TECHNICAL REPORT

Fourth external quality assessment scheme for typing of verocytotoxin-producing *E.coli* (VTEC)

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This report was commissioned by the European Centre for Disease Prevention and Control (ECDC), coordinated by Taina Niskanen (ECDC Food- and Waterborne Diseases Programme) and produced by Statens Serum Institut, Denmark.

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Abbreviations

A/EEC	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*
ECDC	The European Centre for Disease Prevention and Control
EIA	Enzyme immunoassay
EIEC	Enteroinvasive *E. coli*
eltA	G119. Heat labile enterotoxin (LT). Almost identical to cholera toxin
ESBL	Extended Spectrum Beta Lactamase
estA	DAS101. Heat stable enterotoxin (porcine variant) STp (STIa)
ETEC	Enterotoxigenic *E. coli*
FWD	Food- and Waterborne Diseases and Zoonoses
GFN	Global Foodborne Infections Network, Food Safety, WHO
HUS	Haemolytic uremic syndrome
ipaH	WR390. Invasion plasmid antigen. These genes are found in several copies chromosomally as well as on plasmids
NSF	Non-sorbitol fermenting *E. coli*
PCR	Polymerase chain reaction
PFGE	Pulsed Field Gel Electrophoresis
RFLP	Restriction fragment length polymorphism
SF	Sorbitol fermenting *E. coli*
SSI	Statens Serum Institut
STEC	Shiga toxin-producing *E. coli*. STEC is synonymous with VTEC
TESSy	The European Surveillance System
TESSy MSS	EU Molecular Surveillance System, part of the TESSy database
VCA	Vero cell assay
VT1	verocytotoxin 1
VT2	verocytotoxin 2
VTEC	verocytotoxin-producing *E. coli*. VTEC is synonymous with STEC
vtx1	The gene encoding VT1
vtx2	The gene encoding VT2
WHOCC	WHO Collaborating Centre for Reference and Research on *Escherichia* and *Klebsiella*

Executive summary

Main findings

- Twenty-eight public health national reference laboratories from 28 EU/EEA countries signed up for the fourth international external quality assessment (EQA) scheme on typing of VTEC organised by ECDC.
- Seventy-one per cent (20 out of 28) of the laboratories participated in the pulsed-field gel electrophoresis (PFGE) part of the EQA, and 50% 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). Twelve laboratories (60%, 12 out of 20) completed the gel analysis and 50% performed in a fair to good accordance with the guidelines.
- Thirteen (46%) laboratories participated in full O:H serotyping, and 50% of the participating laboratories were able to correctly determine the full O:H serotype of the 15 test strains that were included in the fourth EQA.
- Correct typing of virulence genes was 96% for *eae*, 98% for *vtx1* and 99% for *vtx2* and *ehxA*.
- Subtyping of *vtx* was performed correctly by 94% of the participants for the three *vtx1* subtypes, and by 93% for the four *vtx2* subtypes included in this EQA.
- Correct phenotypic characterisation was high, from 89% for VT production to 100% for ESBL production.

This report presents the results of the fourth round of the external quality assessment scheme for typing of verocytotoxin-producing *E. coli* (VTEC) funded by ECDC. The fourth EQA was carried out from November 2012 to February 2013 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 β-glucuronidase, enterohaemolysin, and extended β-lactamase (ESBL).

Although the majority of reported human VTEC infections are sporadic, serious foodborne outbreaks occur. In 2011, a large foodborne outbreak occurred in Germany, caused by an enteroaggregative VTEC (O104:H4) strain. For VTEC O157 infections, up to 10% of patients develop haemolytic uremic syndrome (HUS), which is the leading cause of acute renal failure in young children.

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

In November 2012, the laboratories of the Food- and Waterborne Diseases Network (FWD-Net) were invited by Statens Serum Institut (SSI) to participate in the fourth ECDC-funded EQA scheme for typing and characterisation of VTEC. Strains for the fourth EQA were selected to cover strains of current public health relevance in Europe. Fifteen strains – 11 VTEC and four non-VTEC strains (A/EEC, EAEC, ETEC and EIEC) – were selected for the fourth EQA, covering the most common serotypes, *vtx* subtypes and acceptable PFGE profiles. In addition to this, non-VTEC strains were included to test for the capacity to detect the relevant virulence genes: *aggR* and *aaiC* (for EAEC), *eltA* and *estA* (for ETEC), *ipaH* (for EIEC), and *eae* (for A/EEC).

A total of 28 laboratories participated in at least one part of the EQA. Twnty laboratories (71%) reported PFGE results, 13 laboratories (46%) participated in full O:H serotyping of all strains (26 laboratories submitted O group results for at least one strain and 18 laboratories submitted H-types for at least one strain. Genotypic detection of *eae*, *vtx1* and *vtx2* was performed by 23-25 laboratories (an average of 85-89%), 20 (71%) for *ehxA*, and 20 (71%) participated in subtyping of *vtx* genes. The average participation in phenotypic detection was 11 laboratories (38%) for VCA (Vero cell assay), 26 (93%) for fermentation of sorbitol, 18-19 (65%) for β-glucuronidase, 13-15 (49%) for enterohaemolysin and 17 (61%) for ESBL.

Twenty laboratories participated in the PFGE part of the EQA, and 9 (45%) were able to produce a PFGE gel of sufficiently high quality to allow comparison with profiles obtained by other laboratories. The subsequent normalisation and interpretation of the profiles were performed using the specialised software suite BioNumerics (BN). Twelve laboratories completed the gel analysis, and 50% performed in fair to good results accordance with the guidelines.

Of 13–18 participants, an average of 80% (range 46–100%) could correctly determine the O:H serotype of the strains (some laboratories only typed a selection of the test strains). The more common serotypes received better

typing results: O157:H7 was mostly typed correctly (94–100%), while O177:H25 was associated with a significantly poorer result (46%).

The results for the genotypic detection of virulence genes were generally very good: *eae* (96%), *vtx1* (98%), *vtx2* (99%) and *ehxA* (99%). False positive results were only reported once for *vtx1*, twice for *vtx2*. Two false negative results were received for *vtx1*, and three for *vtx2*. One laboratory was not able to report correct results for the *eae* gene in 11 strains.

The percentage of correct results for phenotypic detection was 89% for VT, 95% for fermentation of sorbitol, 93% for β-glucuronidase, and 89% for enterohaemolysin. ESBL was correctly determined by all participants.

This fourth EQA scheme marked the first EQA for members of the FWD-Net that also included PFGE typing. The number of participating laboratories in the EQA is encouraging. The molecular surveillance system, which is about to be implemented as part of TESSy, relies on the capacity of the FWD-Net laboratories to produce comparable typing results. At the moment, the molecular typing method used for EU-wide surveillance of VTEC is PFGE. The surveillance of VTEC infections also relies on conventional typing/phenotypic strain characteristics in combination with molecular typing. However, the PFGE results of the fourth EQA show that 50% of the laboratories need to improve their performance in order to produce useful typing profiles for an interlaboratory exchange. However, for the majority of laboratories with identified technical issues, achieving an acceptable quality level is within reach if they optimise procedures, receive trouble-shooting assistance, and additional training.

Thise study was conducted jointly with the network of EU Reference Laboratory for VTEC (EU-RL VTEC) at ISS, Rome, Italy. The aim of this cooperation was the harmonisation of typing methods in order to generate data for a comparison of human and non-human data.

This document presents the results of the fourth EQA of EU/EEA laboratories in the FWD-Net.

The evaluation report of the results produced by the network of VTEC NRLs in the veterinary and food safety fields (Regulation EC 882/2004) is available online: http://www.iss.it/binary/vtec/cont/PT10_Report.pdf.

The report of the first proficiency testing scheme for pulsed-field gel electrophoresis (PFGE) typing of verocytotoxin-producing *E. coli* (VTEC) strains (PT-PFGE1) – 2012–2013 is available at: http://www.iss.it/binary/vtec/cont/Report_PT_PFGE1.pdf

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 an essential part of quality management and evaluates the performance of laboratories through an external evaluator and with materials that are supplied specifically for this purpose.

ECDC's disease-specific networks organise a series of EQA for EU/EEA countries, with the aim to identify needs for improvement in laboratory diagnostic capacity relevant to the surveillance of diseases listed in Decision No 2119/98/EC [2], and to ensure the reliability and comparability of results from laboratories in all EU/EEA countries. The main objectives of external quality assessment schemes include:

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

In 2012, a framework service contract on 'Microbiological characterisation services to support surveillance of *Salmonella*, STEC/VTEC and *Listeria* infections' for the period 2012–2016 was put out to tender by ECDC. The Unit of Foodborne Infections at Statens Serum Institut (SSI) won the three lots covering *Salmonella*, Shiga toxin/verocytotoxin-producing *Escherichia coli* (STEC/VTEC) and *Listeria monocytogenes*. The contract for lot 2 (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 fourth VTEC EQA-exercise of this contract.

1.2 Surveillance of VTEC infections

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

In 2011, the overall EU notification rate of VTEC was 1.93 cases per 100 000 population. The total number of confirmed VTEC cases in the EU was 9 485, which represents an increase of 159% compared with 2010 (N = 3 656). This large increase was the result of an outbreak in 2011 caused by an enteroaggregative verocytotoxin-producing *E. coli* O104:H4 that affected more than 3 816 persons in Germany alone, with linked cases in an additional 15 countries [3]. There was a statistically significant increasing EU trend in the number of reported human cases of VTEC infection during 2008–2011. Even without 2011 data – and thus also excluding the STEC/VTEC O104:H4 outbreak – the EU trend for VTEC infections shows a significant increase between 2008 and 2010 [3].

Outbreaks with non-VTEC strains such as ETEC, A/EEC (including EPEC), EIEC and EAEC are often reported, and four strains representing each of these non-VTEC pathogenic groups were included in the fourth EQA.

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.

Disease surveillance data, including some basic typing parameters for the isolated pathogen, are reported by the Member States to TESSy. Apart from the basic characterisation of pathogens isolated from infections, there is a public health value in using more advanced and more discriminatory typing techniques for the surveillance of foodborne infections. Therefore, in 2012 ECDC initiated a pilot project on enhanced surveillance through

incorporation of molecular typing data ('molecular surveillance'). In the first pilot phase, three selected FWD-Net pathogens were included: *Salmonella*, STEC/VTEC and *L. monocytogenes*, The overall goals of integrating molecular typing in EU level surveillance are to:

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

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

Since 2009, ECDC's FWD Programme has supported EQA schemes for serotyping and antimicrobial resistance testing for *Salmonella* and VTEC. These EQA schemes have contributed to strengthen the laboratory capacity in the Member States and EEA countries and resulted in reliable and valid data for surveillance and research.

As mentioned above, ECDC is now extending its centralised data collection capabilities to include detailed molecular typing data for surveillance of selected pathogens. The technical platform to support this will be molecular typing databases within TESSy. To ascertain that the molecular typing data entered into the surveillance databases is of sufficiently high quality, expert support and EQA schemes covering these methods are needed. Therefore, since 2012 the ECDC FWD Programme has supported EQA schemes with a focus on expert support for molecular typing, namely PFGE and multi-locus-variable-number tandem repeat analysis (MLVA) of *Salmonella* PFGE of (STEC/VTEC, and *L. monocytogenes*. ECDC has also supported EQA activities for virulence gene detection, phage typing and serotyping of the selected pathogens. The fourth EQA scheme was targeted at those public health national reference laboratories in EU/EEA countries and EU acceding and candidate countries that already conduct molecular surveillance at the national level.

1.3 VTEC characterisation methods

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

Finally, some of the existing subtyping methods using a combination of specific polymerase chain reaction (PCR) and restriction fragment length polymorphism (RFLP) are inadequate and may result in misleading conclusions. For example, typing of *vtx2* has been based on the absence of the PstI site as an indicator of the presence of the mucus-activatable *stx*2d subtype [8-11]. However, the PstI site is also absent in six variants of *vtx2a*, in two variants of *vtx2c*, in *stx2f* and in all four variants of subtype *stx2g* [12].

Furthermore, the most commonly detected VTEC serotype – O157:H7 – may be divided into two groups: one with the unusual property of failing to ferment sorbitol within the first 20 hours of incubation (the non-sorbitol fermenters, NSF) and a highly virulent variant of O157 fermenting sorbitol (SF). NSF O157 is most often characterised by failure to produce β-glucuronidase. Approximately 75% of all VTEC produce enterohaemolysin, a toxin which can cause lysis of erythrocytes. Enterohaemolysin may either be detected phenotypically on sheep blood agar plates or by detection of the *ehxA* gene encoding enterohaemolysin.

In May and June 2011, one of the largest outbreaks with VTEC O104:H4 occurred in Germany [13], affecting 15 other countries. Later in June 2011, the same strain was found to be associated with organic fenugreek sprouts in a smaller outbreak in France [14]. The outbreak strain was an unusual hybrid enteroaggregative (*aggR* and *aaiC* positive) O104:H4 strain that had acquired the *vtx2a* encoding bacteriophage. In addition, the strain had acquired the plasmid encoded capacity to produce ESBL [15].

The fourth VTEC EQA 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 (EIA), fermentation of sorbitol,

production of β-glucuronidase , enterohaemolysin and ESBL. Genes for ETEC and EIEC were not specified but *elt*A, *est*A for ETEC, and *ipa*H for EIEC were to be expected as well as *aggR* and *aaiC* genes for EAEC and *eae* for A/EEC.

1.3 Objective of the fourth EQA scheme

1.3.1 Pulsed-field gel electrophoresis (PFGE) typing

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

1.3.2 Serotyping

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

1.3.3 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* (a, c, d), and *vtx2* (a, b, c, d) genes
- Detection of other virulence genes (*aggR* and *aaiC* were expected by public health national reference laboratories)

1.3.4 Phenotypic tests

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

2 Study design

2.1 Organisation

The fourth VTEC EQA was funded by ECDC and arranged by SSI to be conducted November 2012 through February 2013. It included PFGE, O:H serotyping, virulence determination by genotypic methods (detection and typing of virulence genes *eae*, *vtx1*, *vtx2* and *ehxA*, sybtyping of *vtx1* and *vtx2* and by phenotypic detection of VT production, fermentation of sorbitol, production of β-glucuronidase, enterohaemolysin and ESBL. A recently published protocol by conventional gel-based PCR (14) was tested for subtyping of the ten *vtx* subtype genes.

The fourth EQA (without the PFGE part) was coordinated in collaboration with the EU-RL VTEC laboratory in Rome and conducted according to ISO/IEC 17043:2010, entitled *Conformity assessment – General requirements for proficiency testing* (first edition, 1 February 2010) [16].

Invitations were e-mailed to the ECDC contact points in the FWD-Net on 10 October 2012. In addition, the ECDC coordinator sent invitations to the EU acceding and candidate countries Croatia[1], Montenegro, Serbia, the former Yugoslav Republic of Macedonia and Turkey.

Twenty-eight laboratories accepted the invitation and these are listed in Annex 1.

The fourth EQA test strains were sent to the participating laboratories by the end of November 2012.

The participants were asked to submit their PFGE results by e-mail to ecoli.eqa@ssi.dk and report the rest of the results through an online form (Annex 22) by 25 February 2013. In addition, laboratories from the international WHO Global Foodborne Infections Network (GFN) were invited to participate.

