SSI TECHNICAL REPORT

External quality assessment scheme for typing of verocytotoxin-producing *E. coli* (VTEC), self-funded participants, 2014-2015

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Abbreviations

AEEC	Attaching and effacing *E. coli*
aaiC	Chromosomal gene marker for Enteroaggregative *E. coli*
aggR	Gene encoding the master regulator in Enteroaggregative *E. coli*
BN	BioNumerics software suite
eae	CVD434. *E. coli* attaching and effacing gene probe *ehxA*
EAEC	Enteroaggregative *E. coli*
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
ND	Not Done
NSF	Non-sorbitol fermenting *E. coli*
NT	Not Typable
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
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
WHO	World Health Organisation

Executive summary

This report presents the results of external quality assessment (EQA) scheme for typing of verocytotoxin-producing *Escherichia coli* (VTEC) for self-funded participants 2014-2015. The EQA was carried out from January 2015 to May 2015 and included the following methods 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 ESBL.

Twenty-three public health national reference laboratories from 16 countries participated in at least one of the EQA parts. Twelve laboratories (52%) participated in the PFGE part, and among those, eleven (92%) 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 BioNumerics (BN). Nine laboratories completed the gel analysis, and all performed in fair to good accordance with the guidelines.

In the O group determination between 14 to 22 participated (some laboratories only typed a selection of the test strains). An average score of 84% (range 59–100%) was obtained for the O group of the strains, the more common O groups received better typing results: O157 was typed correctly in 100% of attempted, while O174 was associated with a significantly poorer result (59%). An average score of 93% (range 81–100%) was obtained for the H type of the strains (some laboratories only typed a selection of the test strains). Notably, not all laboratories demonstrated the capacity to determine all O groups and H types, and the participation in H typing was low (16/22).

Genotypic detection of *eae* was performed by 20 (87%) laboratories, correct detection (evaluated per strain) was in average 98%. Fifteen (65%) of laboratories submitted results for *ehxA* and all of them did so correctly. Twelve laboratories (52%) participated in the detection of *aggR* and 7 (30%) for *aaiC.* However, do to a new variant of the *aaiC* gene none of participants correctly reported *aaiC*. In addition no false positive *aggR* results were reported. Detection of *vtx1* and *vtx2* were performed by 22 (96%), correct detection (evaluated per strain) was in average 99% and 98% for *vtx*1 *vtx2* respectably.

In comparison to the genotypic methods, the phenotypic methods were in general performed less frequently, however the performance was high. Only 6 laboratories (26%) participated in the phenotypic detection of VCA (Vero cell assay), however 98% of the results in average were correct. Nineteen (83%) participated in fermentation of sorbitol and in average 98% were correct. Ten (43%) participated in the analysis for β-glucuronidase, 97% reported correctly. Nine (39%) participated in the analysis for enterohaemolysin and 98% of the results in average were correct. Eight (35%) participated in the ESBL analysis, 96% in average were correct.

In general the performance within each analysis is high, however full O:H serotyping and subtyping particularly in strain harbouring *vtx2c* and *vtx2d* needs for some participants extra attention. At the moment, the molecular typing method used for surveillance of VTEC is PFGE in combination with conventional typing/phenotyping of strain characteristics. Interlaboratory comparison of results is important for outbreak investigation across borders, this document presents the results of the self-funded laboratories participating in the VTEC EQA 2014-2015.

1 Introduction

1.1 Background

External quality assessment (EQA) is an important aspect of quality management systems. An EQA is a system for an external agency to objectively check a laboratory's performance and has numerous roles of public health importance. An EQA allows for comparison among different test sites, it is an early warning for systematic problems, it provides objective evidence of testing quality, identifies areas needing improvement and specific training needs amongst participants.

Since 2002, the Reference and Research Centre on *Escherichia* and *Klebsiella* at Statens Serum Institut (SSI) has played a leading role in establishing a worldwide international network of quality evaluation and assurance for typing of *E. coli*. From 2002 to 2010 this laboratory arranged annual EQA rounds for the national reference laboratories in the EU/EEA and non-EU countries on serotyping and virulence typing for VTEC. From 2008 onwards the EQA for national reference laboratories in EU were funded by ECDC, however this report only included the self-funded participants. This EQA is the 11th of its kind. The EQAs are conducted according to the International Standard ISO/IEC 17043:2010.

1.2 Surveillance of VTEC infections

VTEC are a group of *E. coli* characterised by the ability to produce toxins designated verocytotoxins (VT). Human pathogenic VTEC often harbour additional virulence factors important in the development of the disease in humans. A large number of serotypes of *E. coli* has 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 often is accompanied by abdominal cramps, usually without fever. VTEC infections can result in haemolytic uremic syndrome (HUS), which is characterised by acute renal failure, anaemia and lowered platelet counts.

