (78%) compared with EQA-5 (69%). The average correct O:H serotyping was not influenced by the H typing but rather by errors in the O typing, particularly O41 (LL12) and O174 (TT20).

However, the general trend (in both EQAs) is that the more common serotypes were identified more reliably. No systematic typing errors were observed except for two laboratories, which reported O121 instead of O41 in strain LL12. Finally, the previously reported cross-reaction between H11 and H21 remains unresolved for some laboratories. Overall, the misreporting of 12 O types and six H types in this EQA is higher than EQA-5 (eight O groups and three H types). The remainder of incorrect typing was submitted as not typable (NT) or not done (ND).

The comparisons (Figure 6 and 7) of the strains that were included in both EQA-4, EQA-5 and EQA-6 show that 10 of 27 of the laboratories had all three strains correctly O grouped in all three EQAs. Three laboratories improved their performance compared with last year. In the H typing (Figure 7), 9 out of 17 of the laboratories had all three strains correctly H typed in all three EQAs. When comparing only EQA-5 and EQA-6, ten laboratories had correct H typing.

None of the participants performed poorer in EQA-6 compared with EQA-5 for the three strains (Figure 6 and Figure 7).

In addition, 85–100% of the of 26 laboratories were able to correctly determine the O group in four of the top five strains (O26, O111, O121, O104 and O157) in the six strains that were included in EQA-6, and which are a part of the suggested minimal requirement for the typing of VTEC at the EU-level by ECDC (17, not published). The O:H serotype of the same six strains was also correctly determined by 76–100% of the participants.

In addition to O grouping, H typing detection is crucial during outbreaks, not only for epidemiological surveillance, but also 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 Public Health National Reference Laboratories to perform complete and reliable O:H serotyping – particular H typing.

5.3 Virulence determination

5.3.1 Genotypic tests

Genotypic detection of virulence genes *eae*, *vtx1, vtx2* and *ehxA* was performed by 19–26 laboratories for all the 10 test strains; results were 97–99% correct. The participation rate varied substantially between the different tests in the sixth EQA, being highest for the genotypic detection of the *vtx* genes (93%) and lowest for the detection of *ehxA* (66%). This is similar to EQA-5. The incorrect results for the *eae* gene originated from errors submitted by one laboratory. The average correct score of 97% in EQA-6 has improved from 96% in EQA-4 and EQA-3, but decreased from 98% in EQA-5. Compared with the EQA-3 and EQA-4, the average correct score of *ehxA* has slightly decreased from 99% to 98% in both EQA-5 and EQA-6.

The correct detection rate of *vtx1* and *vtx2* genes was high (99%) which is similar to the previous EQAs. Notably, the majority of false negative results originated from test strain TT20 (*vtx2b* and *vtx2d*) where six laboratories failed to detect *vtx2d*.

Compared with EQA-5 there was a 20% overall improvement of *vtx2f* detection. Resent cases of HUS caused by strains harbouring *vtx2f* have been described. The importance of awareness of *vtx2f* has been described by Friesema *et al*., 2014 [18] and routine detection of *vtx2f* should be included in the expected repertoire of VTEC in Europe in the future.

Nine laboratories have correctly *vtx* subtyped all three strains (OO15, QQ17 and SS19) in EQA-4, EQA-5 and EQA-6 (Figure 8), including only EQA-5, 12 laboratories obtained a perfect score. Compared with last year two laboratories showed lower performance.

Correct subtyping of both *vtx1* and *vtx2* was obtained at an average of 91% which has been more or less stable during EQA-3–EQA-5 (90%-92%)

The identification of a new variant of the *aaiC* gene in strain NN14 (O104:H7) illustrates the importance of selecting different strains to the EQA programmes in order for the existing procedures to be challenged. In the EQA-6 it was demonstrated that three different primer sets and one probe for for either conventional PCR or RT PCR were unable to detect the variant of the *aaiC* gene not previously described. WGS was useful in the identification of this new variant. A revised procedure for detection of all variants of *aaiC* gene should be developed.

In general, science often only elucidate that 'you only find what you are looking for', and this EQA-6 with the aaiC primer issues shows and that ongoing EQA programmes contributes to development and improvement of molecular detection procedures for European Surveillance.

5.3.2 Phenotypic tests

The participation in the phenotypic detection was between 25% and 83% on average (7–24 laboratories). As in previous years, the lowest participation was for VCA, where only seven laboratories participated (25%). The decrease in VCA participation has gone from 10 laboratories in EQA-3 and EQA-4 to eight in EQA-5 to now seven; despite the same number of laboratories participating.

Arguably, the most important phenotypic test is the fermentation of sorbitol because it can determine the highly virulent SF O157:H7 clone. It is therefore encouraging that the fermentation of sorbitol was performed by 24 laboratories with an average correct score of 98%. The second highest participation was for ESBL and Beta-glucuronidase production (55%) which is a slight decrease for ESBL compared with EQA-5. Participation within enterohaemolysin was slightly higher in EQA-6 (52%) than EQA-5 (48%).

In the detection of enterohaemolysin production all test strains were reported by three laboratories as 'negative', which suggests that only one or a few strains were tested and the 'negative' results were selected because 'not done' was not an option. The reporting of ESBL production was nearly perfect with only one laboratory of sixteen reporting a false positive. Furthermore, sixteen participating laboratories is lower than last year (18 participating laboratories). As the EQA provider we encourage the laboratories to participate as much as they can.

In summary, the performance level for phenotypic characterisation was very high. However, the participation rate was overall lower in this EQA compared to last years.

5.3 General remarks

The inconsistency in the number of performed tests per strain and per laboratory have been a recurrent problem in all VTEC EQAs so far. The participants always fail to explain why a specific test was not performed on all 10-test strains. This was particularly evident for O grouping and H typing where laboratories submitted multiple entries of 'NT'. 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 de-staining and imprecise image capturing - especially training on the last part would be helpful for some of the participants. 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 were 15 (EQA-6), an increase from 12 (EQA-4) but a decrease from 17 EQA-5. Proofreading before submission is also recommended.

As with EQA-5, roughly 60% 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 in order to explore the underlying reasons.

Regarding both genotypic and phenotypic tests, it is evident from the results and discussion that only a limited number of laboratories encountered difficulties. Nonetheless, 88% of the participants incorrectly reported a false negative result for *aaiC* in strain NN14 (O104:H7). Further investigation of this strain resulted in the identification of a hitherto unknown sequence variant of the *aaiC* gene. This emphasises the importance of continued EQA programmes, which should be used to update the design of PCR primers for genes reportable to the TESSy database.

Notably, in this EQA, laboratories that selectively only ran tests (i.e. virulence or serotyping) on a subset of the ten strains, were scored as negative results for 'Non typeable' or 'Not done' reporting.

Additional trouble shooting and training activities should be considered for laboratories with poor performance. Still, some laboratories have difficulties in creating and sending TIFF and XML files of the PFGE results, however laboratories seem to have improved proofreading of the results before submission.

6.2 ECDC and FWD-Net

The PFGE part of the VTEC EQA-6 had a 76% participation rate; 68% of the participating laboratories performed the BN gel analysis. Seventy-two percent of the gels produced were of sufficiently high quality for inter-laboratory comparison, and 87% of the BN analyses were at an acceptable level. Compared with the EQA-4 of VTEC an increase in both the gel quality of 34% and in the BN analysis performance of 20% has been observed. However, there is still a need to improve laboratory procedures, gel analysis, and interpretation with BN software as well as training to get familiar with the electrophoresis equipment and image acquisition equipment.

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

6.3 The EQA provider

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 should consider making a guide on optimising the image acquisition and recommends that the laboratories use the EQA provider's expertise in troubleshooting.

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

In addition, it should be mentioned that standardised comments were added in the evaluation report.

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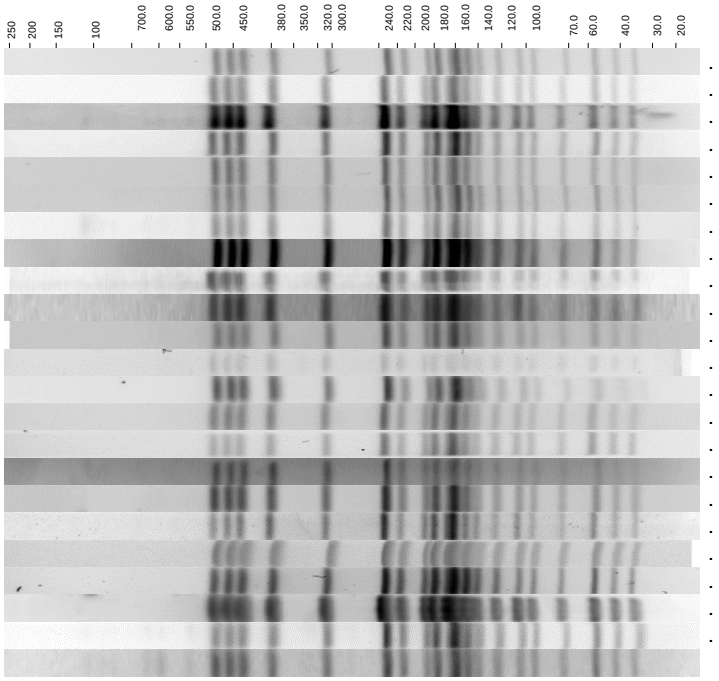
Annex 1. List of participants