2.2 Selection of strains

The strains for the fourth EQA were selected based on representativeness: all strains should be representative for strains reported from Europe. Also, 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	11	BB2, CC3, EE5, FF6, GG7, HH8, II9, JJ10, KK11, LL12 and MM13
O:H serotyping	15	O113:H4, O177:H25, O121:H9, O128:H2/H-, O41:H26, O26:H11, O111:H8/H-, O104:H4, O157:H7 (two strains), O146:H21, O103:H2, O166:H15, O78:H11, O124:H30
Virulence gene determination	15	*eae*, *vtx1a*, *vtx1c, vtx1d, vtx2a, vtx2b, vtx2c* and *vtx2d, ehxA*, *aggR*, *aaiC* , (*eltA*, *estA*p, *ipaH)*
Phenotypic testing	15	VCA, sorbitol, β-glucuronidase, enterohaemolysin, ESBL

Detailed information about the strains is shown in Annex 6.

In addition to the 15 test strains, laboratories participating in the fourth EQA for PFGE could request the *Salmonella* Braenderup H9812 reference strain and reference strains for the *vtx* subtyping (Annex 19).

2.3 Carriage of strains

By the end of November, all strains were blinded, packed and shipped (shipping began on 27 November 2012). Almost all of the participants received their dispatched strains within 1–3 days. One parcel was delayed by customs and not delivered until after 12 days. The parcels were shipped from SSI Copenhagen, labelled as UN 3373 Biological Substance, Category B.

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

[1] Croatia became the 28th EU Member State on 1 July 2013.

On 19 December, instructions on how to submit results were e-mailed to participants. Instructions included a link to a *Google Docs* submission form (see Annex 22), zipped files for the BN database experiment settings (PFGE part), and guidelines on how to export XML files from BN (Annex 20 and 21).

2.4 Testing

In the PFGE part, 11 *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) [17].

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

In the other parts of the fourth EQA, the same 11 strains as in the PFGE part and four 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 [18] 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* to *vtx2d*) were assessed (suggested protocol [19]).

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

For the detection of virulence characteristics related to enteroaggregative VT 2-producing *E. coli* O104:H4 (EAEC-VTEC), e.g. the chromosomally encoded protein gene (*aaiC*) and enteroaggregative adhesion transcription regulator gene (*aggR*), one strain with these characteristics was included. Three strains each representing ETEC, A/EEC and EIEC diarrhoeagenic groups were included in the harbouring genes *eltA* and *estA* (for ETEC), *ipaH* (for EIEC), and *eae* (for A/EEC) (not a requirement for the fourth EQA).

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

2.5 Data analysis

When the results from the individual laboratories were received at SSI, the PFGE results were added to the *E. coli* EQA BN database at SSI. The PFGE gel quality was evaluated according to ECDC FWD MolSurv Pilot SOPs 1.0, (Annex 5), PulseNet US protocol, PFGE image quality assessment,TIFF quality grading guidelines (Annex 3). Scoring of the gels was done with respect to seven parameters (scores in the range of 1–4, with 4 the top score). The BN analysis was based on five parameters (scores in the range of 1–4, with 4 the top score), using the BN gel analysis quality guidelines (Annex 4). After the results from all laboratories were received, 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 on the basis of correct results and a percentage score was calculated.

3 Results

3.1 Participation

Laboratories could choose to participate in the full scheme or a selection of the methods. The methods were PFGE, O:H serotyping, virulence determination including genotyping (virulence gene detection and subtyping) and phenotyping (VT, sorbitol, β-glucuronidase, enterohaemolysin, ESBL). Of the 28 participants, 20 laboratories (71%) participated in the PFGE part, and 13 (46%) participated in the full O:H serotyping of all 15 strains. An additional five laboratories submitted O:H data for only a limited number of the EQA strains. The reasons for omitting some of the strains was not always specified but in some cases was based on the obtained O results. All 18 laboratories (64%) submitted O:H serotype data for strain LL12 (O157:H7). In addition to the FWD-Net participants, 19 laboratories from the international WHO Global Foodborne Infections Network (GFN) participated (results not included in this report).

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

An average of 52% (13–18 laboratories) participated in both O and H serotyping (Table 2). The highest participation, 23–26 laboratories (87% in average), was in the O typing, whereas an average of 52% (13–18 laboratories) participated in the H typing (Table 3).

In the genotyping part (virulence gene detection and subtyping), 23–25 laboratories (average 85–89%) submitted results for *eae* and *vtx* genes, while 20 (71%) laboratories submitted results for *ehxA* genes. Twenty laboratories (71%) submitted results for *vtx* subtypes, and 11–17 laboratories (39–61%) reported results for EAEC (*aggR* and *aaiC*). In the phenotyping part, 26 laboratories (93%) 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]
Number of participants	20	13–18	25	26
% of participants	71	52	89	93

[1] *Participation in O grouping was 23–26 (average 87%) laboratories and 14–18 (average 52%) laboratories in H typing*

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

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

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

Table 3: Detailed participation table

Participants/ method	O:H serotype group	O group	H type	Vero cell assay	ESBL	Haemolysin production	β-glucuronidase production	Sorbitol fermentation	*eae* gene	*ehxA* gene	*vtx1* gene	*vtx2* gen	*vtx* subtypes	Additional genes	
n	13-18	23-26	13-18	10-11	17	13-15	18-19	23-26	23-24	20	25	25	20	11	17
	n=13 for 3 strains, 14 for 6 strains, 15 for 4 strains, 17/18 for II9/LL12 [O157]	n=23 for all 15 strains, 24 for 8 strains, 25 for 4 strains, 26 for II9/LL12 (O157)	n=13 for all 15 strains, 14 for 4 strains, 15/17 for 5/1 strains, 18 for II9/LL12 (H7)	n=10 for all 15 strains, 11 for 10 strains		n=13 for all 15 strains, 14 for 8 strains, 15 for KK11	n=18 for all 15 strains, 19 for DD4	n=25 for DD4, 26 for 14 strains	n=23 for 4 strains, 24 for 11 strains					*aaiC*	*aggR*
%	46-64	82-93	46-64	36-39	61	46-54	64-68	89-93	82-86	71	89	89	71	39	61
Average	52%	87%	52%	38%	61%	49%	65%	93%	85%	71%	89%	89%	71%	39%	61%

Participation in the detection of virulence gene *eae* was an average of 85%, and participation in the detection of *ehx*A was 71%. Participation in the detection of *vtx1* and *vtx2* was 89%, while participation in subtyping was 71% on average (68% for most strains).

Participation in the phenotypic detection was 38–93% (11–26 labs). The lowest participation was for the VT assay: only 10 participants (36%) delivered results for 10 strains. Participation in the sorbitol fermentation was

26 laboratories (93% on average). The test for enterohaemolysin production was performed by 13–15 laboratories (49% on average). The test for production of β-glucuronidase was performed by 18–19 laboratories (65% on average). Seventeen laboratories (61%) submitted results for the production of ESBLs.

3.2 Pulsed-field gel electrophoresis (PFGE)

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

3.2.1 Gel quality

All laboratories except one were proficient in the production of profiles that were recognisable as the profile for the relevant EQA strain. One laboratory seemed to have exchanged two strains, thereby excluding one of the PFGE EQA strains. The gels – and therefore the profiles for individual strains – were inconsistent in quality (Table 4). The gels were graded according to the TIFF quality grading guidelines, where seven parameters are used in the grading (Annex 3). Parameters have to be evaluated individually because a low score in a single category can greatly affect the analysis of the TIFF file and the profile comparison.

A wide variation in quality was seen between laboratories (Table 4). Two parameters, cell suspension and lanes, had a high average score above 3.5, between good and excellent. Two parameters, restriction and DNA degradation, had an average score of 3.1 and 3.2, also between good and excellent. Three parameters, image acquisition and running conditions, bands, and gel background received an average score below 3 (2.2 and 2.9), i.e. between fair and good.

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

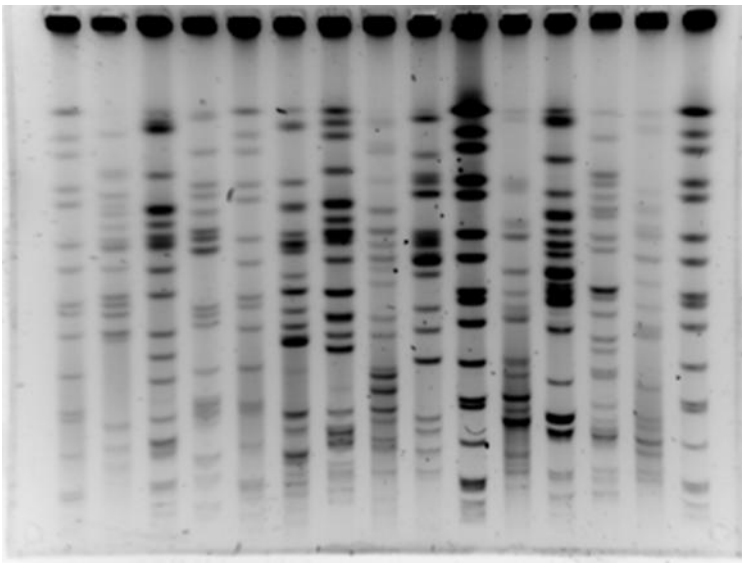
Parameters	1 – poor	2 – fair	3 – good	4 – excellent	Average
Image acquisition and running conditions	40%	20%	20%	20%	2.2
Cell suspension	5%	5%	30%	60%	3.5
Bands	30%	40%	15%	15%	2.2
Lanes	5%	0%	30%	65%	3.6
Restriction	15%	10%	20%	55%	3.2
Gel background	15%	15%	35%	35%	2.9
DNA degradation	25%	5%	10%	60%	3.1

Average scores and percentages of laboratories which obtained scores 1 through 4 for the seven TIFF quality grading guideline parameters

The grading guidelines indicate that a score of 2 (= fair) can still be obtained for image acquisition and running conditions even when band spacing does not match the global standard. Only 40% of the participants were graded good (3) or excellent (4) in the parameter image acquisition and running conditions (Table 4), with 60% of the participants receiving a critically low score (1 and 2). In the bands parameter, 15% of laboratories received the top score of 4; 30% of participants were graded as poor (1), rendering the normalisation and further analysis of the gel in BN impossible. An additional 40% were graded as fair (2), making analysis in BN difficult. In the parameter gel background, 30% of the laboratories were graded poor to fair (1-2). All gel quality scores are listed in Annex 5.

The gel in Figure 1 scored a 1 (poor) in the parameter image acquisition and running conditions. The low score was caused by a combination of different factors; the gel had run a bit too far but most importantly, running conditions were not according to protocol, making the normalisation impossible. The gel also had low scores in all other six parameters, but not all of those are as critical as image acquisition and running conditions.

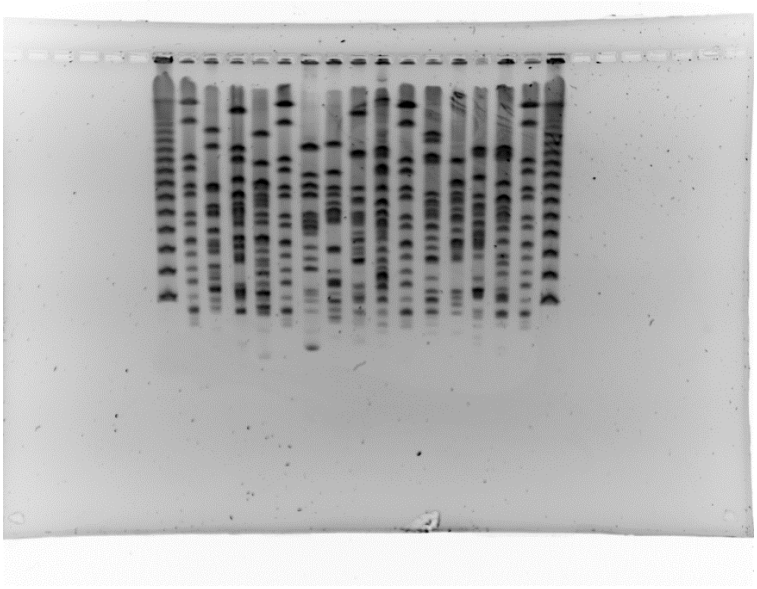
Figure 1: A gel with low scores in all seven parameters



Note: The most critical low score was in the parameter image acquisition and running conditions

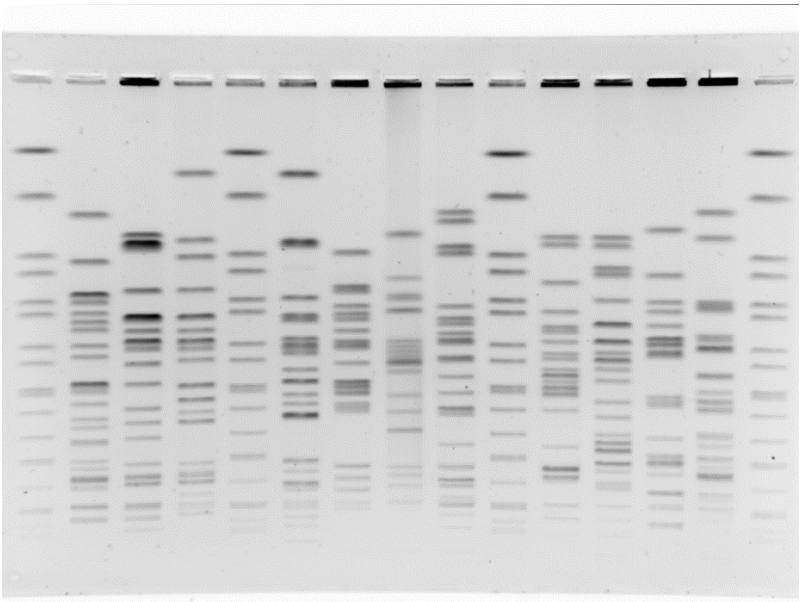
Figure 2 depicts a gel with low scores in the parameters bands and gel background. Scores in Image acquisition and running conditions were also low because the space of the bottom band of the reference strains is not 1–1.5 cm from the bottom of the gel.

Figure 2: A gel with low scores in image acquisition and running conditions, bands, and gel background



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

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



3.2.2 Gel analysis with BioNumerics

Twelve laboratories had analysed their gel and were able to produce XML files according to the protocol attached to the invitation letter (Annex 20 and 21). The gel analysis was evaluated by five parameters according to the BN gel analysis quality guidelines developed at SSI (Annex 4).

Table 5: Results of the BN analysis for 12 laboratories

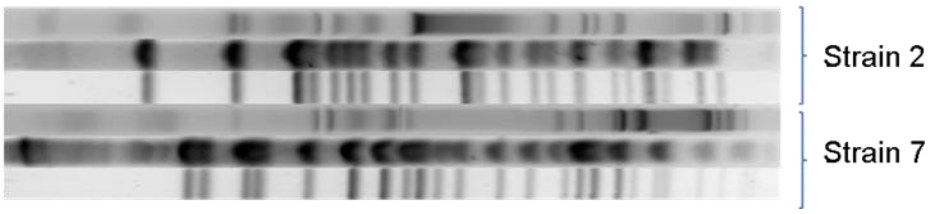
Parameters	1 – poor	2 – fair	3 – good	4 – excellent	Average
Position of the gel	8%	0%	25%	67%	3.5
Strips	8%	0%	0%	92%	3.8
Curves	0%	0%	58%	42%	3.4
Normalisation	25%	8%	33%	33%	2.8
Band assignment	17%	8%	8%	67%	3.3

Table shows five gel analysis grading guideline parameters and the percentage of laboratories that scored in grades 1-4. Also shown is the average score, based on all laboratories.