In 2014, 5,955 confirmed cases of VTEC infections were reported in the EU. The EU notification rate was 1.56 cases per 100,000 population, which was 1.9% lower than the notification rate in 2013. The EU notification rate in the 2 years following the large outbreak in 2011 was higher than before the outbreak and remained so in 2014. [1].

The overall aim of this EQA is the harmonization of the typing methods used for VTEC, in order to produce comparable typing data for VTEC strains between laboratories. This harmonisation and comparability allows for the surveillance of VTEC nationally and internationally and results in the ability to produce better scientific knowledge regarding aetiology, risk factors and burden of food- and waterborne diseases and zoonoses. Standardised laboratory techniques and national and international comparison of results have many benefits for public health including fostering the rapid detection of dispersed international clusters/outbreaks, facilitating the detection and investigation of transmission chains and relatedness of strains globally, detecting the emergence of new evolving pathogenic strains, supporting investigations to trace-back the source of an outbreak and to identify new risk factors and aid in studying the characteristics of a particular pathogen and its behaviour in a community of hosts.

This EQA scheme was open to public health national reference laboratories worldwide and allowed the assessment of countries currently conducting molecular surveillance.

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 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* (AEEC) including enteropathogenic *E. coli* (EPEC). The combination of the toxin genes is clinically relevant in some subtypes of VT2. VT2a in *eae*-positive VTEC and the activatable VT2d subtype in *eae*-negative VTEC seem to be highly associated with the serious sequela HUS [2-4]. VT2c-positive VTEC has also been associated with HUS [5, 6]. Other specific subtypes or variants of VT1 and VT2 are primarily associated with milder course of disease without HUS [4-6], and VT2e-positive VTEC strains are probably not pathogenic to humans [7]. Our understanding of the epidemiology of the VT subtypes is therefore important for reducing the risk of VTEC infection and for the surveillance of VTEC.

Some of the existing VT-subtyping methods using a combination of specific polymerase chain reaction (PCR) and restriction fragment length polymorphism (RFLP) are inadequate and may result in misleading conclusions. For example, typing of *vtx2* has been based on the absence of the PstI site as an indicator of the presence of the mucus-activatable v*tx*2d subtype [6-9]. 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* [10]. 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 causing lysis of erythrocytes. Enterohaemolysin may be detected either phenotypically on sheep blood agar plates or by detection of the *ehxA* gene encoding enterohaemolysin.

This 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 [10], phenotypic detection of VT production through VCA or enzyme immunoassay (EIA), fermentation of sorbitol, production of β-glucuronidase , enterohaemolysin and ESBL.

1.4 Objective of this EQA scheme

1.4.1 Pulsed-field gel electrophoresis (PFGE) typing

The objective of this 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 using the Bionumerics software, and interpretation of the results.

1.4.2 Serotyping

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

1.4.3 Virulence determination

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

- Detection of virulence genes *eae*, *vtx1*, *vtx2* and *ehxA*.
- Subtyping of *vtx1* and *vtx2* genes
- Detection of additional virulence genes

1.4.4 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

This VTEC EQA was arranged by SSI, conducted from November 2014 to June 2015 and was self-funded by all participating laboratories included in this report. It included PFGE, O:H serotyping, virulence determination by genotypic methods (detection and typing of virulence genes *eae*, *vtx1*, *vtx2* and *ehxA*, subtyping of *vtx1* and *vtx2*) and by phenotypic detection of VT production, fermentation of sorbitol, production of β-glucuronidase, enterohaemolysin and ESBL.

The EQA (without the PFGE part) was conducted according to ISO/IEC 17043:2010, entitled '*Conformity assessment – General requirements for proficiency testing'* (first edition, 1 February 2010) [11].

Invitations were e-mailed to previously participating countries and laboratories from the international World Health Organisation (WHO) Global Foodborne Infections Network (GFN) on the 13th of November 2014. Twenty-four laboratories accepted the invitation (Annex 1).

The EQA test strains were sent to the participating laboratories in the first week of February 2015.

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 by the 13th of April 2015.

In addition, laboratories from EU member states were invited to participate and funded by the ECDC FWD programme. The results from these laboratories are published elsewhere.

2.2 Selection of strains

The strains for this EQA were selected based on representativeness: all strains should be representative for strains reported from Europe. The self-funded EQA is based on the same strains as the ECDC-funded EQA. In addition, strains should remain stable during the preliminary testing period at the laboratory of the EQA provider. The selected types should be easy to type, and they should represent the three different subtypes of *vtx1* and cover as many of the seven different subtypes of *vtx2*.