Country	Institute/organisation	Laboratory
Austria	Österreichische Agentur für Gesundheit und Ernährungssicherheit GmbH, Institut für Medizinische Mikrobiologie und Hygiene	Nationale Referenzzentrale für Escherichia coli einschließlich Verotoxin bildender E. coli
Belgium	UZ Brussel	Laboratory of Microbiology and Infection Control
Bulgaria	National Centre 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	E. coli Reference laboratory
Estonia	Health Board	Central Laboratory of Communicable Diseases
Finland	National Institute for Health and Welfare (THL)	Bacteriology Unit
France	Institut Pasteur	Centre National de Référence des Escherichia coli, Shigella et Salmonella
Germany	Robert Koch Institute, Branch Wernigerode	NRC for Salmonella and other Bacterial Enterics
Greece	National schoole of publich health	National reference centre for Salmonella, Shigella and VTEC
Hungary	National Center for Epidemiology	National Reference Laboratory for Enteric Aerob Bacteria
Iceland	Landspitali University Hospital	Dept. of Clinical Microbiology
Ireland	Cherry Orchard hospital, Public Health Laboratory	VTEC-RL
Italia	Istituto Superiore di Sanità	Dipartimento di Sanità Pubblica Veterinaria e Sicurezza Alimentare
Latvian	Riga East University Hospital, Latvian Infectology Center	National Microbiology Reference Laboratory
Lithuania	The State Public Health Service under the Ministry of Health	National Public Health Surveillance Laboratory (NPHSL)NPHSL
Luxembourg	Laboratoire National de Sante	Surveillance Epidemiologique
Norway	Norwegian institute of Public Health	Norwegian Reference laboratory for enteropathogenic bacteria
Poland	National Institute of Public Health- National Institute of Hygiene	Department of Bacteriology, Laboratory of Enteric Rods
Portugal	Instituto Nacional de Saúde Dr. Ricardo Jorge	LNR de Salmonella, E.coli e outras bactérias entéricas
Republic of Macedonia	Faculty of veterinary medicine-Skopje, Food institute	Laboratory for food microbiology
Romania	Cantacuzino National Institute of Research-Development for Microbiology and Immunology	Molecular Epidemiology Laboratory
Slovenia	Centre for Medical Microbiology, National Laboratory of Health, Environment and Food	Department of Public Health Microbiology
Spain	Centro Nacional de Microbiología, Instituto de Salud Carlos III.	Unit of Enterobacteriaceae, Campylobacter and Vibrio
Sweden	MI-PL	Folkhälsomyndigheten
The Netherlands	RIVM, Cib	IDS/BSR
Turkey	Ministry of Health, Public Health Institution of Turkey	National Reference Laboratory for Enteric Pathogens
United Kingdom	Public Health England	Gastrointestinal Bacteria Reference Unit

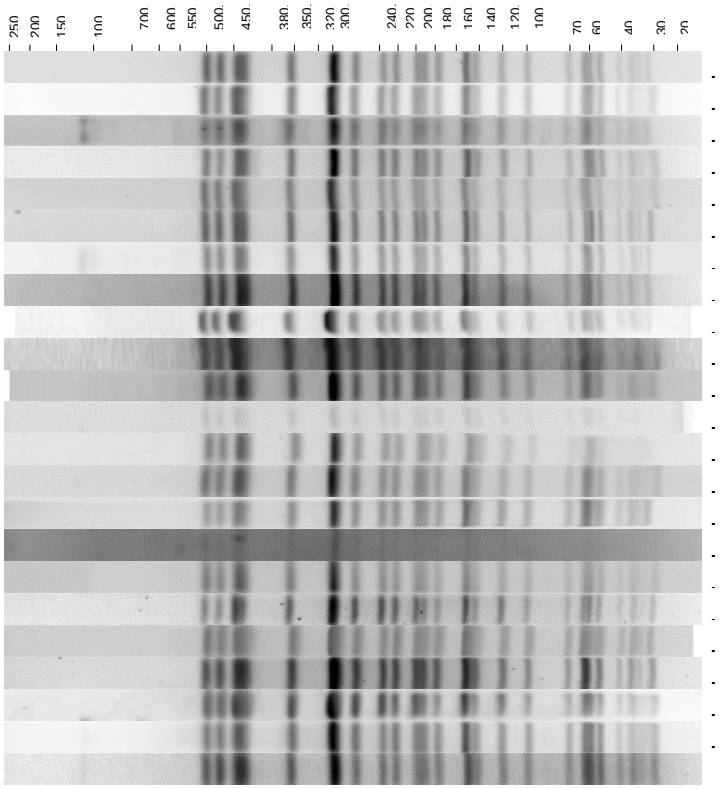
Annex 2. Examples of PFGE profiles

Profiles from the 22 participants in random order

Strain 6



Strain 10



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. Bottom band of standard is not 1–1.5 cm from bottom of gel but analysis 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.

1 ECDC FWD MolSurv Pilot - SOPs 1.0 – Annex 5 – PulseNet US protocol PFGE Image Quality Assessment, modified in 2013-2014

Annex 4. 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 5. Scores of the PFGE results

Gel quality

Parameters\laboratory	123	124	19	129	139	34	130	131	132	133	222	138	145	134	180	135	153	90	100	136	108	114
Image acquisition and running conditions	4	4	3	4	2	4	3	2	2	3	3	4	3	2	3	4	4	4	3	4	3	4
Cell suspension	4	3	4	3	4	4	3	3	4	2	4	2	4	4	3	4	4	4	4	4	4	4
Bands	4	4	4	2	1	4	2	1	1	2	2	1	2	4	3	3	3	4	4	3	1	3
Lanes	4	4	4	4	3	4	4	4	2	2	4	4	2	4	4	4	4	4	4	4	4	4
Restriction	4	2	4	2	3	4	4	2	4	1	4	4	4	4	2	4	4	4	2	4	4	2
Gel background	4	2	4	4	3	4	3	4	2	4	4	2	4	4	4	4	4	4	4	4	4	4
DNA degradation	4	4	4	4	4	4	4	2	4	2	4	3	4	4	3	3	3	4	4	4	4	2

Scored according to Annex 3 (TIFF quality grading guidelines)

BN analysis

Parameters\laboratory	123	124	19	129	34	130	132	133	222	134	135	153	90	100	108
Position of gel	3	2	3	3	3	2	2	2	3	2	3	1	3	3	3
Strips	3	2	3	3	2	2	2	3	3	2	3	3	3	3	2
Curves	2	3	3	3	3	2	2	2	3	2	3	3	3	2	3
Normalisation	3	1	3	3	3	2	2	2	2	3	3	3	3	3	3
Band assignment	2	3	3	2	2	3	2	2	2	3	2	2	2	3	2

Scores according to Annex 4 (BN gel analysis quality guidelines)

Annex 6. 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*	*aggR* gene	*aaiC* gene	Pathogenic group
KK11	O26	H11	+	-	+	+	+	+	+	-	+	*vtx2a*	-	-	STEC/VTEC
LL12	O41	H26	+	-	-	+	+	-	-	+	-	*vtx1d*	-	-	STEC/VTEC
MM13	O63	H6	+	-	-	+	-	+	-	-	+	*vtx2f*	-	-	STEC/VTEC
NN14	O104	H7	+	-	-	+	+	-	-	+	-	*vtx1c*	-	+	STEC/VTEC-EAEC
OO15	O111	H-	+	-	+	+	+	+	+	+	-	*vtx1a*	-	-	STEC/VTEC
PP16	O121	H19	+	-	+	+	+	+	+	-	+	*vtx2a*	-	-	STEC/VTEC
QQ17	O157	H7	+	-	+	-	-	+	+	-	+	*vtx2a + vtx2c*	-	-	STEC/VTEC
RR18	O157	H7	+	-	+	-	-	+	+	+	+	*vtx1a + vtx2a*	-	-	STEC/VTEC
SS19	O166	H15	+	+	-	+	+	-	-	-	+	*vtx2d*	-	-	STEC/VTEC
TT20	O174	H21	+	-	-	+	+	-	-	-	+	*vtx2b + vtx2d*	-	-	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.