Two parameters, position of the gel and strips (Table 5), had a high average score above 3.5. Two parameters, curves and band assignment, were received an average score of 3.4 and 3.3, respectively. Only one parameter, normalisation, received an average score of below 3, and 25% of laboratories were unable to perform a proper normalisation. Eight percent of laboratories were unable to correctly position the gel and make strips. Band assignment was poor for 17% of participants.

Normalisation of the gel in BN is crucial. This dependends on the running condition score from the TIFF quality grading guidelines (Annex 4) and the assignment of the correct bands in the *S.* Braenderup reference strain. In Figure 4, the comparison of two strains from three different participants illustrates differences in normalisation. The bottom lanes in both strain 2 and strain 7 are from a gel with high scores in all parameters (Figure 3). The top lanes are from the gel in Figure 1, the middle lanes were taken from the gel in Figure 2. Only the bottom lanes are useful for analysis in BN and further comparisons with other profiles. In the middle lanes the bands are fuzzy and thick, while the top lanes cannot be used for analyses.

Figure 4: Comparison of six strains from three participants in BN



3.3 Serotyping

An average of 78% of the 23–26 participating laboratories could correctly perform O grouping of the 15 EQA test strains (Table 6). Results were lowest (39%) for serotype O177 (BB2) and highest for serotype O157 (II9), which was correctly typed by all laboratories. High correct scores above 92% were obtained for serotypes O26, O103, O111, O121, O128 and O157, which together represent approximately 80% of all VTEC O groups reported to TESSy.

H-typing was correctly performed by an average of 85% of the 13–18 participants, which represents only 52% of the total number of participants. Results were lowest (69%) for the EE5 (H26) and highest (100%) for JJ8 (H4), II9 and LL12 (H7). Correct scores above 80% were obtained for the H types H2, H4, H7, H11, H15, H19, and H21, which together represent approximately 77% of all motile VTEC H types reported to TESSy.

Table 6: Average scores for the O:H serotyping

Strain/method		O:H Serotype	O group	H type
n		13-18	23-26	13-18
		n= 13 for 3 strains, 14 for 6 strains, 15 for 4 strains, 17/18 for II9/LL12 [O157)	n=23 for all 15 strains, 24 for 8 strains, 25 for 4 strains, 26 for II9/LL12 (O157)	n= 13 for all, 14 for 4 strains, 15/17 for 5/1 strains, 18 for II9/LL12 (H7)
AA1	O113:H4	79%	71%	86%
BB2	O177:H25	46%	39%	69%
CC3	O121:H19	86%	92%	86%
DD4	O128:H2/H-	86%	92%	86%
EE5	O41:H26	62%	42%	69%
FF6	O26:H11	87%	100%	87%
GG7	O111:H8/H-	79%	92%	79%
HH8	O104:H4	100%	88%	100%
II9	O157:H7	94%	100%	94%
JJ10	O146:H21	79%	75%	86%
KK11	O103:H2	87%	92%	87%
LL12	O157:H7	100%	96%	100%
MM13	O166:H15	67%	46%	87%
NN14	O78:H11	73%	67%	80%
OO15	O124:H30	79%	75%	79%
Average		**80%**	**78%**	**85%**

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

An average of 80% (46–100%) of laboratories could correctly identify O:H serotype in the 15 test strains. Correct O:H serotyping ranged from 100% for serotypes O104:H4 and O157:H7 to 46% for serotype O177:H25 (Table 6). The two test strains (LL12 and II9) for serotype O157:H7 were correctly serotyped by 100% and 94% of the laboratories, respectively. Serotypes O103:H2 (KK11) and O26:H11 (FF6) were correctly typed by 87% of the participants, and serotypes O128:H2/H- (DD4) and O121:H19 (CC3) were correctly identified by 86% of the participants. The lowest scores were obtained for O177:H25 (BB2) (46% correct) and O41:H26 (EE5) (62% correct). These last two strains with rare serotypes were included because of the presence of *vtx1c* and *vtx1d*. This leads to the conclusion that it is more difficult for laboratories to serotype a strain correctly if the serotypes are less common.

The complete results are listed in Annexes 7 and 8.

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 20–25 laboratories for all the 15 test strains, with high average scores (96–99% correct) (Table 7). With regard to the detection of *eae*, a perfect score was obtained for four strains. Eleven strains (of 15) were incorrectly identified, with both false positive and false negative results. Two participants detected false negative *eae* in strain BB2 and CC3 O177:H25 and O121:H19). Incidentally, most incorrect *eae* results were reported by only one laboratory.

Table 7: Average scores for virulence determination

Strain/method	*eae* gene	*vtx*1 gene	*vtx*2 gene	*ehx*A gene
n	23-24	25	25	20
	n=23 for 4 strains, 24 for 11 strains			
AA1	100%	96%	92%	95%
BB2	91%	100%	96%	100%
CC3	92%	100%	100%	95%
DD4	96%	96%	100%	100%
EE5	96%	84%	96%	100%
FF6	96%	100%	100%	100%
GG7	96%	100%	100%	100%
HH8	96%	100%	100%	100%
II9	96%	100%	100%	100%
JJ10	100%	100%	100%	100%
KK11	96%	100%	100%	100%
LL12	96%	100%	96%	100%
MM13	100%	100%	100%	100%
NN14	100%	100%	100%	95%
OO15	96%	100%	100%	100%
Average	**96%**	**98%**	**99%**	**99%**

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

Detection of *vtx*1 and *vtx*2 genes had high average correct score of 98% and 99% respectively. One laboratory detected two false positive *vtx1* genes in strain AA1 and DD4 (O113:H4 and O128:H2/H-). Additionally, three participants reported false negative results in the strain EE5 (O41:H26) and one of the participants also detected a false negative *vtx*2 gene in strain AA1 (O113:H4). One laboratory reported both a false positive *vtx1* and a false negative *vtx*2 for strain LL12 (O157:H7). Two laboratories reported one false positive *vtx*2 each for strain BB2 or EE5 (O177:H25 or O41:H26). In total, *vtx1* and *vtx2* were misidentified 11 times: *vtx*1 (five false negatives, one false positive), *vtx2* (three false negatives, two false positives).

An average score of 99% was reported for the detection of the *ehx*A. Two false negative results were obtained for strain AA1 (O113:H4) and CC3 (O121:H19), and one false positive for strain NN14 (O78:H11).

Then complete results are presented in Annexes 14, 15, 16 and 17.

3.4.2 Subtyping of *vtx1* and *vtx2*

The number of laboratories participating in subtyping of *vtx* genes ranged from 18 to 20 (average: 71% of the enrolled participates). The average subtyping results of *vtx* genes were calculated based on the number of participants, excluding laboratories which reported false negatives for *vtx1* or *vtx2*. The results indicate that detection of *vtx1* and *vtx2* was used as initial screening, and some laboratories only subtyped strains if the screening was positive. *vtx1* was correctly subtyped by an average of 94% of the participants. The range was 89–95%(*vtx1d* in strain EE5 (O41:H26)) and 100% for *vtx1c* in strains AA1 (O113:H4) and DD4 (O128:H-); *vtx1a* in strain GG7 (O111:H-) and KK11 (O103:H2) was correctly subtyped in 90–95% of all instances.

vtx2 was correctly typed by an average of 93% of the participants. The range was from 80% for *vtx2d* in strain JJ10 (O146:H21) to 100% in strain MM13 (O166:H15). For the combination of *vtx2a* and *vtx2c* in strain ii9 (O157:H7) the range was 85–95%, for *vtx2a* it was 100%, and 95% for *vtx2b*.

False positive *vtx1c* results were reported five times in three strains. One false positive was reported for *vtx1d*. Three false positive results in two strains were reported for *vtx2a*; one false positive result in one strain was reported for*vtx2b*. Eight false positive results in two strains were reported for *vtx2c* and; five false positives in three strains were reported for *vtx2d*. Further false negatives were reported in the following strains: *vtx1a* (2), *vtx1c* (1), *vtx1d* (1), *vtx2a* (1), *vtx2b* (2), two *vtx2c* (2 in one strain) and *vtx2d* (2 in two strains) (Table 8). Thirteen of the 14 (93%) false positive *vtx2* results showed a lack of capability to distinguish between subtypes *vtx2a*, *vtx2c* and *vtx2d*. Eight of the 11 (73%) false negative *vtx2* subtyping results came from one laboratory.

The strains *vtx*2d (JJ10 and MM13) were the strains with the lowest percentage of correct results (70%). The combination strains with two *vtx*2 genes (II9; *vtx2a* and *vtx2c*) also had a low percentage of correct results (75%). The results for strain (AA1) with *vtx1a* and *vtx2b* were very high: 94% of the submitted results were correct.

The average *vtx* subtyping results were calculated to 90%, based on the number of participants and excluding laboratories that reported false negatives for *vtx1* or *vtx2* in order to get less distorted, unskewed test results. The results indicate that detection of *vtx1* and *vtx2* were used as initial screening; errors in the detection of *vtx1* and *vtx2* resulted in further subtyping errors.

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

Strain/method	Original	*vtx1* subtyping[a]			*vtx2* subtyping[a]			*vtx* subtyping[b]
n		**18-20**			**18-20**			**18-20**
		Found *vtx1* gene	False positive	False negative	Found *vtx2* gene	False positive	False negative	% correct
AA1	*vtx1c* and *vtx2b*	18 (95%)	1 *vtx1d*	1	18 (95%)		2	94
BB2								100
CC3	*vtx2a*				18 (95%)		1	95
DD4	*vtx1c*	19 (100%)						100
EE5	*vtx1d*	17 (89%)	3 *vtx1c*	1				79
FF6	*vtx2a*				19 (95%)	2 *vtx2d*		90
GG7	*vtx1a*	18 (90%)	1 *vtx1c*	2				85
HH8								100
II9	*vtx2a* and *vtx2c*				17 (85%)	2 *vtx2d*	2 *vtx2c*	75
JJ10	*vtx2d*				16 (80%)	1 *vtx2a* 1 *vtx2a* and *vtx2c* 1 *vtx2b* 1 *vtx2c* 1 *vtx2c* and *vtx2d*	1	70
KK11	*vtx1a*	19 (95%)	1 *vtx1c*					90
LL12	*vtx2a*				19 (100%)			100
MM13	*vtx2d*				19 (100%)	4 *vtx2c* 1 *vtx2a* and *vtx2c*	1	70
NN14								100
OO15								100
Average		**94%**			**93%**			**90%**

[a] *Percentages for* vtx1 *and* vtx2 *are calculated on the basis of the actual number of performed tests.*

[b] *Total percentages per strain are calculated for the combined* vtx1 *and* vtx2 *subtyping results on the basis of the number of participants, excluding laboratories that reported false negative detection of* vtx1 *or* vtx2*.*

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

Note: See Annex 18 for complete results.

Sensitivity and specificity of the subtyping of the three vtx1 *and four* vtx2 *subtypes are given in Table 9. Sensitivity was 1.00 for* vtx1a, vtx1d *and* vtx2a, *0.95 for* vtx1c *and between 0.89 and 0.92 for* vtx2b, vtx2c *and* vtx2d*. Specificity was 0.96 to 1.00 for all subtypes.*

Table 9: Sensitivity and specificity of *vtx* subtyping results

	vtx1a	*vtx1c*	*vtx2a*	*vtx2b*	*vtx2c*	*vtx2d*
Sensitivity	1.00	0.95	1.00	0.90	0.89	0.92
Specificity	1.00	0.97	0.99	0.99	0.96	0.98

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

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

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

Seventeen laboratories detected *aggR* correctly in strain HH8 (O104:H4). Eleven laboratories also detected the *aaiC* gene, four laboratories detected the *aatA* gene. Three laboratories detected a false positive *aggR* gene in three strains. ETEC-related genes *estA* and *elt*A in strain NN14 (O78:H11) were correctly determined by ten and nine laboratories, respectively; these genes were also correctly identified by eight laboratories in strain MM13 (O166:H15). The *eltA* gene was reported as a false positive in strain KK11 (O103:H2) by one laboratory. Fourteen laboratories correctly detected the marker gene *ipaH* for EIEC in strain OO15 (O124:H30). The gene for heat-stable enterotoxin (EAST1) *astA* was detected by eight laboratories in three strains, but a number of false positives (12 in

five strains) and one false negative result in strain CC3 suggest that many laboratories experienced difficulties in detecting this gene.

Table 10: Detection of additional virulence genes, including false positive and false negative results

Strain No	False positive results (n)[b]	False negative results (n)[a]	*aggR*	*aaiC*	*aatA*	*astA*	*eltA*	*estA*	*ipaH*
AA1						8			
BB2	*astA* (2)								
CC3		*astA* (1)				3			
DD4						8			
EE5	*astA* (1)								
FF6									
GG7	*aggR* (1)								
HH8			17	11	4				
II9	*aggR* (1)					1			
JJ10	astA (1)								
KK11	*elt* (1)								
LL12	*aggR* (1)					3			
MM13						8	8		
NN14	*astA* (7) [b)]						10	9	
OO15	*astA* (1)								14

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

[a] *The true number of false negative results is unknown because the number of laboratories performing these tests was not recorded in the fourth EQA.*

[b] *The high number of false positive results for* astA *suggests a possible cross-reaction with the heat-stable enterotoxin estA.*

WHOCC recorded both positive and negative results while testing the strains for stability.

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

Designations for accepted heat-stable enterotoxin were: est, est1a (ST1a) *or* est1b, estA (sta1), estAp, estIa

Other additional virulence genes detected by the participating laboratories are shown in Table 11 and are only included for future reference. These genes were not tested by the EQA provider.

Table 11: Additional virulence genes in the fourth EQA test strains

Strain no.	Positive gene results (n)
AA1	*cma; cba; celb; eaaA; espI; iha; senB* *subAB* (2) *invE*
BB2	*ehaA* *ent* (2) *escV* (2) *espB; efa1; espA; espF; espI; espJ; lpfA; nleA; nleB; nleC; tccP; tir*_O103H2; *tsh*
CC3	*cba; cma; espB; efa1; espA; espF; espJ; etpD; iha; lpfA; nleA; nleB; nleC; tccP; tir*_O103:H2*; toxB; iss* *ent (2)* *escV (2)* *etp, toxB, ehaA*
DD4	*eaaA; cma; espI; iha; lpfA; iroN; iss; mchB; mchC; mchF* *subAB* (2) *ehaA* *escV*
EE5	*cma; mchB; mchC; mchF*
FF6	escV (2) *espP* (2) *katP* (2) *invE* *ent* *espB; cif; efa1; espA; espF; espJ; iha; lpfA; nleA; nleB; nleC; tccP; tir_O103H2; iss* *etp, toxB, ehaA*
GG7	*ent* (2)

Strain no.	Positive gene results (n)
	escV (2) *cba; celb; cif; efa1; espA; espF; espJ; iha; lpfA; nleA; nleB; tccP*; *tir*_O111 ehaA
HH8	*pic* (4) *aggA* (3) *aap* (2) *pet* *aggC* (2) *aggD* *ehaA* *iha; lpfA; sepA; sigA; mchB; mchC; mchF*
II9	*ent* (2) *escV* (2) *espP* (2) *katP* (2) *espF; espJ; etpD; iha; nleA; nleB; nleC; tccP; tir*_O157:H7*; toxB* *etp, toxB, ehaA*
JJ10	*subAB* (2) *celb; eaaA; iha; lpfA; iss* *ehaA, eibG* hlyA
KK11	*escV* (2) *katP* (2) *ent* *espB; cif; efa1; espF; espJ; nleA; nleB; tccP; tir*_O103:H2 *etp, ehaA*
LL12	*ent* (2) *escV* (2) *katP* (2) *espP* (2) *espF; espJ; etpD; iha; nleA; nleB; nleC; tccP; tir*_O157:H7 *toxB*(2) *etp, ehaA* bla-TEM1; bla-CTX-M-15
MM13	*iss* (ltcA) invE bla-CTX-M-15
NN14	
OO15	*iha; pic; sigA; ipaD* *ial* EIEC

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

Genes detected by participating laboratories: aap, aggA, aggC, aggD, bfpA, bfpB., bla-CTX-M-15, bla-TEM1, cba, celb, cif, cma, cma, DA, eaaA, efa1, ehaA, eibG, EIEC, ent, escV, espA, espB, espF, espI, espJ, espP, etp, etpD, fliCH7, hlyA, ial, iha, invE, ipaD, ipaH, iroN, iss, katP, lpfA, mchB, mchC, mchF, nleA, nleB, nleC, pet, pic, senB, sepA, sigA, stb, subAB, tccP, tir_O103:H2, tir_O111, tir_O157:H7, toxB and tsh.

3.4.4 Phenotypic test

Participation in phenotypic detection ranged from 36% (VCA/EIA) to 93% (sorbitol fermentation). Correct results of 100% were reported for the production of ESBL, a test that was newly introduced to the fourth EQA (Table 12). One laboratory submitted the ESBL-positive strain LL12 as ESBL negative but the result was accepted because plasmid loss was observed once during the stability test.

Table 12: Average scores of the phenotypic tests

Strain/method	VCA/EIA	ESBL production	Haemolysin production	β-glucuronidase production	Sorbitol fermentation
n	10-11	17	13-15	18-19	25-26
	n=10 for all 15 strains, 11 for 10 strains		n=13 for all 15 strains, 14 for 8 strains, 15 for KK11	n=18 for all 15 strains, 19 for DD4	n=25 for DD4, 26 for 14 strains
AA1	91%	100%	79%	94%	96%
BB2	90%	100%	79%	89%	96%
CC3	91%	100%	79%	94%	96%
DD4	90%	100%	100%	95%	96%
EE5	91%	100%	100%	94%	92%
FF6	91%	100%	79%	94%	92%
GG7	91%	100%	79%	89%	96%
HH8	90%	100%	100%	94%	92%
II9	91%	100%	79%	94%	96%
JJ10	82%	100%	100%	94%	92%
KK11	91%	100%	80%	89%	96%
LL12	91%	100%	79%	94%	92%
MM13	82%	100%	100%	94%	92%
NN14	90%	100%	100%	94%	96%
OO15	90%	100%	100%	94%	100%
Average	**89%**	**100%**	**89%**	**93%**	**95%**

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 89% for both VCA/EA and enterohaemolysin production, 93% for β-glucuronidase production, 95% for fermentation of sorbitol,l and 100% for ESBL (Table 12).

Most of the errors in the detection of sorbitol fermentation were submitted by a single laboratory, and we suspect that the positive (1) and negative (2) results were swapped during result submission. With regard to the detection of VCA/EIA production, one laboratory submitted only positive results for all 15 strains, which might also be a submission error. Three laboratories submitted errors in β-glucuronidase production: one laboratory reported 13 errors, while the other two submitted three and two errors, respectively.

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

4 Conclusions

Twenty-eight laboratories signed up for the fourth EQA on VTEC typing funded by ECDC. For the first time, the EQA also included PFGE, and a total of 20 laboratories participated in the PFGE exercise. Nine (45%) of the laboratories were able to produce a PFGE gel of sufficiently high quality to allow comparison with profiles obtained by other laboratories. (Comparability primarily relies on the use of correct running conditions, good quality image acquisition, and distinct bands.) The BN software suite was used for the normalisation and interpretation of profiles. Twelve (60%) laboratories analysis the resulting gels, and 50% of these laboratories performed in good accordance with the guidelines.

About half of the laboratories (52%) participated in full O:H serotyping, and an average of 80% of the laboratories were able to correctly serotype the fifteen test strains (11 VTEC strains and one strain each of non-VTEC strains A/EEC, EAEC, ETEC and EIEC). Correct O grouping was performed by an average of 78% of the participants; 85% of the H typing results were also correct.

Gene detection of *eae, vtx1*, *vtx2* and *ehxA* was 96–99% correct. One laboratory submitted largely incorrect results for *eae*; this was thought to be due to incorrect laboratory procedures or submission errors. Participation rate was 85% for *eae*, 89% for both *vtx1* and *vtx2,* and 71% for *ehxA.* Subtyping for *vtx* was performed by 71% of the participants, with an average of 90% of correct results.

Phenotypic characterisation generally showed very good results: 89% correct results for VT and enterohaemolysin production, 95% for fermentation of sorbitol, 93% for β-glucuronidase production, and 100% for detection of ESBL production. Phenotypic characterisation was not performed as often as genotypic characterisation: detection of VT production (36% of the participants), enterohaemolysin production (49%), β-glucuronidase production (65%), sorbitol (93%), and ESBL (61%).

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

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

5 Discussion

The WHO Collaborating Centre for Reference and Research on *Escherichia* and *Klebsiella* (WHOCC), Unit of Foodborne Infections at the SSI in Copenhagen, Denmark, has played a leading role in establishing a worldwide international network of quality evaluation and assessment for the typing of *E. coli* since 2002. The first two serotyping ring trials were conducted in 2002 and 2003. In 2005, the third ring trial was launched, including serotyping, virulence typing, and PFGE typing of *E. coli*. The PFGE part of the ring trial was jointly coordinated with PulseNet Europe as part of the 'PulseNet Europe Feasibility Study' of VTEC [20]. The fourth international ring trial in 2006 was, apart from the regular O:H serotyping, centred on the capacity to detect the *vtx* genes. In 2007, the fifth ring trial EQA scheme for sero- and virulence typing of VTEC was arranged. In 2008, the WHOCC was awarded the tender launched by ECDC for the typing of VTEC (fourth EQA).

5.1 Pulsed-field gel electrophoresis (PFGE)

Twenty laboratories participated in the PFGE part of the fourth EQA. All laboratories were able to produce a PFGE gel and generate an image of the gel (TIFF file). We graded the gel quality according to the TIFF quality grading guidelines which evaluate seven parameters. Scores were given between 1 and 4 (poor, fair, good and excellent). The majority (70%) of laboratories were able to produce gels with sufficiently high quality (above poor score of 1; sufficient quality to be compared to gel profiles obtained by other laboratories) for five parameters: cell suspension, lanes, restriction, gel background and DNA degradation. These five parameters are therefore not the most problematic in this EQA, but it is still desirable to improve the laboratories' capacity in these areas. In the remaining two parameters, overall results were acceptable: bands received scores of 3 or 4 (30%, good–excellent), while 40% of image acquisition and running conditions were given a score of 3 and 4 (40%, good–excellent).

Three laboratories had low scores in several parameters, including bands and image acquisition and running conditions. In general, for a highly sensitive method such as PFGE, it is paramount 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 DNA lysis step, the recommended time of restriction for the relevant enzyme, washing plugs six times as recommended, and de-staining the gel adequately after dying.

Major improvements can be achieved in capturing the image of the gel and producing a TIFF file. Many laboratories seem to increase the contrast of the image in order to enhance weak bands, which leads to reduced grey levels, thicker bands, and blurs differences between double bands. This practice, together with the overloading of plugs with DNA, contributed significantly to the low scores in the bands category.

Many laboratories had problems with the category image acquisition and running conditions. This is crucial, as incorrect running conditions render the gel useless as it cannot be compared with gels run at correct conditions. It is important to adjust running conditions as described for the relevant organism because they vary significantly between species. It is also important to make sure that all laboratory equipment functions properly. Temperatures need to be set as described in the protocol.

The grading guidelines indicate that a score of 2 (fair) can be obtained for image acquisition and running conditions even if band spacing does not match the global standard because poor results for this parameter can be compensated by higher scores in other parameters. However, this does not necessarily mean that gels which have obtained an overall score of 2 or better are suitable for interlaboratory comparison. During the fourth VTEC EQA some of the gels with a score of 2 were still dismissed as 'non-comparable'.

Another common deviations from the protocol is image acquisition and production: many laboratories fail to fill the entire image with the gel, include wells, and left 1 to 1.5 cm of space below the smallest gel band. This is less critical than using incorrect running conditions, but still has an impact on the ability to assign bands correctly.

Only 60% of the laboratories that performed PFGE did the subsequent gel analysis, i.e. the normalisation and band assignment that 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. Six of the 12 laboratories that submitted gel analysis data analysed PFGE gels in fair to excellent (2–4) accordance with the guidelines.

5.2 Serotyping

Participation in O:H serotyping was roughly the same in the third and fourth EQA (>50%). An average of 80% of the 23–26 participating laboratories could correctly perform O grouping of the 15 test strains, which is an increase compared to the third EQA (68%).

Participation in the fourth EQA was also higher (87% versus 83%) in O group typing, but somewhat lower (52% versus 56%) in H typing.

This fourth EQA had 28 EU/EEA participants, which is three more than the last EQA. Correct O:H serotyping ranged from 100% correct typing of one of the two O157:H7 and O104:H4 strains to 46% correct typing of serotype O177:H25. Correct results for O:H serotyping in the fourth EQA were higher (80%) than in the third EQA (63%). This may in part be explained by one additional laboratory now performing H typing.

The inclusion of non-VTEC O:H serotypes did not seem to influence the level of performance. However, the general trend (in both EQAs) was that the more common serotypes are identified more reliably. No systematic typing errors were observed. Serotype O177 was reported as O26, O118 and O145 by three laboratories; serotype O41 was identified as O115 and O150 by two laboratories; serotype O26 was once misidentified as O6 (most likely a submission error where '2' was omitted); and serotype O166 was reported as O111 by one laboratory. The remainder of incorrect typing was submitted as unknown (UNK) for 59 results in 13 strains, seven were reported as non-typeable (NT) in five strains, and six were identified as non-O157s in five strains.

Incorrect H types were H5 instead of H4, H30 instead of H25, H41 instead of H26, H21 instead of H11 (in two strains by the same laboratory), H8 instead of H21, H44 instead of H2, and H4 instead of H30. The only known cross-reaction is the H11*H21 cross-reaction, and only one laboratory could not resolve this. Some of the results could represent submission errors rather than errors in methodology.

In summary, 18 of 26 (69%) laboratories were able to correctly determine the O group of the seven strains that were suggested as a minimal requirement for the typing of VTEC in the EU-level by ECDC (not published). Half of these laboratories (nine out of 18) were able to correctly determine the O:H serotype in the same seven strains. The addition of non-VTEC strains did not affect the general level of performance.

5.3 Virulence determination

5.3.1 Genotypic tests

Genotypic detection of virulence genes *eae*, *vtx1, vtx2* and *ehxA* was performed by 20–25 laboratories for all the 15 test strains; results were 96–99% correct. The participation rate varied substantially between the different tests in the fourth EQA, being highest for the genotypic detection of *eae* and the *vtx* genes (range 85–89%) and lowest for the detection of *ehxA* (71%). In general, the percentage of correct results was very high (96–99%). The incorrect results for the *eae* gene originated primarily in one laboratory which reported 11 incorrect results. Otherwise, the *eae* gene was only missed twice by two different laboratories. The average correct score is the same as in the third EQA (96%).

Detection of *vtx1* and *vtx2* genes was achieved with a high percentage of correct results (average 98% and 99%, respectively), very similar to the third EQA (*vtx1*: 98%; *vtx2*: 98%). In the third EQA, the major problem in subtyping the *vtx* genes was to distinguish between *vtx2a*, *vtx2b*, *vtx2c* and *vtx2d*. A revised protocol for subtyping of *vtx* genes was published in September 2012 [19]. Previously recorded problems with significant sensitivity to different PCR cycler equipment and use of different DNA polymerases seem to have been resolved in the majority of participating laboratories, mainly by adherence to the revised protocol. The new protocol was used by 71% of the participants, and correct results ranged from 89–100% for subtyping *vtx1* and from 80–100% for subtyping *vtx2*. Correct subtyping of both *vtx1* and *vtx2* was obtained at an average of 90%. The majority of false positive results originated from two laboratories, while false negative results came from one laboratory. Laboratories received advice on how to test and calibrate their PCR cyclers in order to improve their performance. Compared to the third EQA, the average correct score of *ehxA* was the same in the fourth EQA – 99% of the results were correct, and only two false negative results and one false positive result were obtained.

5.3.2 Phenotypic tests

The participation in the phenotypic detection was between 38 and 93% on average (11–26 laboratories). The lowest participation was for VCA/EIA, where only 10 participants (36%) delivered results for 10 strains. The same number of participants delivered results in the third EQA.

In general, the most important phenotypic test is the sorbitol fermentation, which is used to screen for the highly virulent SF O157:H7 clone. It is therefore encouraging that the fermentation of sorbitol was performed more often (93% compared to 78% in the third EQA) than any other phenotypical test. The average results (95% correct)

were influenced by submission errors made by a single laboratory which mostly submitted incorrect results (93%, 14 out of 15). The remaining six incorrect results were submitted by six different laboratories on six different strains. Participation in the other phenotypic detection tests was relatively low. The second highest participation was for β-glucuronidase activity (65% compared with 58% in the third EQA), followed by ESBL (61%), enterohaemolysin (49%, compared with 42% in the third EQA) and VCA/EAI (38%, compared with 40% in the third EQA) production. Thirteen strains out of 15 (87%) had incorrect results for β-glucuronidase production; all incorrect results were submitted by a single laboratory as 'negative', which suggests that 'negative' was merely confused with 'not done'. The average percentage of correct results was 93%. All 24 incorrect results for haemolysin production were reported by three laboratories. Three to six incorrect results on VCA production came from four laboratories. ESBL was always correctly detected (correct results by all 17 participating laboratories) because we accepted one negative result due to plasmid loss, which had been observed earlier by the EQA provider.

In summary, the performance level for phenotypic characterisation was very high but some laboratories need to ensure that results are correctly verified before submission.

5.3 General remarks

The inconsistency in the number of performed tests per strain and per laboratory was notable in all VTEC EQAs so far. Laboratories never explained why a specific test was not performed on all 15 test strains (or 11 for PFGE). This was particularly evident for O grouping where three laboratories submitted multiple instances of 'not done' strains (13, 9, 1). A similar situation was encountered for H typing (13, 10, 3 strains marked as 'not done') and *eae* detection (one laboratory officially signed up but submitted 'not done' for four of the 15 strains). These inconsistencies reduce comparability between the tests and the laboratories and complicate the analyses.

6 Recommendations

6.1 Laboratories

This EQA was instrumental in identifying a number of technical issues that negatively affected the quality of typing results.

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

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

A number of avoidable errors were made. Many errors could have been avoided if laboratory personnel had carefully read the instructions on how to produce and submit TIFF and XML files of the PFGE results. Other errors were caused by losing track of numbering and strain numbers and by failing to proofread the results before submission. For this first EQA on molecular typing, some errors in procedures were accepted and forwarding extra results/information/corrections after the deadline was accepted in some instances.