Gene abbreviations			
eae	CVD434. *E. coli* attaching and effacing gene probe	*aaiC*	Chromosomal gene marker for enteroaggregative *E. coli*
ehxA	CVD419. Plasmid-encoded O157-enterohaemolysin	*aggR*	Gene encoding the master regulator in enteroaggregative *E. coli*
vtx1	NTP705. Verotoxin1; almost identical with Shiga toxin		
vtx2	DEP28. Verotoxin2; variants exist. Approximately 60% homology to *vtx1*		

Annex 7. O group serotyping results

Strain/laboratory	Ori.	19	34	80	88	100	108	114	123	124	125	127	128	129	130	131	132	133	134	135	136	137	138	139	145	153	222
KK11	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	O26
LL12	O41	O41	O41	NT	NT	O41	O41	O126	O41	NT	O115	O41	O41	O41	NT	O41	ND	NON-O157	NT	O41	O41	O41	O121	O41	O121	NT	NT
MM13	O63	O63	O63	NT	O63	O63	O63	O63	O63	NT	O63	O63	ND	NT	NT	O63	ND	NON-O157	O63	O63	O63	O63	Rough	O63	O125	NT	NT
NN14	O104	O104	O104	O104	O157	O104	O104	O104	O104	O104	O104	O104	O104	O104	O104	O104	ND	O104	O104	O104	O104	O104	ND	O104	O109	O104	O104
OO15	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	O111
PP16	O121	O121	O121	O121	O121	O121	O121	O121	O121	O121	NT	O121	ND	O121	O121	O121	ND	O121	O121	O121	O121	O121	O121	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	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
SS19	O166	O166	O166	NT	NT	O166	O166	O23	O166	NT	O166	O166	O166	O166	NT	O166	ND	NON-O157	O166	O166	O166	O166	O86	O166	ND	NT	NT
TT20	O174	O174	O174	O174	O174	NT	O174	O174	O174	NT	NT	O174	ND	NT	NT	NT	ND	NON-O157	NT	O174	O174	NT	Rough	O174	O104	NT	O174

Incorrect result

Strains included from the EQA-4 and EQA-5

Annex 8. H type serotyping results

Strain/laboratory	Ori.	19	34	80	108	114	123	124	125	127	129	131	134	135	136	137	138	139
KK11	H11	H11	H11	H11	H11	H11	H11	NT	NT	H11	H11	H21	H11	H11	H11	H11	ND	H11
LL12	H26	H26	H26	NT	H26	H5	H26	NT	NT	H26	H26	H26	H26	H26	H26	H27	ND	H26
MM13	H6	H6	H6	NT	H6	H6	H6	NT	NT	H6	H6	H6	H6	H6	H6	H6	ND	H6
NN14	H7	H7	H7	H7	H7	H7	H7	H7	NT	H7	H7	H7	H7	H7	H7	H7	H7	H7
OO15	H-	H-	H-	H8	H-	H-	H-	H-	NT	H-	H-	H-	H8	H-	H-	H-	ND	H-
PP16	H19	H19	H19	H19	H19	H19	H19	NT	NT	H19	H19	H19	H4	H19	H19	H19	ND	H19
QQ17	H7	H7	H7	H7	H7	H-	H7	H7	NT	H7	H7	H7	H7	H7	H7	H7	H7	H7
RR18	H7	H7	H7	H7	H7	H7	H7	H7	H7	H7	H7	H7	H7	H7	H7	H7	H7	H7
SS19	H15	H15	H15	NT	H15	H15	H15	NT	NT	H15	H15	H15	H15	H15	H15	H15	ND	H15
TT20	H21	H21	H21	H21	H21	H21	H21	NT	NT	H21	H21	H21	H21	H21	H21	H11	ND	H21

H- was accepted as a correct result.

Incorrect result

Strains included from the EQA-4 and EQA-5

Annex 9. VCA results

Strain/laboratory	Ori.	19	114	123	126	127	131	222
KK11	+	+	+	+	+	+	+	+
LL12	+	+	+	+	+	+	+	+
MM13	+	+	+	+	+	+	+	+
NN14	+	+	+	+	+	+	+	+
OO15	+	+	+	+	+	+	+	+
PP16	+	+	+	+	+	+	+	+
QQ17	+	+	+	+	+	+	+	+
RR18	+	+	+	+	+	+	+	+
SS19	+	+	+	+	+	+	+	+
TT20	+	+	+	+	+	+	Intermediate	+

Intermediate result noted in the Vero cell assay is accepted as a positive result.
+= Positive, - = Negative

Annex 10. ESBL production results

Strain/laboratory	Ori.	19	34	80	100	114	123	124	126	128	130	131	132	134	136	145	153
KK11	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
LL12	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-
MM13	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
NN14	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
OO15	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
PP16	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
QQ17	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
RR18	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
SS19	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
TT20	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

+ = Positive, -. = Negative

Neg. = Negative

Annex 11. Enterohaemolysin production results

Strain/laboratory	Ori.	19	34	100	114	123	125	126	127	129	131	133	136	137	145	153
KK11	+	+	+	+	+	+	-	+	+	+	+	+	+	-	-	+
LL12	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
MM13	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
NN14	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
OO15	+	+	+	+	-	+	-	+	+	+	+	+	+	-	-	+
PP16	+	+	+	+	+	+	-	+	+	+	+	-	+	-	-	+
QQ17	+	+	+	+	+	+	-	+	+	+	+	+	+	-	-	+
RR18	+	+	+	+	+	+	-	+	+	+	+	+	+	-	-	+
SS19	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
TT20	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

+= Positive, -= Negative

Incorrect result

Annex 12. Beta-glucuronidase production results

Strain/laboratory	Ori.	19	34	80	100	114	123	124	127	128	129	130	131	136	139	145	153
KK11	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
LL12	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
MM13	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
NN14	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
OO15	+	+	-	+	+	+	+	+	+	+	+	-	-	+	+	-	-
PP16	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
QQ17	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-
RR18	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-
SS19	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
TT20	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+

+= Positive, -= Negative

Incorrect result

Annex 13. Sorbitol fermentation results

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

+= Positive, -= Negative

Incorrect result

Annex 14. *eae* gene detection results

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

+= Positive, -= Negative

Incorrect result

Annex 15. *ehxA* gene detection results

Strain/laboratory	Ori	19	34	80	90	100	108	114	123	124	127	128	129	130	131	133	134	136	153	222
KK11	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
LL12	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-
MM13	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
NN14	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
OO15	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
PP16	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+
QQ17	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
RR18	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
SS19	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
TT20	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

Incorrect result

+= Positive, -= Negative

Annex 16. *vtx1* gene detection results

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

Incorrect result

+= Positive, -= Negative

Annex 17. *vtx*2 gene detection results

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

+= Positive, -= Negative

Incorrect result

Annex 18. *vtx* subtyping results

Strain/laboratory	Ori.	19	34	80	88	90	100	108	114	123	124	127
KK11	*vtx2a*	*vtx2a*	*vtx2a*	*vtx2a*	*vtx2a*	*vtx2a*	*vtx2a*	*vtx2a*	*vtx2a*	*vtx2a*	*vtx2a*	*vtx2a*
LL12	*vtx1d*	*vtx1d*	*vtx1d*	*vtx1d*	*vtx1d*	*vtx1d*	*vtx1d*	*vtx1d*	*vtx1d*	*vtx1d*	*vtx1d*	*vtx1d*
MM13	*vtx2f*	*vtx2f*	*vtx2f*	*vtx2f*	*vtx2f*	*vtx2f*	*vtx2f*	*vtx2f*	*vtx2f*	*vtx2f*	*vtx2f*	*vtx2f*
NN14	*vtx1c*	*vtx1c*	*vtx1c*	*vtx1c*	*vtx1c*	*vtx1c*	*vtx1c*	*vtx1c*	*vtx1c*	*vtx1c*	*vtx1c*	*vtx1c*
OO15	*vtx1a*	*vtx1a*	*vtx1a*	*vtx1a*	*vtx1a*	*vtx1a*	*vtx1a*	*vtx1a*	*vtx1a*	*vtx1a*	*vtx1a*	*vtx1a*
PP16	*vtx2a*	*vtx2a*	*vtx2a*	*vtx2a*	*vtx2a + vtx2f*	*vtx2a*	*vtx2a*	*vtx2a*	*vtx2a*	*vtx2a*	*vtx2a*	*vtx2a*
QQ17	*vtx2a + vtx2c*	*vtx2a + vtx2c*	*vtx2a + vtx2c*	*vtx2a + vtx2c*	*vtx2a + vtx2c*	*vtx2a + vtx2c*	*vtx2a + vtx2c*	*vtx2a + vtx2c*	*vtx2a + vtx2c*	*vtx2a + vtx2c*	*vtx2a + vtx2c*	*vtx2a + vtx2c*
RR18	*vtx1a + vtx2a*	*vtx1a + vtx2a*	*vtx1a + vtx2a*	*vtx1a + vtx2a*	*vtx1a + vtx2a*	*vtx1a + vtx2a*	*vtx1a + vtx2a*	*vtx1a + vtx2a*	*vtx1a + vtx2a*	*vtx1a + vtx2a*	*vtx1a + vtx2a*	*vtx1a + vtx2a*
SS19	*vtx2d*	*vtx2d*	*vtx2d*	*vtx2d*	*vtx2c + vtx2d*	*vtx2d*	*vtx2d*	*vtx2d*	*vtx2d*	*vtx2d*	*vtx2d*	*vtx2d*
TT20	*vtx2b + vtx2d*	*vtx2b + vtx2d*	*vtx2b + vtx2d*	*vtx2b*	*vtx2b + vtx2d*	*vtx2b*	*vtx2b + vtx2d*	*vtx2b + vtx2d*	*vtx2b + vtx2d*	*vtx2b + vtx2d*	*vtx2b + vtx2d*	*vtx2b*
Strain/laboratory	**Ori.**	**129**	**130**	**131**	**133**	**134**	**136**	**137**	**139**	**145**	**153**	**222**
KK11	*vtx2a*	*vtx2a*	*vtx2a*	*vtx2a*	*vtx2a*	*vtx2a*	*vtx2a*	-	*vtx2a*	*vtx2a*	*vtx2a*	*vtx2a*
LL12	*vtx1d*	*vtx1d*	*vtx1d*	*vtx1d + vtx2d*	*vtx1d*	*vtx1d*	*vtx1d*	*vtx1d*	*vtx1d*	*vtx1d*	*vtx1d*	*vtx1d*
MM13	*vtx2f*	*vtx2f*	*vtx2f*	*vtx2f*	*vtx2f*	*vtx2f*	*vtx2f*	-	*vtx2f*	*vtx2f*	*vtx2f*	*vtx2f*
NN14	*vtx1c*	*vtx1c*	*vtx1c*	*vtx1c*	*vtx1c*	*vtx1c*	*vtx1c*	*vtx1c*	*vtx1c*	*vtx1c*	*vtx1c*	*vtx1c*
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*
QQ17	*vtx2a + vtx2c*	*vtx2a + vtx2c*	*vtx2a + vtx2c*	*vtx2a + vtx2c*	*vtx2a + vtx2c*	*vtx2a + vtx2c*	*vtx2a + vtx2c*	*vtx2a + vtx2c*	*vtx2a + vtx2c*	*vtx2c*	*vtx2a + vtx2c*	*vtx2a + vtx2c*
RR18	*vtx1a + vtx2a*	*vtx1a + vtx2a*	*vtx1a + vtx2a*	*vtx1a + vtx2a*	*vtx1a + vtx2a*	*vtx1a + vtx2a*	*vtx1a + vtx2a*	*vtx1a + vtx2a*	*vtx1a + vtx2a*	*vtx1a + vtx2a*	*vtx1a + vtx2a*	*vtx1a + vtx2a*
SS19	*vtx2d*	*vtx2c + vtx2d*	*vtx2c*	*vtx2d*	*vtx2d*	*vtx2d*	*vtx2d*	*vtx2d*	*vtx2c + vtx2d*	*vtx2d*	*vtx2d*	*vtx2c + vtx2d*
TT20	*vtx2b + vtx2d*	*vtx2b + vtx2d*	*vtx2b + vtx2c*	*vtx2b + vtx2d*	*vtx2b + vtx2d*	*vtx2a + vtx2d*	*vtx2b*	*vtx2b + vtx2d*	*vtx2b + vtx2d*	*vtx2d*	*vtx2b*	*vtx2b + vtx2d*

Incorrect result

Strains included from the EQA-4 and EQA-5

Annex 19. Virulence genes *aggR* and *aaiC*

aggR

Strain/laboratory	Ori.	19	34	80	88	90	100	108	114	123	124	127	129	130	131	133	136	137	153	222
KK11	-	-	-	-	-	-	-	-	-	-	-	ND	-	-	-	-	-	-	-	-
LL12	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
MM13	-	-	-	-	-	-	-	-	-	-	-	ND	-	-	-	-	-	-	-	-
NN14	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
OO15	-	-	-	-	-	-	-	-	-	-	-	ND	-	-	-	-	-	-	-	-
PP16	-	-	-	-	-	-	-	-	-	-	-	ND	-	-	-	-	-	-	-	-
QQ17	-	-	-	-	-	-	-	-	-	-	-	ND	-	-	-	-	-	-	-	-
RR18	-	-	-	-	-	-	-	-	-	-	-	ND	-	-	-	-	-	-	-	-
SS19	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
TT20	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

+= Positive, -= Negative

Incorrect result

aaiC

Strain/laboratory	Ori.	19	34	80	90	100	114	123	124	127	130	131	133	136	137	153	222
KK11	-	-	-	-	-	-	-	-	-	ND	-	-	-	-	-	-	-
LL12	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
MM13	-	-	-	-	-	-	-	-	-	ND	-	-	-	-	-	-	-
NN14	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-
OO15	-	-	-	-	-	-	-	-	-	ND	-	-	-	-	-	-	-
PP16	-	-	-	-	-	-	-	-	-	ND	-	-	-	-	-	-	-
QQ17	-	-	-	-	-	-	-	-	-	ND	-	-	-	-	-	-	-
RR18	-	-	-	-	-	-	-	-	-	ND	-	-	-	-	-	-	-
SS19	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
TT20	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

+= Positive, -= Negative

Incorrect result

Annex 20. 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	

Reference strains of vtx *subtypes*
ND: Not done

Annex 21. 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).

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

The database is packaged in the zip archive called "Listeria EQA-2 BN<6/7>.zip" "E coli EQA-5 BN<6/7>.zip" or "salmonella EQA-5 BN<6/7>.zip". Note that there are two versions of each, one for version 6 and one for version 7 of BioNumerics.

Choose the correct file and unzip it into the folder where you would like to have your database. The archive contains the complete ready-made database (one file and one folder).

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

B. Setting up a database from scratch

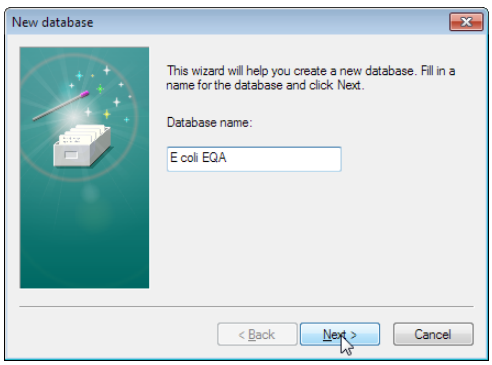
All the images in this instruction refer to E. coli so just exchange 'E coli' for either 'Salmonella' or 'Listeria' when setting up the databases.

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.

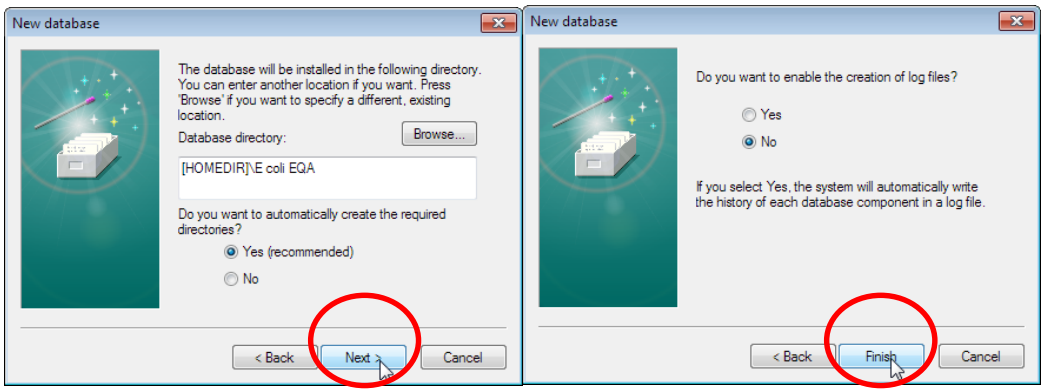
Setting up the empty database

1. Choose 'Create a new database'.

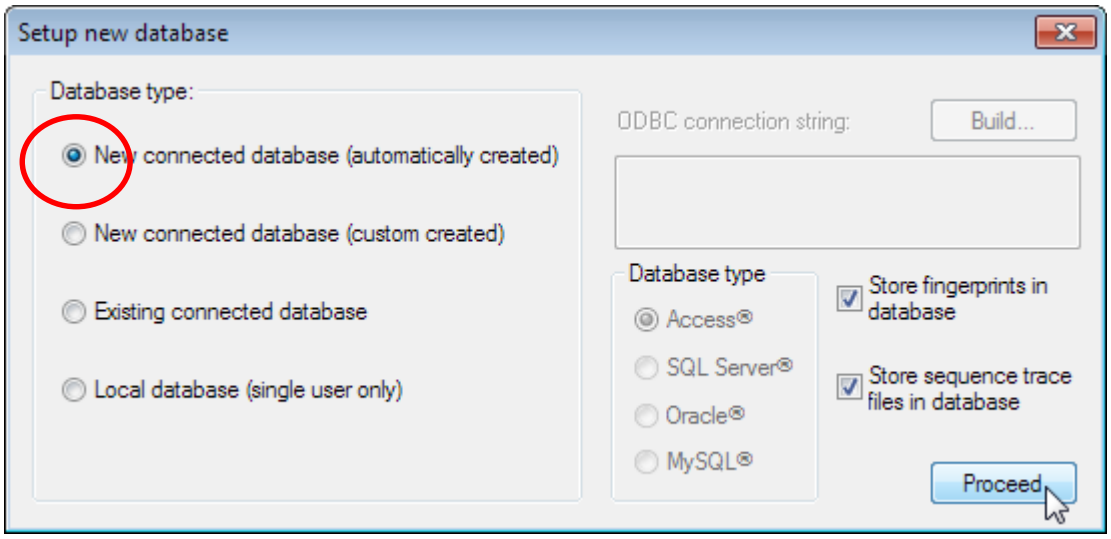
2. Enter a database name. Remember to Enter a database name, "Salmonella "Listeria " or "E coli EQA"



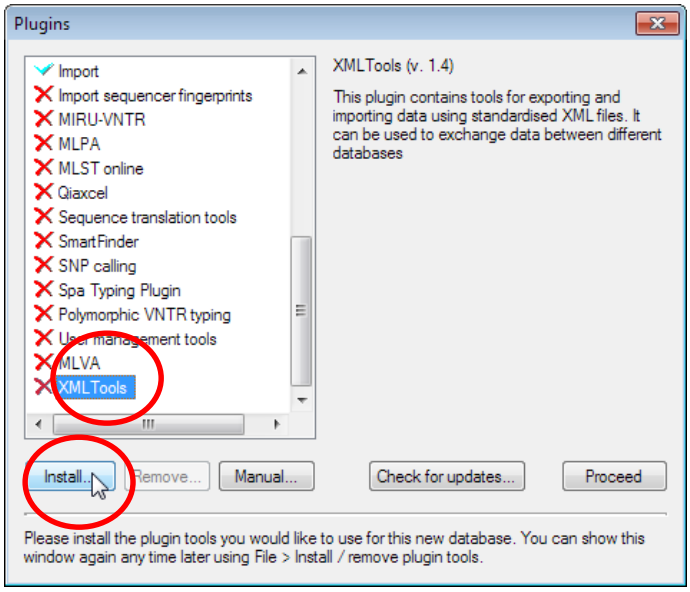
3. Use default values.