Only half of the laboratories participated in O:H serotyping and it will be a challenge to increase participation. Serotyping is essential for the characterisation of *E. coli* and VTEC. A survey among non-participating laboratories could explore the underlying reasons.

Regarding both genotypic and phenotypic tests, it is evident from the results and discussion that only a small number of laboratories encountered difficulties. If these laboratories are excluded from the overall results, the level of performance is very high. Additional trouble shooting and training activities should be considered for laboratories with poor performance.

6.2 ECDC and FWD-Net

The PFGE part of the fourth VTEC EQA had a 71% participation rate; 60% of the participating laboratories performed the BN gel analysis. Less than half (45%) of the gels produced were of sufficiently high quality for inter-laboratory comparison, and half of the BN analyses were at an acceptable level. Compared with the *Salmonella* and *Listeria* EQAs, the gel quality scores of the fourth VTEC EQA were lower, demonstrating the need to improve laboratory procedures, gel analysis, and interpretation with BN software.

The relatively low levels of participation in O grouping and H typing need to be explored and the reasons for the low participation rates have to be addressed. Similarly, the phenotypic tests for VT production and β-glucuronidase production are only performed by a small number of laboratories.

6.3 The EQA provider

The methodology for grading the quality of PFGE gels is part of the ECDC SOP for EU molecular surveillance (TESSy MSS database) and was adopted from PulseNet. This methodology does not automatically result in a score which clearly indicates whether a gel is suitable for interlaboratory comparisons: occasionally, a fair overall score would be given to a gel that could not be compared with other gels. Future EQAs will make sure that gels which cannot be used for comparisons with gels from other laboratories will be rated 'poor' by default.

Future EQAs will continue to not penalise laboratories if O group detection renders an 'unknown' result. 'Not done', however, will be considered as a negative result and scored accordingly. Participants will be informed of this and other changes in a letter and in the accompanying protocol.

Many laboratories informed the EQA organiser that they would prefer a more relaxed schedule. As much as the EQA organiser would like to give more time to the laboratories, time is limited by the fact that the evaluation of EQA results is time-consuming (visual evaluation of the PFGE gels and additional analysis) and that the production of the individual evaluation reports and the final report also require a considerable amount of time. Also, the EQA organiser has to provide individual feedback on the molecular methods, which also cuts into the available time.

This first EQA on molecular typing methods used a rather lenient scoring procedure; part of this lenience has to be attributed to our guidelines which were not sufficiently detailed. For example, it was not properly explained how to set up and use a BN database for the EQA data or how to create and name the files for submission.

These shortcomings will be corrected for the next round of the EQA; at the same time, all deadlines for submissions will be strictly enforced.

7 References

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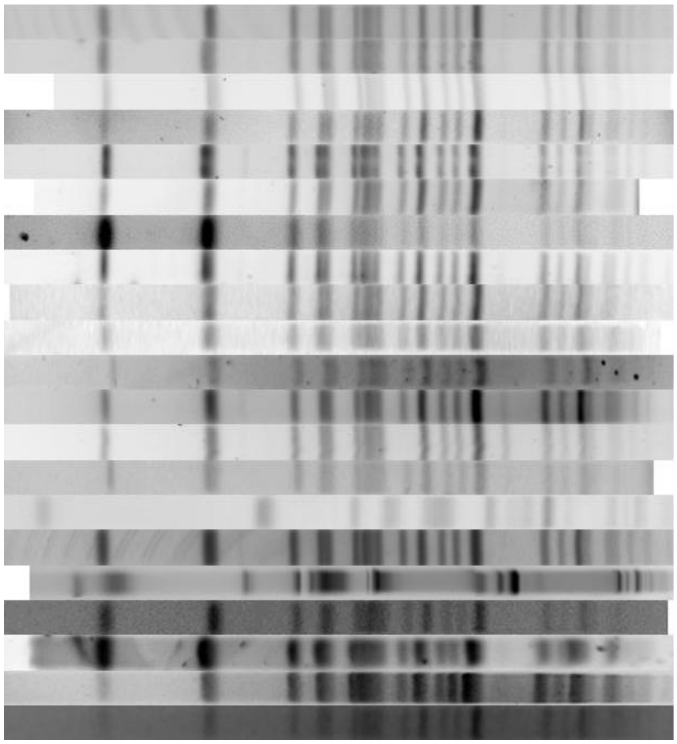
Annex 1. List of participants

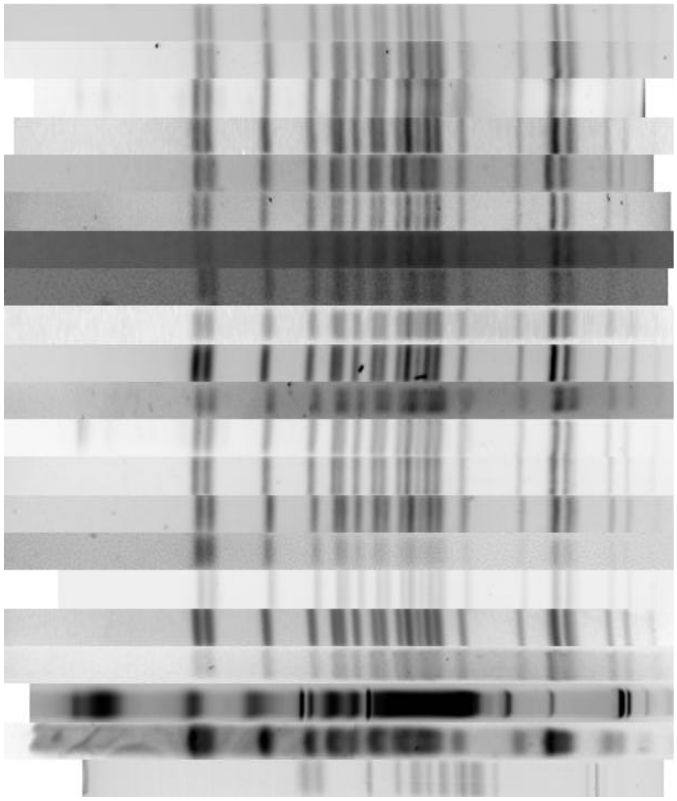
Country	Institute/organisation	Laboratory
Austria	Bereich Humanmedizin, Institut für medizinische Mikrobiologie und Hygiene	Österreichische Agentur für Gesundheit und Ernährungssicherheit GmbH
Belgium	Universitair Ziekenhuis Brussel	Dienst microbiologie en ziekenhuishygiëne
Bulgaria	National Center of Infectious and Parasitic Diseases	NRL for enteric pathogens
Cyprus	Nicosia General Hospital	Microbiology department of Nicosia General Hospital
Czech Republic	National Institute of Public Health	NRL for *E. coli* and *shigella*
Denmark	Statens serum institut	Unit of Foodborne Infections
Estonia	Health board	Central Laboratory of Communicable Diseases
Finland	THL, Institute of Health and Welfare	Bacteriology unit
France	Unité des bactéries pathogènes entériques	Centre national de référence des escherichia coli, des *shigella* et *Salmonella*
Germany	Robert Koch Institute, Bereich Wernigerode	National reference centre for *Salmonella* and other bacterial enterics
Greece	National School of Public Health clbh/hcdcp	National reference centre for *Salmonella* and other enteropathogens
Hungary	National Center for Epidemiology, országos epidemiológiai központ	Bacteriology II Department
Iceland	Landspitali University Hospital	Department of clinical microbiology
Ireland	Cherry Orchard Hospital	Public health laboratory
Italy	Istituto superiore di sanità, national institute of health	Dipartimento di sanità pubblica veterinaria e sicurezza alimentare istituto
Latvia	Riga East Clinical University Hospital	Bacteriology department
Luxembourg	Laboratoire national de sante	Department of microbiology
Netherlands	National Institute for Public Health and the Environment	Laboratory for infectious diseases and perinatal screening
Norway	Nasjonalt folkehelseinstitutt	Avdeling for næringsmiddelbårne infeksjoner
Poland	National Public Health Institute, National Institute of Hygiene	Department of bacteriology
Portugal	Instituto nacional de saúde dr. Ricardo Jorge	Departamento de doenças infecciosas
Romania	Cantacuzino National Institute of Research-Development for Microbiology & Immunology	Molecular epidemiology laboratory
Serbia	Military Medical Academy, Institute of Epidemiology	Laboratory for molecular genetics
Slovenia	National Institute of Public Health	Department of medical microbiology
Spain	National Center of Microbiology, Institute of Health Carlos III	Laboratory of enterobacteriaceae, campylobacter and vibrio
Sweden	Smittskydsinstitutet	Food and water unit
Turkey	Public Health Institution of Turkey	National reference laboratory for enteric pathogens
United Kingdom	Gastrointestinal Bacteria Reference Unit (GBRU)	*E. coli*, *Shigella*, *Yersinia* & *Vibrio* (ESYV) Reference Services

Annex 2. Examples of PFGE profiles

Figure A2-1: Sample profile from participants



Strain 5



Strain 8

Annex 3. TIFF quality grading guidelines[2]

Parameter	TIFF Quality Grading Guidelines			
	Excellent	**Good**	**Fair**	**Poor**
Image Acquisition and Running Conditions	By protocol, for example: - Gel fills whole TIFF - Wells included on TIFF - Bottom band of standard 1-1.5 cm from bottom of gel	- Gel doesn't fill whole TIFF but band finding is not affected	Not protocol; for example, one of the following: - Gel doesn't fill whole TIFF and band finding is affected - Wells not included on TIFF - Bottom band of standard not 1-1.5 cm from bottom of gel - Band spacing of standards doesn't match global standard	Not protocol; for example, >1 of the following: - Gel doesn't fill whole TIFF and this affects band finding - Wells not included on TIFF - Bottom band of standard not 1-1.5 cm from bottom of gel - Band spacing of standards doesn't match global standard
Cell Suspensions	The cell concentration is approximately the same in each lane	1-2 lanes contain darker or lighter bands than the other lanes	- >2 lanes contain darker or lighter bands than the other lanes, or - At least 1 lane is much darker or lighter than the other lanes, making the gel difficult to analyze	The cell concentrations are uneven from lane to lane, making the gel impossible to analyze
Bands	Clear and distinct all the way to the bottom of the gel	- Slight band distortion in 1 lane but doesn't interfere with analysis - Bands are slightly fuzzy and/or slanted - A few bands (e.g., ≤3) difficult to see clearly (e.g., DNA overload), especially at bottom of gel	- Some band distortion (e.g., nicks) in 2-3 lanes but still analyzable - Fuzzy bands - Some bands (e.g., 4-5) are too thick - Bands at the bottom of the gel are light, but analyzable	- Band distortion that makes analysis difficult - Very fuzzy bands. - Many bands too thick to distinguish - Bands at the bottom of the gel too light to distinguish
Lanes	Straight	- Slight smiling (higher bands in the outside lanes vs. the inside) - Lanes gradually run longer toward the right or left - Still analyzable	- Significant smiling - Slight curves on the outside lanes - Still analyzable	- Smiling or curving that interferes with analysis
Restriction	Complete restriction in all lanes	- One to two faint shadow bands on gel	- One lane with many shadow bands - A few shadow bands spread out over several lanes	- Greater than 1 lane with several shadow bands - Lots of shadow bands over the whole gel
Gel Background	Clear	- Mostly clear background - Minor debris present that doesn't affect analysis	- Some debris present that may or may not make analysis difficult (e.g., auto band search finds too many bands) - Background caused by photographing a gel with very light bands (image contrast was "brought up" in photographing gel-makes image look grainy)	- Lots of debris present that may or may not make analysis difficult (i.e., auto band search finds too many bands)
DNA Degradation (smearing in the lanes)	Not present	- Minor background (smearing) in a few lanes but bands are clear	- Significant smearing in 1-2 lanes that may or may not make analysis difficult - Minor background (smearing) in many lanes	- Significant smearing in >2 lanes that may or may not make analysis difficult - Smearing so that a lane is not analyzable (except if untypeable [thiourea required])

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

Annex 4. BioNumerics (BN) gel analysis quality guidelines

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

Annex 5. Scores of the PFGE results

Gel quality

	123	124	127	34	19	136	129	139	130	131	133	132	222	134	135	153	90	108	100	114
Image acquisition and running conditions	3	1	1	4	4	4	2	2	4	2	2	1	1	1	1	1	3	3	1	3
Cell suspension	4	1	4	4	4	3	4	2	3	3	4	4	4	4	3	4	4	4	3	3
Bands	1	1	2	2	4	3	1	1	2	3	4	1	2	1	3	2	4	2	2	2
Lanes	4	1	4	3	4	3	4	3	4	4	4	4	4	3	4	3	4	4	4	3
Restriction	4	2	3	4	4	4	4	1	4	3	4	2	3	1	1	4	4	4	4	3
Gel background	2	1	3	4	4	3	3	1	3	2	4	1	2	4	3	4	4	4	3	3
DNA degradation	4	1	1	3	4	4	4	1	2	4	4	1	4	1	3	4	4	4	4	4

Scored according to Annex 3 (TIFF quality grading guidelines)

BN analysis

	123	127	34	19	129	130	132	222	134	135	153	90
Position of the gel	4	3	4	4	4	3	3	1	4	4	4	4
Strips	4	4	4	4	4	4	4	1	4	4	4	4
Curves	4	3	3	4	3	4	3	4	3	3	3	4
Normalisation	1	2	4	1	3	4	3	4	3	4	1	3
Band assignment	4	2	1	4	4	4	1	3	4	4	4	4

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 production	Sorbitol fermentation	*eae* gene	*ehxA* gene	*vtx1* gene	*vtx2* gene	*vtx* Subtypes			Additional virulence genes	Pathogenic group
AA1	O113	H4	Pos.	Neg.	Pos.	Pos.	Pos.	Neg.	Pos.	Pos.	Pos.	*vtx1c*		*vtx2b*		STEC/VTEC
BB2	O177	H25	Neg.	Neg.	Pos.	Pos.	Pos.	Pos.	Pos.	Neg.	Neg.					AEEC
CC3	O121	H19	Pos.	Neg.	Pos.	Pos.	Pos.	Pos.	Pos.	Neg.	Pos.		*vtx2a*			STEC/VTEC
DD4	O128	H2/H-	Pos.	Neg.	Neg.	Pos.	Pos.	Neg.	Neg.	Pos.	Neg.	*vtx1c*				STEC/VTEC
EE5	O41	H26	Pos.	Neg.	Neg.	Pos.	Pos.	Neg.	Neg.	Pos.	Neg.	*vtx1d*				STEC/VTEC
FF6	O26	H11	Pos.	Neg.	Pos.	Pos.	Pos.	Pos.	Pos.	Neg.	Pos.		*vtx2a*			STEC/VTEC
GG7	O111	H8/H-	Pos.	Neg.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Neg.	*vtx1a*				STEC/VTEC
HH8	O104	H4	Neg.	Neg.	Neg.	Pos.	Pos.	Neg.	Neg.	Neg.	Neg.				*aggR, aaiC , aatA*	EAggEC
II9	O157	H7	Pos.	Neg.	Pos.	Neg.	Neg.	Pos.	Pos.	Neg.	Pos.		*vtx2a*	*vtx2c*		STEC/VTEC
JJ10	O146	H21	Pos.	Neg.	alfa	Pos.	Pos.	Neg.	Neg.	Neg.	Pos.			*vtx2d*		STEC/VTEC
KK11	O103	H2	Pos.	Neg.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Neg.	*vtx1a*				STEC/VTEC
LL12	O157	H7	Pos.	Pos.[b]	Pos.	Neg.	Neg.	Pos.	Pos.	Neg.	Pos.		*vtx2a*			STEC/VTEC
MM13	O166	H15	Pos.	Pos.	Neg.	Pos.	Pos.	Neg.	Neg.	Neg.	Pos.			*vtx2d*	*elt*	STEC/VTEC
NN14	O78	H11	Neg.	Neg.	Neg.	Pos.	Pos.	Neg.	Neg.	Neg.	Neg.				*estAp, elt*	ETEC
OO15 [a]	O124	H30	Neg.	Neg.	Neg.	Pos.	Pos.	Neg.	Neg.	Neg.	Neg.				*ipaH*	EIEC

Pos. = Positive, Neg. = Negative, alfa = positive for alfahaemolysin, but entero/alfahaemolysin results were accepted for all strains.