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



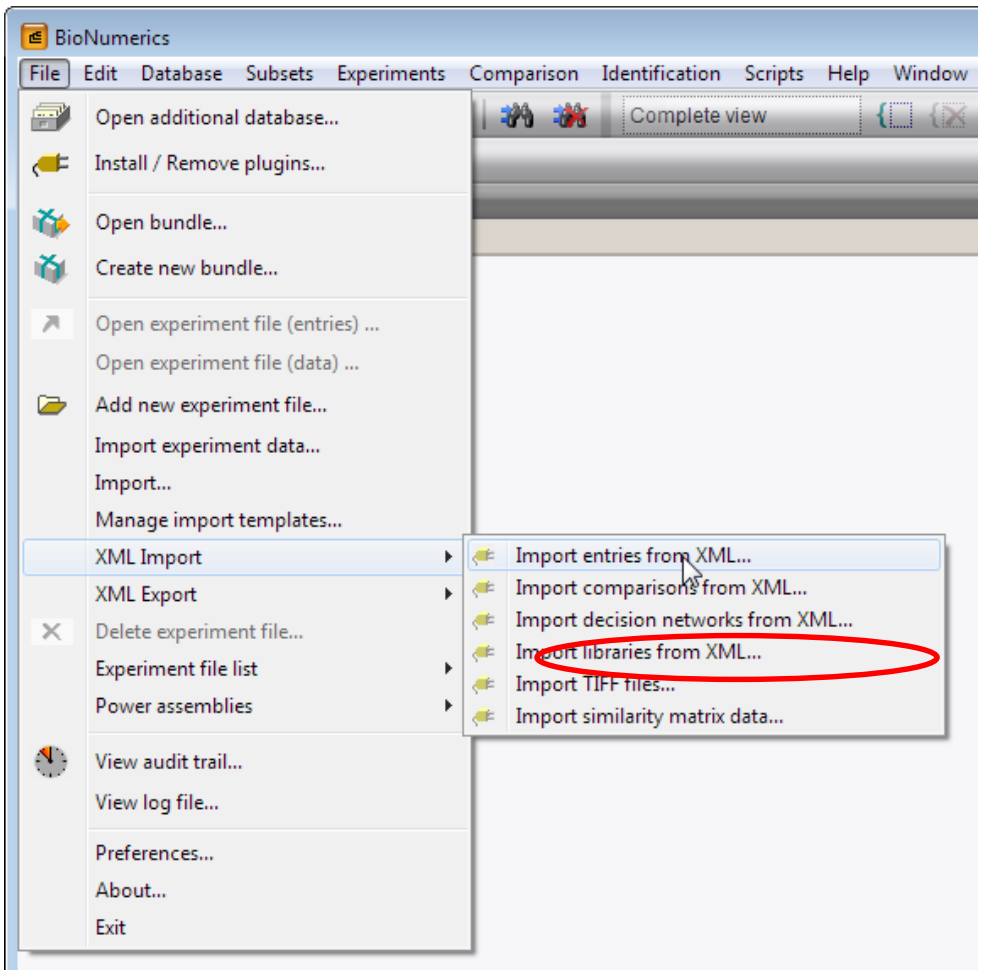
5. When choosing plugins, add the XML Tools plugin by selecting the plugin from the list and pressing 'Install…'



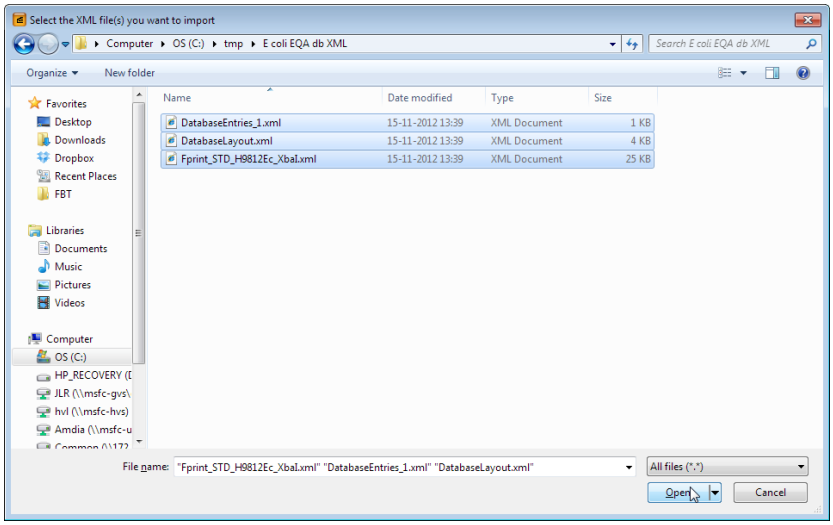
6. Proceed to the next window. The database is now set up and ready to import the database definitions.

Importing the XML structure

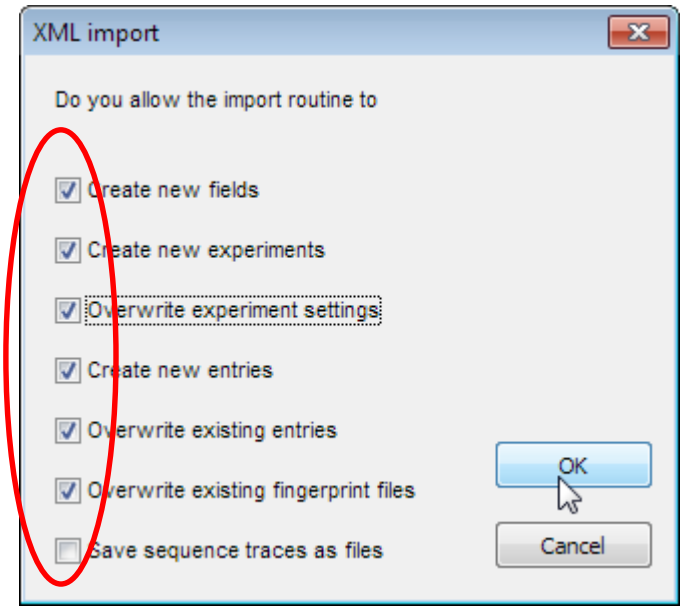
1. Unzip the contents of the supplied file 'Listeria EQA db XML.zip' or 'Salmonella EQA db XML.zip'. into the folder where you would like to place the files.
2. Select 'Import entries from XML' in the menu.



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



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



5. Restart the database.

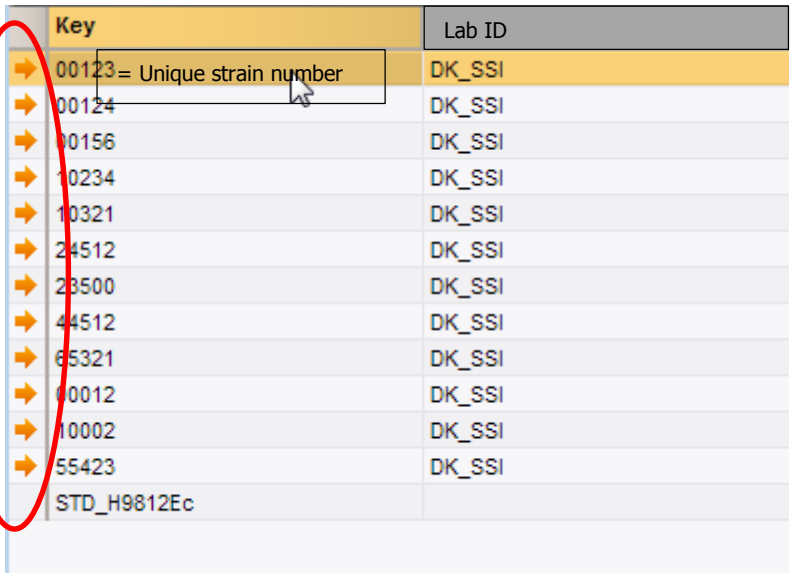
Annex 22. Guide to XML export

After analysing your 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.

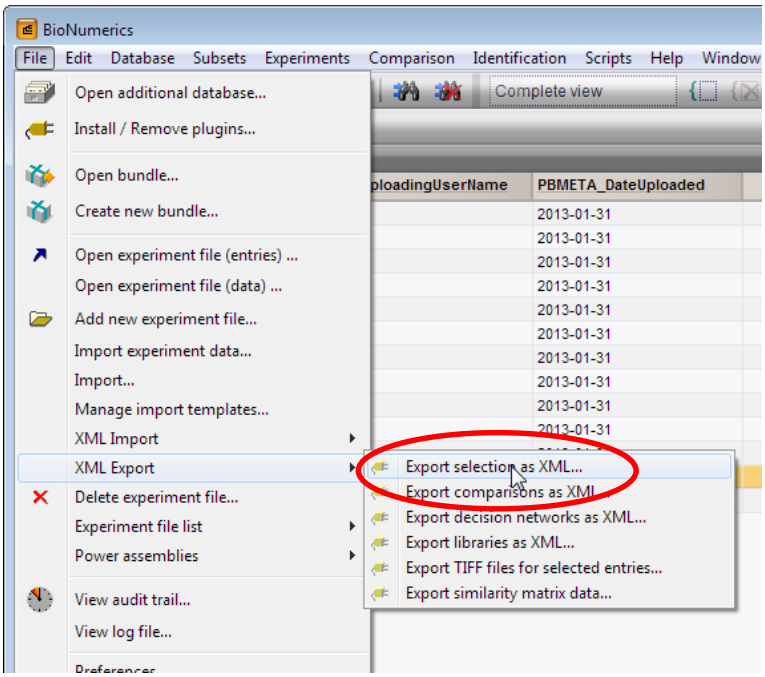
A. Exporting XML data from your database BN version 6

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

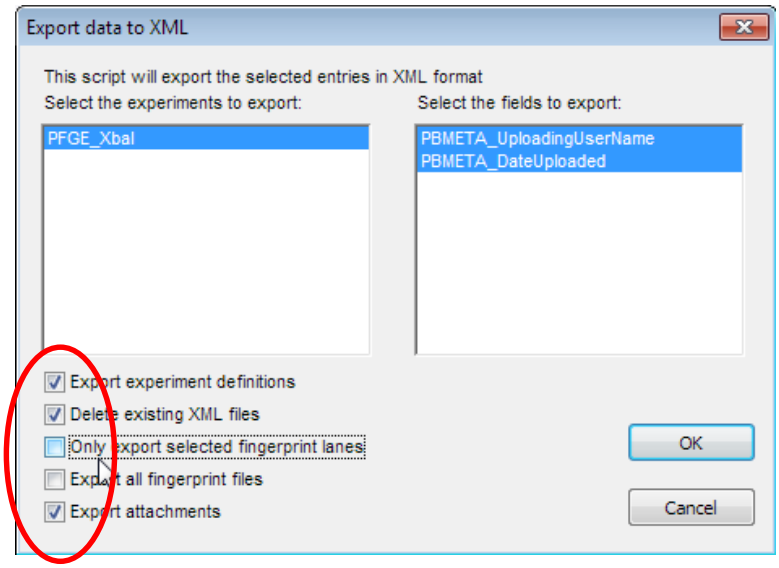
1. After analysing your data, select all the isolates that you would like to export



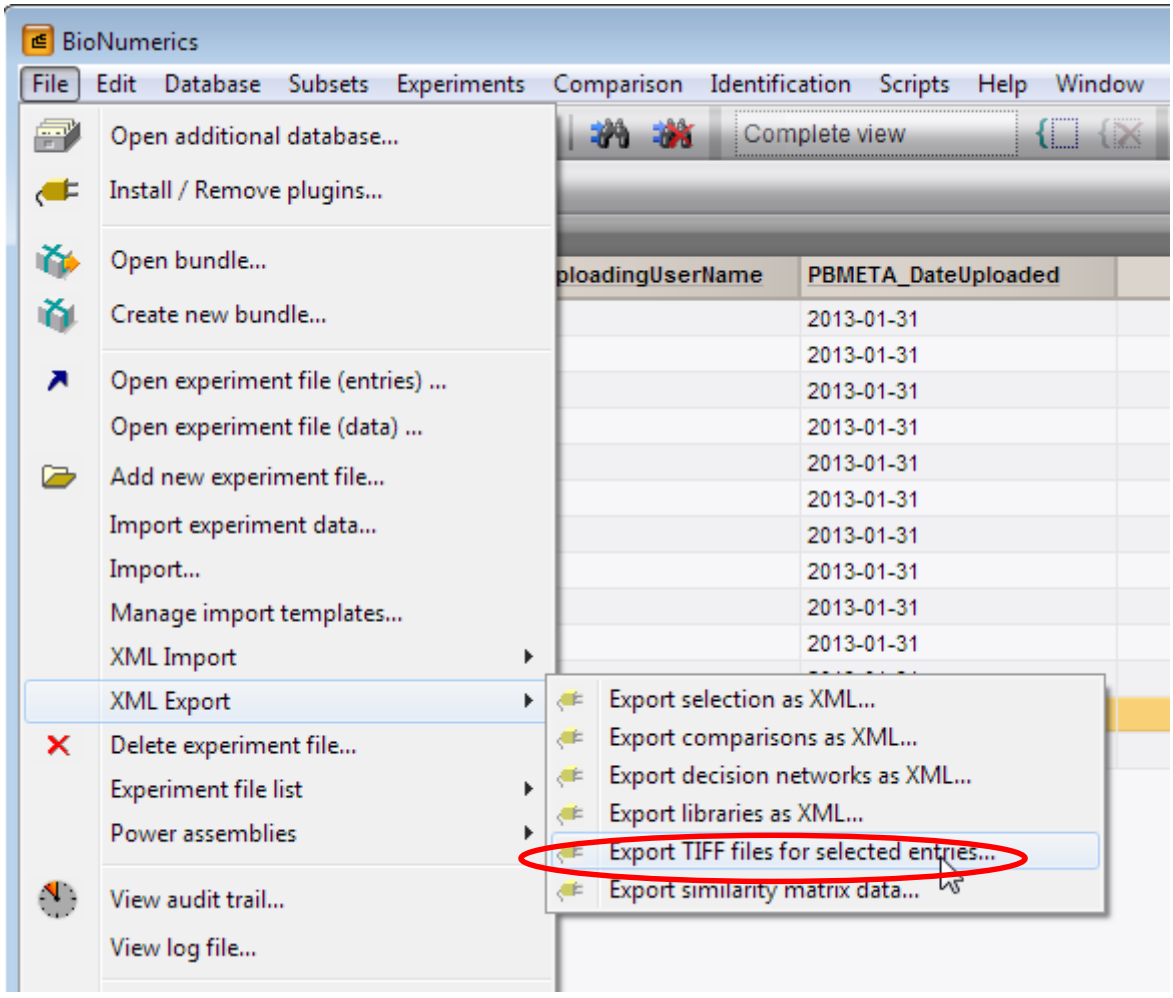
2. Export selection as XML.



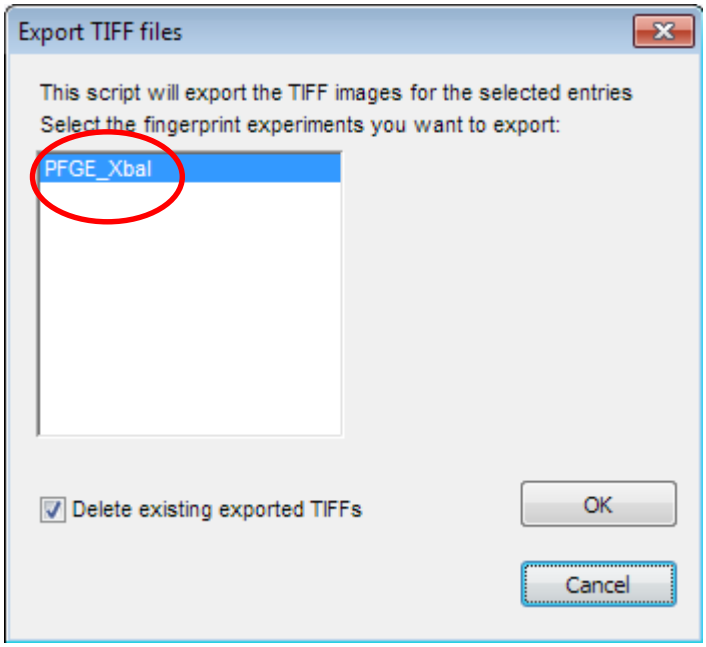
3. De-select the check box 'Only export selected fingerprint lanes'.