Intermediate result noted in the VCA was accepted as a positive result. Other additional virulence genes are described in Table 10.

a) Lactose negative

b) The strain lost the ESBL plasmid. Therefore, both results were accepted.

Gene abbreviations			
eae	CVD434. *E. coli* attaching and effacing gene probe	*eltI*	G119. Heat-labile enterotoxin (LT). Almost identical to cholera toxin
ehxA	CVD419. Plasmid-encoded O157-enterohaemolysin	*aatA*	PCR fragment. The gene encodes the dispersin (aap) transporter protein, which is a good plasmid marker for enteroaggregative *E. coli*
vtx1	NTP705. Verotoxin1; almost identical with Shiga toxin	*estA p*	DAS101. Heat-stable enterotoxin (porcine variant) ST_p (STIa)
vtx2	DEP28. Verotoxin2; variants exist. Approximately 60% homology to *vtx1*	*ipaH*	WR390. Invasion plasmid antigen. These genes are found in several copies, chromosomally as well as on plasmids.
aggR	Gene encoding the master regulator in enteroaggregative *E. coli*	*aaiC*	Chromosomal gene marker for enteroaggregative *E. coli*

Annex 7. O group serotyping results

Strain/ laboratory	Original	19	34	80	88	94	100	108	114	123	124	125	127	128	129	130	131	132	133	134	135	136	137	138	139	153	222	%
AA1	O113	O113	O113	O113	O113	UNK	O113	O113	O113	O113	UNK	O113	UNK	ND	O113	O113	O113	ND	UNK	UNK	O113	O113	O113	NON-O157	O113	UNK	O113	71
BB2	O177	O177	O177	O177	O177	UNK	O177	O177	O145	O177	O26	O145	UNK	ND	UNK	UNK	UNK	ND	UNK	UNK	O177	O177	ND	UNK	O118	UNK	UNK	39
CC3	O121	O121	O121	O121	O121	UNK	O121	O121	O121	O121	O121	UNK	O121	ND	O121	O121	O121	ND	O121	O121	O121	O121	O121	O121	O121	O121	O121	92
DD4	O128	O128	O128	O128	O128	O128	O128	O128	O128	O128	UNK	O128	O128	ND	O128	O128	O128	ND	UNK	O128	O128	O128	O128	O128	O128	O128	O128	92
EE5	O41	O41	O41	UNK	NON-O157	UNK	O41	O41	O150	O41	UNK	O115	UNK	ND	UNK	UNK	O41	ND	UNK	UNK	O41	O41	O41	NON-O157	O41	UNK	UNK	42
FF6	O26	O26	O26	O26	O26	O26	O26	O26	O26	O26	O26	O26	O26	O26	O26	O26	O26	ND	O26	O26	O26	O26	O26	O26	O26	O26	O26	100
GG7	O111	O111	O111	O111	O111	UNK	O111	O111	O111	O111	O111	O111	O111	O111	O111	O111	O111	ND	O111	O111	O111	O111	O111	NON-O157	O111	O111	O111	92
HH8	O104	O104	O104	O104	NON-O157	UNK	O104	O104	O104	O104	O104	O104	O104	O104	O104	O104	O104	ND	O104	O104	O104	O104	O104	UNK	O104	O104	O104	88
II9	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	100
JJ10	O146	O146	O146	O146	O146	UNK	O146	O146	O146	O146	UNK	O146	O146	ND	O146	O146	O146	ND	UNK	UNK	O146	O146	O146	NON-O157	O146	UNK	O146	75
KK11	O103	O103	O103	O103	O103	UNK	O103	O103	O103	O103	O103	O103	O103	O103	O103	O103	O103	ND	UNK	O103	O103	O103	O103	O103	O103	O103	O103	92
LL12	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	UNK	O157	96
MM13	O166	O166	O166	UNK	NT	UNK	O166	O166	O166	O166	UNK	O86	O166	ND	UNK	UNK	O166	ND	UNK	UNK	O166	O166	O166	O111	NT	UNK	UNK	46
NN14	O78	O78	O78	O78	O78	UNK	O78	O78	O78	O78	UNK	O78	O78	ND	UNK	UNK	O78	ND	UNK	O78	O78	O78	O78	UNK	O78	UNK	UNK	67
OO15	O124	O124	O124	O124	NT	O124	O124	O124	O124	O124	UNK	O124	O124	ND	O124	UNK	O124	ND	UNK	O124	O124	O124	O124	UNK	O124	O124	UNK	75

Incorrect result	ND: Not done; corrected result accepted after deadline (approved by ECDC)

Annex 8. H type serotyping results

Strain/laboratory	Original	19	34	80	100	108	114	123	124	125	127	129	131	134	135	136	137	138	139	%
AA1	H4	H4	H4	H4	ND	H4	H4	H4	H4	ND	ND	H4	H4	UNK	H4	H4	H5	ND	H4	86
BB2	H25	H25	H25	H-	ND	H25	H25	H25	UNK	ND	ND	H30	H-	H25	H25	H25	ND	ND	H25	69
CC3	H19	H19	H19	H19	ND	H19	H1	H19	UNK	ND	H19	H19	H19	H19	H19	H19	ND	ND	H19	86
DD4	H-/H2	H-	H2	H2	ND	H2	H2	H-	UNK	ND	H2	H2	H-	NT	H-	H2	ND	ND	H2	86
EE5	H26	H26	H26	UNK	ND	H26	H5	H26	UNK	ND	ND	H26	H26	H41	H26	H26	ND	ND	H26	69
FF6	H11	H11	H11	H11	H11	H11	H11	H11	UNK	ND	H11	H11	H21	H11	H11	H11	ND	ND	H11	87
GG7	H-/H8	H-	H8	H8	ND	H8	H-	H-	UNK	ND	H8	H8	NT	UNK	H-	H8	ND	ND	H8	79
HH8	H4	H4	H4	H4	H4	H4	H4	H4	H4	ND	H4	H4	H4	H4	H4	H4	ND	ND	H4	100
II9	H7	H7	H7	H7	H7	H7	H7	H7	H7	UNK	H7	H7	H7	H7	H-	H7	ND	H7	H7	94
JJ10	H21	H21	H21	H8	ND	H21	H21	H21	UNK	ND	H21	H21	H21	H21	H21	H21	ND	ND	H21	86
KK11	H2	H2	H2	H2	ND	H2	H2	H2	UNK	ND	H2	H2	H2	H2	H2	H2	ND	ND	H2	87
LL12	H7	H7	H7	H7	H7	H7	H7	H7	H7	H7	H7	H7	H7	H7	H7	H7	H7	H7	H7	100
MM13	H15	H15	H15	UNK	ND	H15	H15	H15	UNK	ND	H15	H15	H15	H15	H15	H15	H15	ND	H15	87
NN14	H11	H11	H11	H11	ND	H11	H11	H11	UNK	ND	UNK	H11	H21	H11	H11	H11	H11	ND	H11	80
OO15	H30	H30	H30	UNK	ND	H30	H30	H30	H4	ND	UNK	H30	H30	H30	H30	H30	ND	ND	H30	79

H- was accepted as a correct result.

Incorrect result	ND: Not done

Annex 9. VCA results

Strain/laboratory	Original	19	94	100	114	123	125	126	127	128	131	222	%
AA1	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Neg.	Pos.	Pos.	Pos.	91
BB2	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Pos.	Neg.	ND	Neg.	Neg.	Neg.	90
CC3	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Neg.	Pos.	Pos.	91
DD4	Pos.	Pos.	Neg.	Pos.	Pos.	Pos.	Pos.	Pos.	ND	Pos.	Pos.	Pos.	90
EE5	Pos.	Pos.	Neg.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	91
FF6	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Neg.	Pos.	Pos.	91
GG7	Pos.	Pos.	Neg.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	91
HH8	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Pos.	Neg.	ND	Neg.	Neg.	Neg.	90
II9	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Neg.	Pos.	Pos.	91
JJ10	Pos.	Pos.	Pos.	3	Pos.	Pos.	Pos.	Pos.	Neg.	Neg.	3	Pos.	82
KK11	Pos.	Pos.	Neg.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	91
LL12	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Neg.	Pos.	Pos.	91
MM13	Pos.	Pos.	Pos.	3	Pos.	Pos.	Pos.	Pos.	Neg.	Neg.	3	Pos.	82
NN14	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Pos.	Neg.	ND	Neg.	Neg.	Neg.	90
OO15	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Pos.	Neg.	ND	Neg.	Neg.	Neg.	90

Intermediate result (3) noted in the Vero cell assay is accepted as a positive result.

Pos. = Positive, Neg. = Negative

Incorrect result	ND: Not done

Annex 10. ESBL production results

Strain/laboratory	Original	19	94	100	114	123	124	125	126	128	129	130	131	132	133	134	136	138	%
AA1	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	100
BB2	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	100
CC3	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	100
DD4	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	100
EE5	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	100
FF6	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	100
GG7	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	100
HH8	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	100
II9	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	100
JJ10	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	100
KK11	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	100
LL12	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Neg.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	100
MM13	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	100
NN14	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	100
OO15	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	100

The strain LL12 has been observed to loss the ESBL plasmid. Therefore, positive and negative results will be accepted.

Pos. = Positive, Neg. = Negative

Neg. = Negative

Annex 11. Enterohaemolysin production results

Strain/laboratory	Original	19	34	88	94	100	114	123	125	126	127	128	129	134	136	153	%
AA1	Pos.	Pos.	Pos.	Neg.	Pos.	Pos.	Pos.	Pos.	Neg.	Pos.	alfa.	ND	Pos.	Neg.	Pos.	Pos.	79
BB2	Pos.	Pos.	Pos.	Neg.	Pos.	Pos.	Pos.	Pos.	Neg.	Pos.	Pos.	ND	Pos.	Neg.	Pos.	Pos.	79
CC3	Pos.	Pos.	Pos.	Neg.	Pos.	Pos.	Pos.	Pos.	Neg.	Pos.	Pos.	ND	Pos.	Neg.	Pos.	Pos.	79
DD4	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	ND	ND	Neg.	Neg.	Neg.	Neg.	100
EE5	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	ND	ND	Neg.	Neg.	Neg.	Neg.	100
FF6	Pos.	Pos.	Pos.	Neg.	Pos.	Pos.	Pos.	Pos.	Neg.	Pos.	Pos.	ND	Pos.	Neg.	Pos.	Pos.	79
GG7	Pos.	Pos.	Pos.	Neg.	Pos.	Pos.	Pos.	Pos.	Neg.	Pos.	Pos.	ND	Pos.	Neg.	Pos.	Pos.	79
HH8	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	ND	ND	Neg.	Neg.	Neg.	Neg.	100
II9	Pos.	Pos.	Pos.	Neg.	Pos.	Pos.	Pos.	Pos.	Neg.	Pos.	alfa.	ND	Pos.	Neg.	Pos.	Pos.	79
JJ10	Pos.	Pos.	Pos.	Pos.	Pos.	alfa.	alfa.	alfa.	Pos.	Pos.	ND	Pos.	alfa.	Pos.	alfa.	alfa.	100
KK11	Pos.	Pos.	Pos.	Neg.	Pos.	Pos.	Pos.	Pos.	Neg.	Pos.	Pos.	Pos.	Pos.	Neg.	Pos.	Pos.	80
LL12	Pos.	Pos.	Pos.	Neg.	Pos.	Pos.	Pos.	Pos.	Neg.	Pos.	Pos.	ND	Pos.	Neg.	Pos.	Pos.	79
MM13	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	ND	ND	Neg.	Neg.	Neg.	Neg.	100
NN14	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	ND	ND	Neg.	Neg.	Neg.	Neg.	100
OO15	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	ND	ND	Neg.	Neg.	Neg.	Neg.	100

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

Pos. = Positive, Neg. = Negative

Incorrect result	ND: Not done; corrected result accepted after deadline (approved by ECDC)

Annex 12. β-glucuronidase production results

Strain/laboratory	Original	19	34	80	88	94	100	114	123	124	125	127	128	129	130	131	134	136	139	153	%
AA1	Pos.	Pos.	Pos.	Pos.	Neg.	Pos.	Pos.	Pos.	Pos.	Pos.	ND	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	94
BB2	Pos.	Pos.	Pos.	Pos.	Neg.	Pos.	Pos.	Pos.	Pos.	Pos.	ND	Pos.	Pos.	Pos.	Pos.	Neg.	Pos.	Pos.	Pos.	Pos.	89
CC3	Pos.	Pos.	Pos.	Pos.	Neg.	Pos.	Pos.	Pos.	Pos.	Pos.	ND	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	94
DD4	Pos.	Pos.	Pos.	Pos.	Neg.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	95
EE5	Pos.	Pos.	Pos.	Pos.	Neg.	Pos.	Pos.	Pos.	Pos.	Pos.	ND	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	94
FF6	Pos.	Pos.	Pos.	Pos.	Neg.	Pos.	Pos.	Pos.	Pos.	Pos.	ND	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	94
GG7	Pos.	Pos.	Pos.	Pos.	Neg.	Pos.	Pos.	Pos.	Pos.	Pos.	ND	Pos.	Pos.	Pos.	Pos.	Neg.	Pos.	Pos.	Pos.	Pos.	89
HH8	Pos.	Pos.	Pos.	Pos.	Neg.	Pos.	Pos.	Pos.	Pos.	Pos.	ND	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	94
II9	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	ND	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Pos.	Neg.	94
JJ10	Pos.	Pos.	Pos.	Pos.	Neg.	Pos.	Pos.	Pos.	Pos.	Pos.	ND	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	94
KK11	Pos.	Pos.	Pos.	Pos.	Neg.	Pos.	Pos.	Pos.	Pos.	Pos.	ND	Pos.	Pos.	Pos.	Pos.	Neg.	Pos.	Pos.	Pos.	Pos.	89
LL12	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	ND	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Pos.	Neg.	94
MM13	Pos.	Pos.	Pos.	Pos.	Neg.	Pos.	Pos.	Pos.	Pos.	Pos.	ND	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	94
NN14	Pos.	Pos.	Pos.	Pos.	Neg.	Pos.	Pos.	Pos.	Pos.	Pos.	ND	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	94
OO15	Pos.	Pos.	Pos.	Pos.	Neg.	Pos.	Pos.	Pos.	Pos.	Pos.	ND	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	94