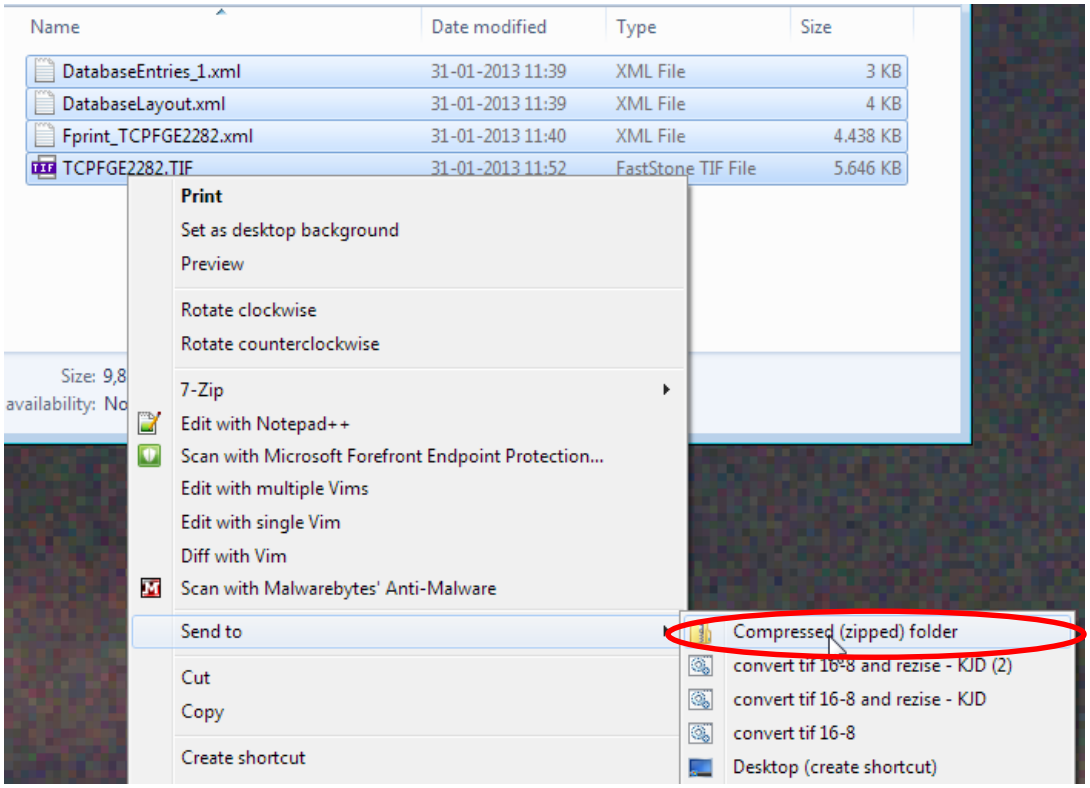
4. Now export the TIFF file(s).



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



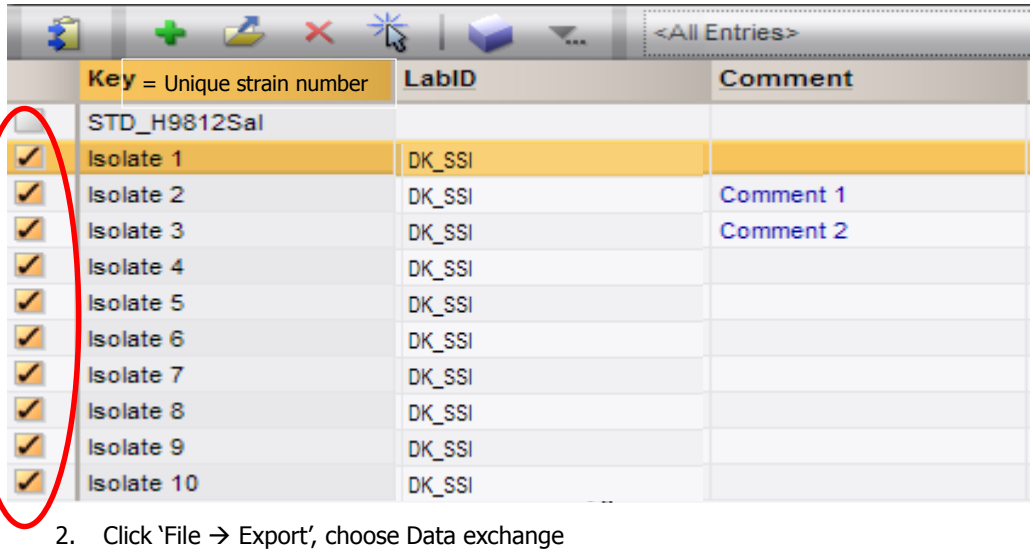
6. Now locate the EXPORT directory in your database directory. **Remember to check that the TIFF file is included**
7. Send all XML and TIFF files located there via mail.
8. Please compress them into a zip archive. One way of creating the zip archive is to mark all the XML and TIFF files, right click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



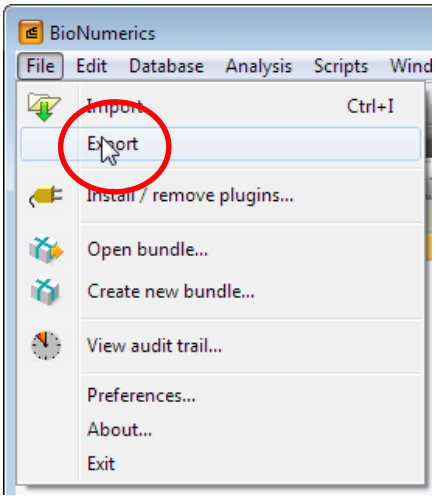
B. Exporting XML data from your database BN version 7

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

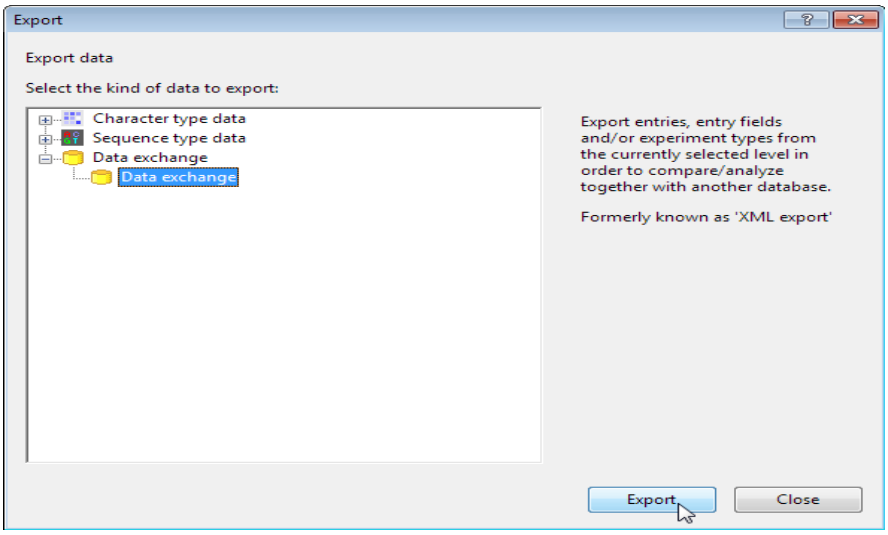
1. Select all isolates that you would like to export



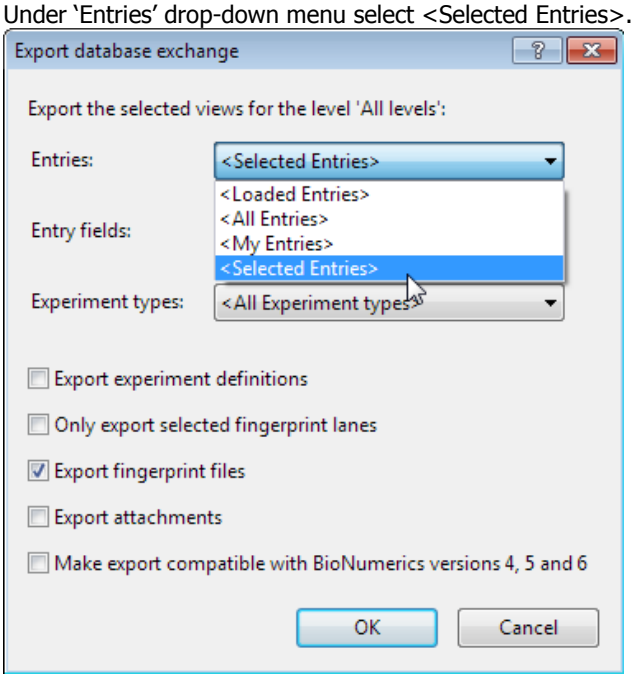
2. Click 'File → Export', choose Data exchange



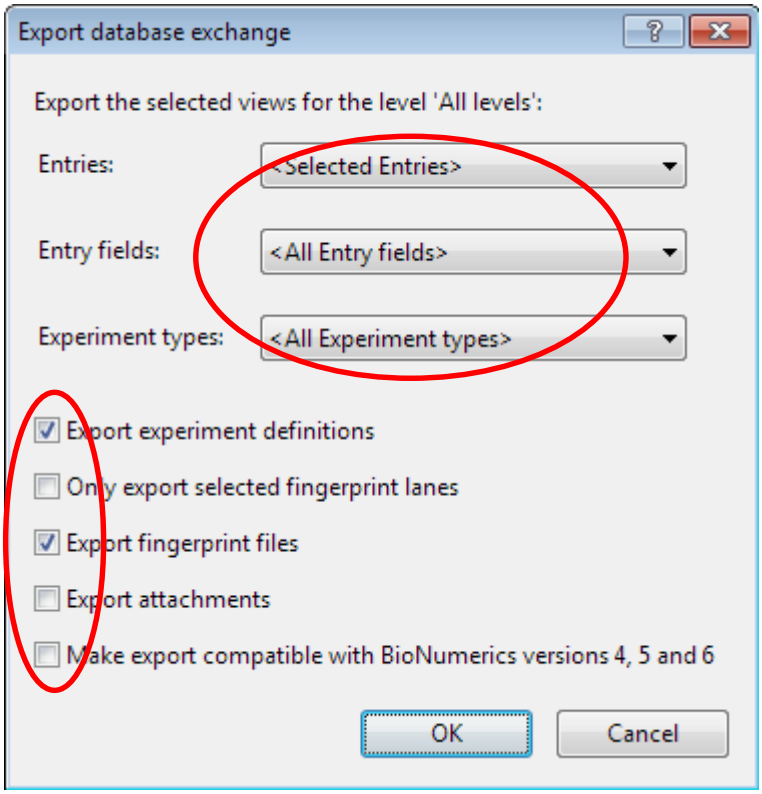
3. and click 'Export'