Incorrect result	ND: Not done

Annex 13. Sorbitol fermentation results

Strain/laboratory	Original	19	34	80	88	94	100	108	114	123	124	125	127	128	129	130	131	132	133	134	135	136	137	138	139	153	222	%
AA1	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Neg.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	96
BB2	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Neg.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	96
CC3	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Neg.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	96
DD4	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	ND	Pos.	Pos.	Pos.	Pos.	Pos.	Neg.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	96
EE5	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Neg.	Pos.	Pos.	Pos.	Pos.	Pos.	Neg.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	92
FF6	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Neg.	Pos.	Pos.	Neg.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	92
GG7	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Neg.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	96
HH8	Pos.	Pos.	Pos.	Pos.	Neg.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Neg.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	92
II9	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Pos.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	96
JJ10	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Neg.	Pos.	Pos.	Pos.	Pos.	Pos.	Neg.	Pos.	Pos.	Pos.	92
KK11	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Neg.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	96
LL12	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Pos.	Neg.	Pos.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	92
MM13	Pos.	Pos.	Pos.	Neg.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Neg.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	92
NN14	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Neg.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	96
OO15	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	100

Incorrect result	ND: Not done

Annex 14. *eae* gene detection results

Strain/laboratory	Original	19	34	80	88	90	94	100	108	114	123	124	127	129	130	131	133	134	135	136	137	138	139	153	222	%
AA1	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	100
BB2	Pos.	Pos.	Pos.	Pos.	Neg.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	ND	Neg.	Pos.	Pos.	91
CC3	Pos.	Pos.	Pos.	Pos.	Neg.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Neg.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	92
DD4	Neg.	Neg.	Neg.	Neg.	Pos.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	96
EE5	Neg.	Neg.	Neg.	Neg.	Pos.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	96
FF6	Pos.	Pos.	Pos.	Pos.	Neg.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	96
GG7	Pos.	Pos.	Pos.	Pos.	Neg.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	96
HH8	Neg.	Neg.	Neg.	Neg.	Pos.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	ND	Neg.	Neg.	Neg.	96
II9	Pos.	Pos.	Pos.	Pos.	Neg.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	96
JJ10	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	100
KK11	Pos.	Pos.	Pos.	Pos.	Neg.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	96
LL12	Pos.	Pos.	Pos.	Pos.	Neg.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	96
MM13	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	100
NN14	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	ND	Neg.	Neg.	Neg.	100
OO15	Neg.	Neg.	Neg.	Neg.	Pos.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	ND	Neg.	Neg.	Neg.	96

Incorrect result	ND: Not done

Annex 15. *ehx*A gene detection results

Strain/laboratory	Original	19	34	80	90	94	100	108	114	123	124	127	129	131	133	134	135	136	139	153	222	%
AA1	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Neg.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	95
BB2	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	100
CC3	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Neg.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	95
DD4	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	100
EE5	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	100
FF6	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	100
GG7	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	100
HH8	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	100
II9	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	100
JJ10	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	100
KK11	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	100
LL12	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	100
MM13	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	100
NN14	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Pos.	Neg.	95
OO15	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	100

Incorrect result

Annex 16. *vtx*1 gene detection results

Strain/laboratory	Original	19	34	80	88	90	94	100	108	114	123	124	127	129	130	131	132	133	134	135	136	137	138	139	153	222	%
AA1	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Neg.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	96
BB2	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	100
CC3	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	100
DD4	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Neg.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	96
EE5	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Neg.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Neg.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Neg.	Pos.	Neg.	Pos.	Pos.	84
FF6	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	100
GG7	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	100
HH8	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	100
II9	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	100
JJ10	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	100
KK11	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	100
LL12	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	100
MM13	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	100
NN14	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	100
OO15	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	100

Incorrect result

Annex 17. *vtx*2 gene detection results

Strain/laboratory	Original	19	34	80	88	90	94	100	108	114	123	124	127	129	130	131	132	133	134	135	136	137	138	139	153	222	%
AA1	Pos.	Pos.	Pos.	Pos.	Neg.	Pos.	Neg.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	92
BB2	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Pos.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	96
CC3	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	100
DD4	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	100
EE5	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Pos.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	96
FF6	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	100
GG7	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	100
HH8	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	100
II9	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	100
JJ10	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	100
KK11	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	100
LL12	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Neg.	Pos.	Pos.	Pos.	96
MM13	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	100
NN14	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	100
OO15	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	100

Incorrect result

Annex 18. *vtx* subtyping results

Strain/laboratory	Original	19	34	80	88	90	100	108	114	123	124
AA1	*vtx1c* and *vtx2b*	*vtx1c* and *vtx2b*	*vtx1c* and *vtx2b*	*vtx1c* and *vtx2b*	*vtx1d*	*vtx1c* and *vtx2b*	*vtx1c* and *vtx2b*	*vtx1c* and *vtx2b*	*vtx1c* and *vtx2b*	*vtx1c* and *vtx2b*	*vtx1c* and *vtx2b*
BB2		NO	NO	NO	NO	NO	NO	NO	NO	NO	NO
CC3	*vtx2a*	*vtx2a*	*vtx2a*	*vtx2a*	*vtx2a*	*vtx2a*	*vtx2a*	*vtx2a*	*vtx2a*	*vtx2a*	*vtx2a*
DD4	*vtx1c*	*vtx1c*	*vtx1c*	*vtx1c*	*vtx1c*	*vtx1c*	*vtx1c*	*vtx1c*	*vtx1c*	*vtx1c*	*vtx1c*
EE5	*vtx1d*	*vtx1d*	*vtx1d*	*vtx1d*	*vtx1d*	*vtx1d*	*vtx1d*	*vtx1d*	*vtx1d*	*vtx1d*	*vtx1d*
FF6	*vtx2a*	*vtx2a*	*vtx2a*	*vtx2a*	*vtx2a*	*vtx2a*	*vtx2a* and *vtx2d*	*vtx2a*	*vtx2a*	*vtx2a*	*vtx2a*
GG7	*vtx1a*	*vtx1a*	*vtx1a*	*vtx1a*	*vtx1a*	*vtx1a*	*vtx1a*	*vtx1a*	*vtx1a*	*vtx1a*	*vtx1a*
HH8		NO	NO	NO	NO	NO	NO	NO	NO	NO	NO
II9	*vtx2a* and *vtx2c*	*vtx2a* and *vtx2c*	*vtx2a* and *vtx2c*	*vtx2a* and *vtx2c*	*vtx2a*	*vtx2a* and *vtx2c*	*vtx2a* and *vtx2c*	*vtx2a* and *vtx2c*	*vtx2a* and *vtx2c*	*vtx2a* and *vtx2c*	*vtx2a* and *vtx2c*
JJ10	*vtx2d*	*vtx2d*	*vtx2d*	*vtx2d*	*vtx2b*	*vtx2d*	*vtx2d*	*vtx2d*	*vtx2d*	*vtx2d*	*vtx2d*
KK11	*vtx1a*	*vtx1a*	*vtx1a*	*vtx1a*	*vtx1a*	*vtx1a*	*vtx1a*	*vtx1a*	*vtx1a*	*vtx1a*	*vtx1a*
LL12	*vtx2a*	*vtx2a*	*vtx2a*	*vtx2a*	*vtx2a*	*vtx2a*	*vtx2a*	*vtx2a*	*vtx2a*	*vtx2a*	*vtx2a*
MM13	*vtx2d*	*vtx2d*	*vtx2d*	*vtx2d*	*vtx2d*	*vtx2c* and *vtx2d*	*vtx2d*	*vtx2d*	*vtx2d*	*vtx2d*	*vtx2d*
NN14		NO	NO	NO	NO	NO	NO	NO	NO	NO	NO
OO15		NO	NO	NO	NO	NO	NO	NO	NO	NO	NO

Strain/ laboratory	Original	127	129	130	131	133	134	136	138	153	222	%
AA1	*vtx1c* and *vtx2b*	*vtx2b*	*vtx1c* and *vtx2b*	*vtx1c* and *vtx2b*	*vtx1c* and *vtx2b*	*vtx1c* and *vtx2b*	*vtx1c* and *vtx2b*	*vtx1c* and *vtx2b*	*vtx1c*	*vtx1c* and *vtx2b*	*vtx1c* and *vtx2b*	*94*
BB2		NO	NO	NO	NO	NO	NO	NO	NO	NO	NO	*100*
CC3	*vtx2a*	*vtx2a*	*vtx2a*	*vtx2a*	*vtx2a*	*vtx2a*	*vtx2a*	*vtx2a*	*NO*	*vtx2a*	*vtx2a*	*95*
DD4	*vtx1c*	*NO*	*vtx1c*	*vtx1c*	*vtx1c*	*vtx1c*	*vtx1c*	*vtx1c*	*vtx1c*	*vtx1c*	*vtx1c*	*100*
EE5	*vtx1d*	*vtx1d*	*vtx1d*	*NO*	*NO*	*vtx1c* and *vtx1d*	*vtx1d*	*vtx1c* and *vtx1d*	*vtx1c*	*vtx1d*	*vtx1d*	*79*
FF6	*vtx2a*	*vtx2a*	*vtx2a*	*vtx2a*	*vtx2a*	*vtx2a* and *vtx2d*	*vtx2a*	*vtx2a*	*NO*	*vtx2a*	*vtx2a*	*90*
GG7	*vtx1a*	*vtx1a*	*NO*	*vtx1a*	*vtx1a*	*vtx1a* and *vtx1c*	*vtx1a*	*vtx1a*	*NO*	*vtx1a*	*vtx1a*	*85*
HH8		*NO*	*NO*	*NO*	*NO*	*NO*	*NO*	*NO*	*NO*	*NO*	*NO*	*100*
II9	*vtx2a* and *vtx2c*	*vtx2a* and *vtx2c*	*vtx2a* and *vtx2c*	*vtx2a* and *vtx2c*	*vtx2a* and *vtx2c*	*vtx2a* and *vtx2c* and *vtx2d*	*vtx2a* and *vtx2c*	*vtx2a* and *vtx2c* and *vtx2d*	*NO*	*vtx2a*	*vtx2a* and *vtx2c*	*75*
JJ10	*vtx2d*	*vtx2d*	*vtx2d*	*vtx2c*	*vtx2d*	*vtx2c* and *vtx2d*	*vtx2d*	*vtx2a* and *vtx2c* and *vtx2d*	*NO*	*vtx2a*	*vtx2d*	*70*
KK11	*vtx1a*	*vtx1a*	*vtx1a*	*vtx1a*	*vtx1a*	*vtx1a* and *vtx1c*	*vtx1a*	*vtx1a*	*NO*	*vtx1a*	*vtx1a*	*90*
LL12	*vtx2a*	*vtx2a*	*vtx2a*	*vtx2a*	*vtx2a*	*vtx2a*	*vtx2a*	*vtx2a*	*NO*	*vtx2a*	*vtx2a*	*100*
MM13	*vtx2d*	*vtx2d*	*vtx2d*	*vtx2c* and *vtx2d*	*vtx2d*	*vtx2c* and *vtx2d*	*vtx2c* and *vtx2d*	*vtx2a* and *vtx2c* and *vtx2d*	*NO*	*vtx2d*	*vtx2d*	*70*
NN14		NO	NO	NO	NO	NO	NO	NO	NO	NO	NO	100
OO15		NO	NO	NO	NO	NO	NO	NO	NO	NO	NO	100

Incorrect result

False negative results due to errors in the initial screening of *vtx1* and *vtx2*. Results not included in the overall percentages.

Annex 19. Reference strains of *vtx* subtypes

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

Replacement strains added to the fourth EQA shipment of vtx *subtypes*

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

Removed from the fourth EQA shipment for the following reasons:

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

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

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

ND: Not done

Annex 20. Guide to BN database

Setting up an EQA database

There are two possibilities for setting up an EQA database. If you have BioNumerics version 6 or above you can just use the ready-made databases that have been sent out together with this instruction. The database is packaged in the zip archive called 'Listeria EQA db.zip' or 'Salmonella EQA db.zip'. If you have a BioNumerics version prior to 6.0 or wish to set up the database yourself, please use the instructions below.

Setting up a new database

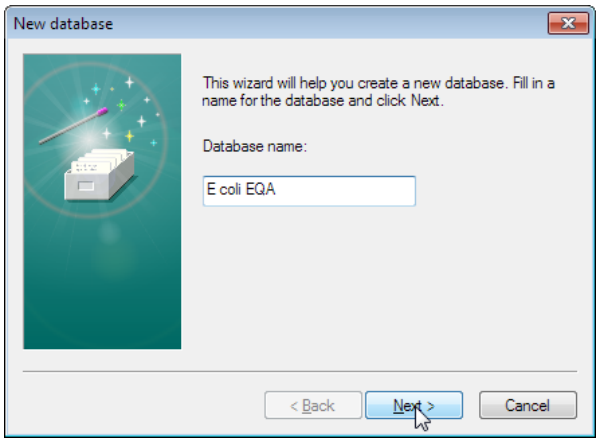
All the images in these instructions refer to *E. coli*. Please modify if you work on *Salmonella* or *Listeria*.

A new database is established up by first setting up an empty database followed by the importation of an XML file containing experiment settings and field definitions.

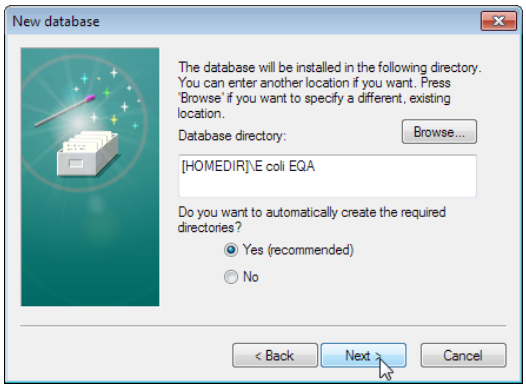
Setting up the empty database

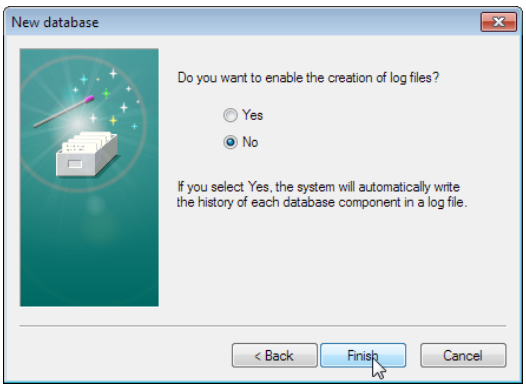
Select 'Create a new database'

Enter a database name, e.g. 'Salmonella EQA' or 'Listeria EQA'.

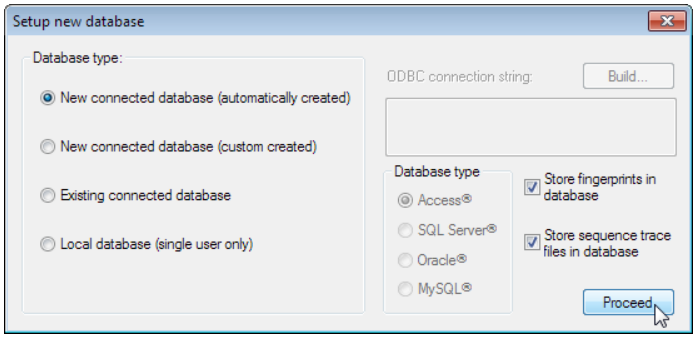


Use default values.

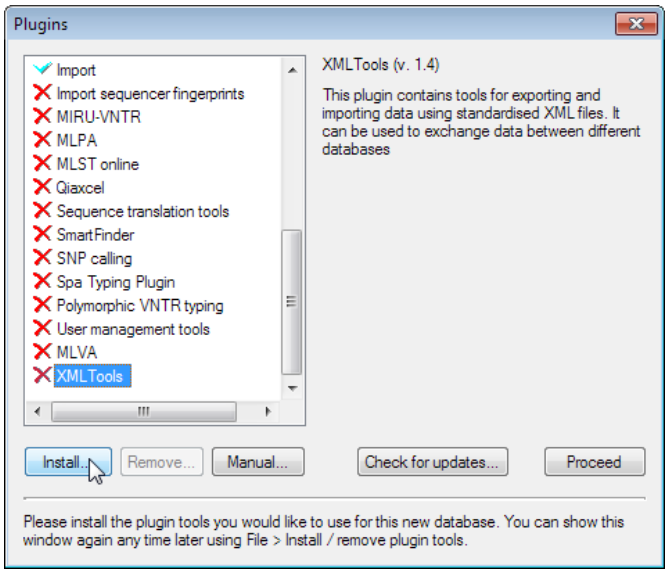




Choose a new connected Access database.



When choosing plugins, add the XML Tools plugin by selecting the plugin in the list followed by 'Install…'.

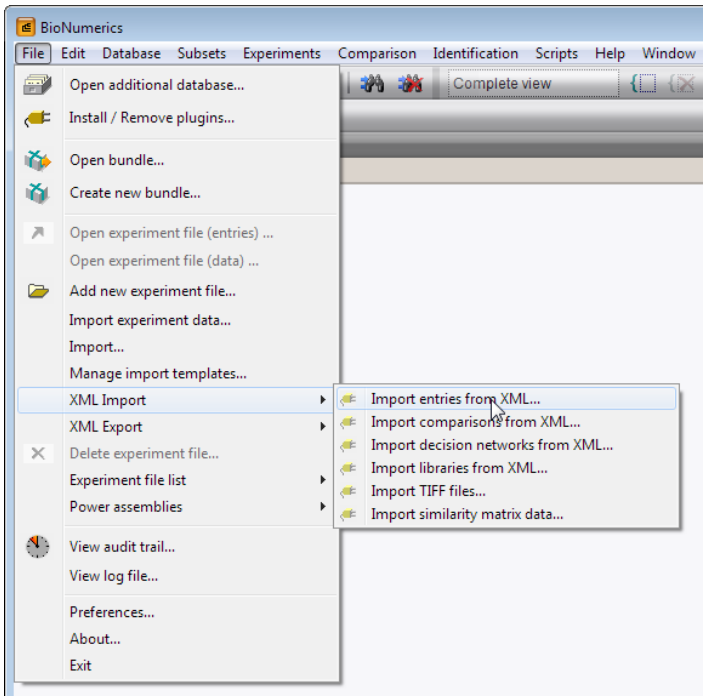


Proceed to the next window. The database is now set up and ready to import the database definitions.

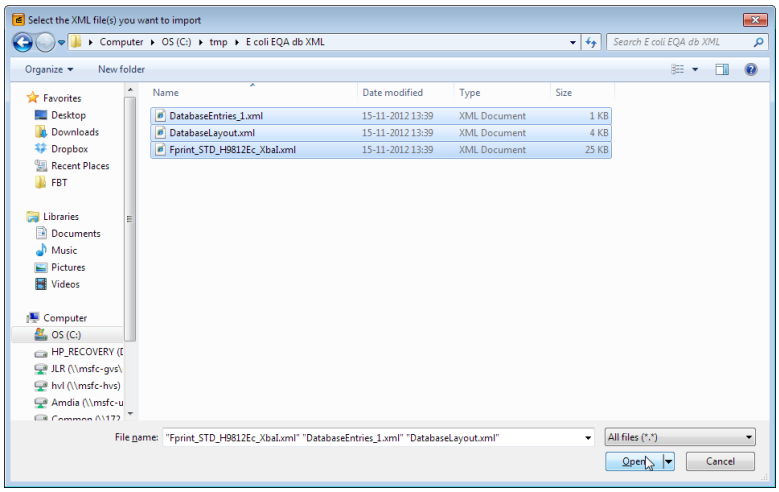
Importing the XML structure

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

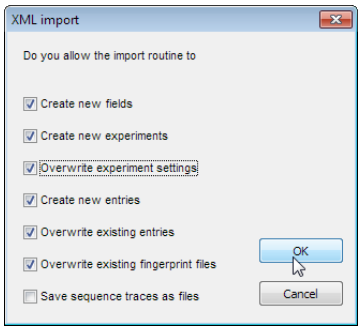
Select 'Import entries from XML'.



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



Tick 'Overwrite experiment settings' and click OK.

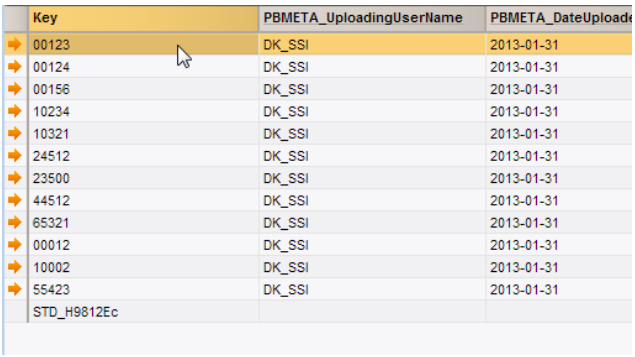


Restart the database.

Annex 21. Guide to XML export

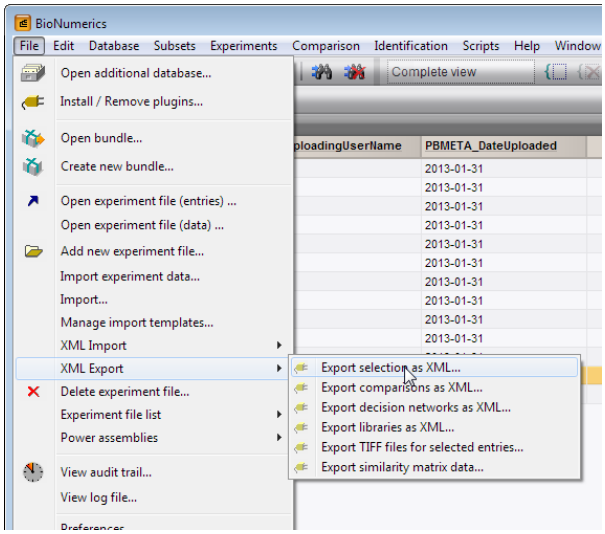
Exporting XML data from your database

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

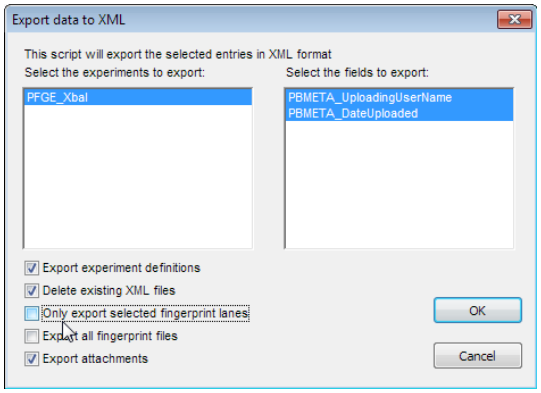


Key	PBMETA_UploadingUserName	PBMETA_DateUploade
00123	DK_SSI	2013-01-31
00124	DK_SSI	2013-01-31
00156	DK_SSI	2013-01-31
10234	DK_SSI	2013-01-31
10321	DK_SSI	2013-01-31
24512	DK_SSI	2013-01-31
23500	DK_SSI	2013-01-31
44512	DK_SSI	2013-01-31
65321	DK_SSI	2013-01-31
00012	DK_SSI	2013-01-31
10002	DK_SSI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

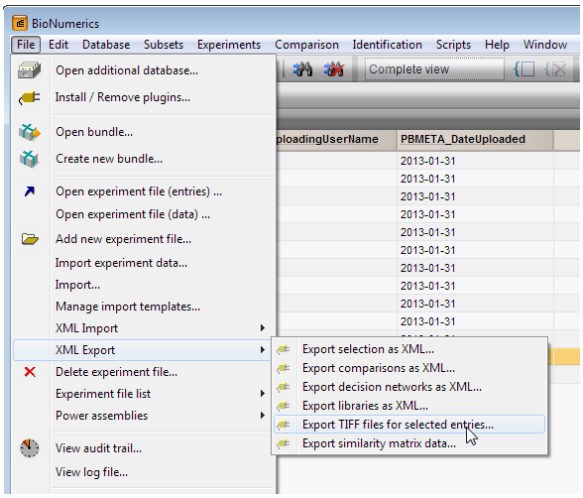
Export selection as XML.



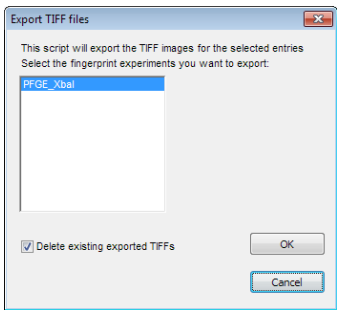
Untick 'Only export selected fingerprint lanes'.



Now export the TIFF files.



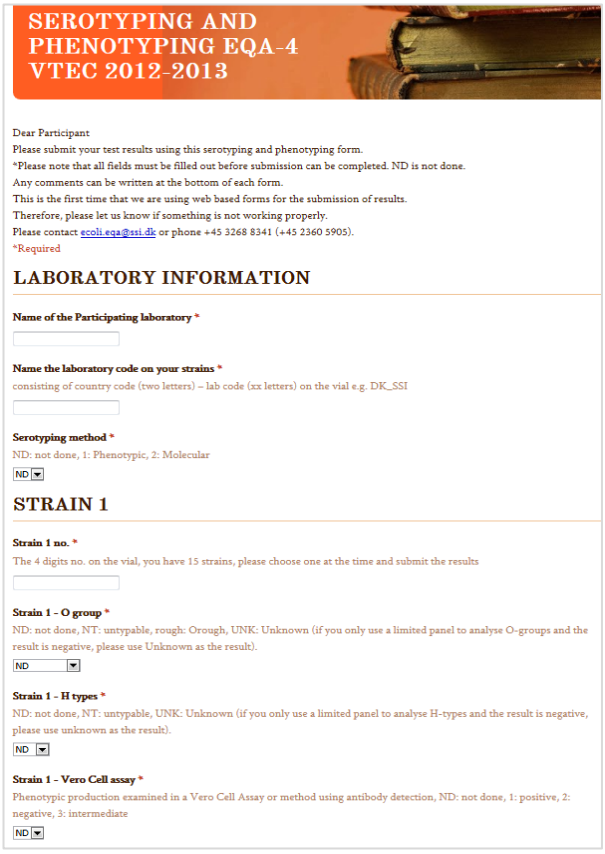
Select which experiments to export; for Listeria you can export both enzymes at the same time.



Now locate the EXPORT directory in your database directory. Send all XML and TIFF files in this directory via mail. Before sending files, please save them to a zip archive.

Annex 22. Online submission

Online submission form available from:
https://docs.google.com/spreadsheet/viewform?usp=drive_web&formkey=dGtzRkZaYkc3ZHVxbndRaC1ybEJuZkE6MA#gid=0



SEROTYPING AND PHENOTYPING EQA-4 VTEC 2012-2013

Dear Participant
Please submit your test results using this serotyping and phenotyping form.
*Please note that all fields must be filled out before submission can be completed. ND is not done.
Any comments can be written at the bottom of each form.
This is the first time that we are using web based forms for the submission of results.
Therefore, please let us know if something is not working properly.
Please contact ecoli.eqa@ssi.dk or phone +45 3268 8341 (+45 2360 5905).
*Required

LABORATORY INFORMATION

Name of the Participating laboratory *

Name the laboratory code on your strains *
consisting of country code (two letters) – lab code (xx letters) on the vial e.g. DK_SSI

Serotyping method *
ND: not done, 1: Phenotypic, 2: Molecular
ND

STRAIN 1

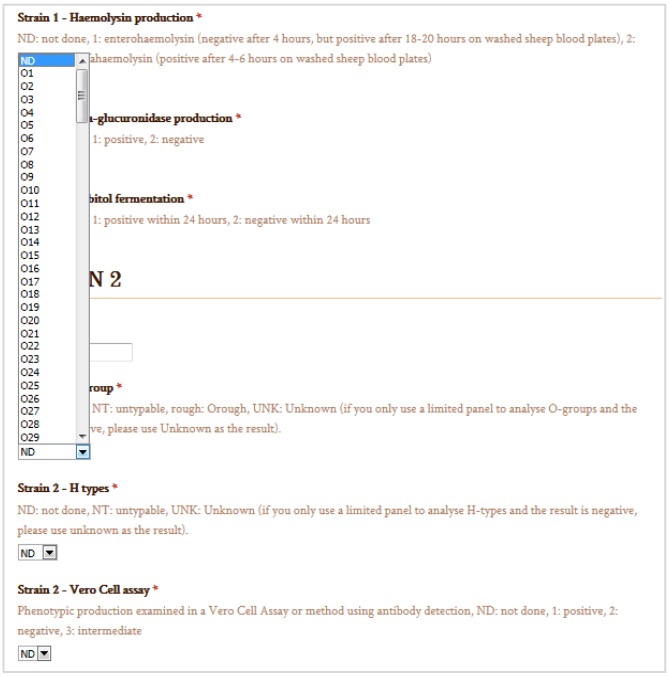
Strain 1 no. *
The 4 digits no. on the vial, you have 15 strains, please choose one at the time and submit the results

Strain 1 - O group *
ND: not done, NT: untypable, rough: Orough, UNK: Unknown (if you only use a limited panel to analyse O-groups and the result is negative, please use Unknown as the result).
ND

Strain 1 - H types *
ND: not done, NT: untypable, UNK: Unknown (if you only use a limited panel to analyse H-types and the result is negative, please use unknown as the result).
ND

Strain 1 - Vero Cell assay *
Phenotypic production examined in a Vero Cell Assay or method using antibody detection, ND: not done, 1: positive, 2: negative, 3: intermediate
ND

Online selection of strains through drop-down menus



Strain 1 - Haemolysin production *
ND: not done, 1: enterohaemolysin (negative after 4 hours, but positive after 18-20 hours on washed sheep blood plates), 2: ahaemolysin (positive after 4-6 hours on washed sheep blood plates)
ND
O1
O2
O3
O4
O5
O6
O7
O8
O9
O10
O11
O12
O13
O14
O15
O16
O17
O18
O19
O20
O21
O22
O23
O24
O25
O26
O27
O28
O29
-glucuronidase production *
1: positive, 2: negative
bitol fermentation *
1: positive within 24 hours, 2: negative within 24 hours
N 2
roup *
NT: untypable, rough: Orough, UNK: Unknown (if you only use a limited panel to analyse O-groups and the ve, please use Unknown as the result).
ND
Strain 2 - H types *
ND: not done, NT: untypable, UNK: Unknown (if you only use a limited panel to analyse H-types and the result is negative, please use unknown as the result).
ND
Strain 2 - Vero Cell assay *
Phenotypic production examined in a Vero Cell Assay or method using antibody detection, ND: not done, 1: positive, 2: negative, 3: intermediate
ND