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

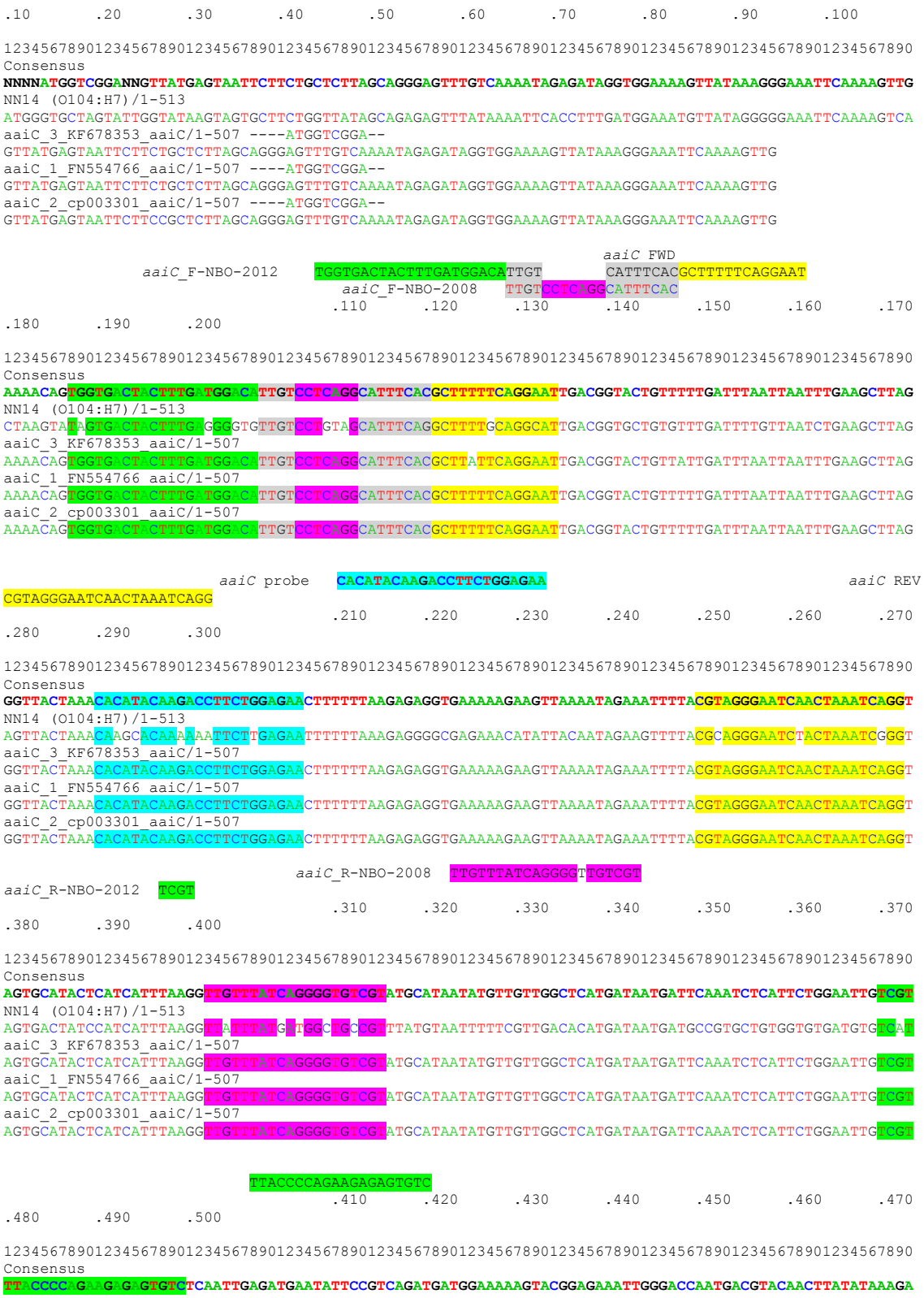


5. Under 'Entry fields' drop-down menu select <All Entry Fields>.
6. Under 'Experiments types' drop-down menu select <All experiment types>.
7. In the checkboxes tick **ONLY** the alternative 'Export fingerprint files'



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 23. Alignment of *aaiC* variants known before EQA-6 and the new *aaiC* variant identified in strain NN14 during EQA-6



```
NN14 (O104:H7)/1-513
TTATTCCAGAAATGAGTGTCTCAATTGAGATGAATATTCCATCAGAGGACGGGAAAAGTACAGAAAAGTTGGGGCCAATAACGTATAGCTTATCTAAAGA
aaiC_3_KF678353_aaiC/1-507
TTACCGCAGAAGAGTGTCTCAATTGAGATGAATATTCCGTCAGATGATGGAAAAAGTACGGAGAAATTGGGACCAATGACGTACAACTTATATAAAGA
aaiC_1_FN554766_aaiC/1-507
TTACCCCAGAAGAGTGTCTCAATTGAGATGAATATTCCGTCAGATGATGGAAAAAGTACGGAGAAATTGGGACCAATGACGTACAACTTATATAAAGA
aaiC_2_cp003301_aaiC/1-507
TTACCCCAGAAGAGTGTCTCAATTGAGATGAATATTCCGTCAGATGATGGAAAAAGTACGGAGAAATTGGGACCAATGACGTACAACTTATATAAAGA
```

```
                                .510
                          1234567890123
Consensus                 GGCTCTTGTATAA
NN14 (O104:H7)/1-513      GGAACTTGTGTAA
aaiC_3_KF678353_aaiC/1-507 GGCTCTTGTATAA
aaiC_1_FN554766_aaiC/1-507 GGCTCTTGTATAA
aaiC_2_cp003301_aaiC/1-507 GGCTCTTGTATAA
```

Accession numbers are shown for the three known variants and aligned with the new variant found in strain NN14 (O104:H7), which is 513 bp compared to the three known variants, which are 507 bp.

Mismatches are not coloured and are three and five respectively for the Boisen et al. 2008 primers, five in both forward and reverse for Boisen et al. 2012 primers, and one and three for NN14 for EU RL_Method_05_Rev 1 primers and seven for the probe. Two mismatches are also seen for acc. no. KF678353 in both the forward (*aaiC* FWD) and reverse (*aaiC*_R-NBO-2012) primers. Finally, the original publication by Boisen et al. 2008 includes an added A at position 7, which is not present in the consensus sequence of *aaiC*.

Primers used in EQA-6 for the detection of *aaiC*

Boisen et al. 2008; Forward primer coloured grey and purple, reverse coloured purple

*aaiC*_F-NBO-2008 5'-ATTGTCCTCAGGCATTTCAC-3'
*aaiC*_R-NBO-2008 5'-ACGACAACCCCTGATAAACAA-3' (complementary sequence: TTGTTTATCAGGGGTTGTCGT

Boisen et al. 2012; Forward primer coloured green and grey, reverse coloured green

*aaiC*_F-NBO-2012 5'-TGGTGACTACTTTGATGGACATTGT-3'
*aaiC*_R-NBO-2012 5'-GACACTCTCTTCTGGGGTAAACGA-3' (complementary sequence: TCGTTTACCCCAGAAGAGAGTGTC)

EU RL_Method_05_Rev 1; Forward primer coloured grey and yellow, reverse coloured yellow, probe coloured blue

aaiC FWD 5'-CATTTCACGCTTTTTCAGGAAT-3'
aaiC REV 5'-CCTGATTTAGTTGATTCCCTACG-3' (complementary sequence: CGTAGGGAATCAACTAAATCAGG)
aaiC probe 5-CACATACAAGACCTTCTGGAGAA-3'

References

1. Boisen N, Struve C, Scheutz F, Krogfelt KA, Nataro JP. 2008. New adhesin of enteroaggregative *Escherichia coli* related to the Afa/Dr/AAF family. Infect.Immun. 76:3281-3292.
2. Boisen N, Scheutz F, Rasko DA, Redman JC, Persson S, Simon J et al. 2012. Genomic characterization of enteroaggregative *Escherichia coli* from children in Mali. J.Infect.Dis. 205:431-444.
3. EU Reference Laboratory for *E. coli*. Department of Veterinary Public Health and Food Safety, Unit of Foodborne Zoonoses, Istituto Superiore di Sanità. Detection of Enteroaggregative *Escherichia coli* in food by Real Time PCR amplification of the *aggR* and *aaiC* genes. EU RL_Method_05_Rev 1; 05/10/2013.

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