Table 1: Test strains

Method	No. of test strains	Characterisation
PFGE	10	AA1, BB2, CC3, DD4, EE5, FF6, GG7, HH8, II9 and JJ10
O:H serotyping	10*	KK11, LL12, MM13, NN14, OO15, PP16, QQ17, RR18, SS19 and TT20 O26:H11, O41:H26, O63:H6, O104:H7, O111:H-/H8 O121:H19, O157:H7, O157:H7, O166:H15, O174:H21
Virulence gene determination	10*	*eae*, *vtx1a*, *vtx1c*, *vtx1d*, *vtx2a, vtx2b, vtx2c, vtx2d* and *vtx2f, ehxA*, *aggR*, *aaiC*
Phenotypic testing	10*	VCA, sorbitol, β-glucuronidase, enterohaemolysin, ESBL

**Same 10 strains*
Detailed information (Annex 5).

In addition to the 20 test strains, laboratories participating in the 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 4th of February 2015, all strains were blinded, packed and sent. Almost all of the participants received their dispatched strains within 4–5 days. Two parcels were delayed by customs and were not delivered for over a month. The parcels were shipped from SSI Copenhagen, labelled as UN 3373 Biological Substance, Category B.

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

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

2.4 Testing

In the PFGE part, ten *E. coli* strains representing different serotypes were tested, and participants could opt only to 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 PFGE [12].

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

In the other parts of the EQA, ten additional *E. coli* strains were included. All results were submitted online to *Google Docs*. The participants' ability to obtain the correct serotype, both O group and H type, by either serological methods (suggested protocol [13] or molecular typing (no international standard but the applied methods should be submitted together with the results) was tested.

In the genotyping part, the participants' ability to detect the virulence genes *eae*, *vtx1*, *vtx2* and *ehxA* genes and the ability to subtype *vtx1* (*vtx1a)* and *vtx2* (*vtx2a, vtx2b, vtx2d and vtx2f*) were assessed (suggested protocol [14]).

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

Participants were requested to test for additional virulence genes at their own convenience and capacities. This voluntary and additional testing was not a core part of the EQA 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 laboratories were received, the PFGE results were added to a dedicated *E.coli* BN database at SSI. For PFGE, the gel quality was evaluated according to a modified version of the PulseNet US protocol PFGE Image Quality Assessment (TIFF Quality Grading Guidelines 2015) by scoring the gel with respect to seven parameters (scores in the range 1–4, 4 being the top score, Annex 2). The BN analysis was evaluated according to a modified version of the BN Gel Analysis Quality Guidelines 2015 (Annex 3) with respect to five parameters (scores in the range 1–3, 3 being the top score). After the results from all laboratories were submitted in the online forms, SSI exported a copy of all results to an Excel spreadsheet. Results were then analysed; scores of the serotyping, genotyping, and phenotyping tests were evaluated based on correct results and a percentage score was calculated.

3 Results

3.1 Participation

Laboratories could choose to participate in the full scheme or a selection of the methods. The methods were PFGE, O:H serotyping, virulence determination including genotyping (virulence gene detection and subtyping) and phenotyping (VT, sorbitol, β-glucuronidase, enterohaemolysin, ESBL). Twenty-three laboratories submitted results; an additional laboratory registered to take part in the EQA but withdrew before submitting any result. Twelve (52%) participated in the PFGE part, 9 out of the twelve (75%) also in the BN analysis. Eleven (47%) participated in the full O:H serotyping of all 10 strains. An additional five laboratories (22%) submitted O:H data for only a limited number of the EQA strains. The reasons for omitting some of the strains were not always specified, but in some cases, it was based on the obtained O results. Sixteen (70%) submitted full O:H serotype data for strain QQ17 and RR18 (O157:H7).

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

The highest participation, 22 laboratories participated in the in the O grouping of O157, However only 14 participated for O174. In addition 16 laboratories participated in the H typing (Table 3).

For the genotyping component (virulence gene detection and subtyping), 22 laboratories (96%) submitted results for the *vtx* genes, 20 for *eae* (87%) and 15 (65%) submitted results for *ehxA*. Between 18 and 20 laboratories (78-87%) submitted results for *vtx* subtypes. A total of 12 and 7 laboratories (52% and 30%) reported results for EAEC (*aggR* and *aaiC* respectively*)*.

For the phenotyping component of the EQA, 19 laboratories (83) participated in one or more of the phenotyping methods. Participation is presented in Table 2, details are listed in Table 3. Participation in the phenotypic detection was 26–83% (6–19 labs). The lowest participation was for the VT assay: only 6 participants (26%) delivered results for 10 strains. Participation in the sorbitol fermentation was the highest and included 19 laboratories (83%). The test for enterohaemolysin production was performed by 9 laboratories (39%). The test for production of β-glucuronidase was performed by 10 laboratories (43%). Eight laboratories (35%) submitted results for the production of ESBLs.

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

Methods	PFGE		Full O:H serotyping[1]	Virulence determination[2]	Phenotypic test[3]
	TIFF	XML			
Number of participants	12	9	16	22	19
% of participants	52	75*	70	96	83

[1] *Participation in O grouping was 14–22 laboratories and 15–16 laboratories in H typing*
[2] *Participation in one or more of the virulence gene determination parts (*eae, vtx1, vtx2 *or* ehxA*)*
[3] *Participation in one or more of the phenotypic test parts (VCA, sorbitol, enterohaemolysin, β-glucuronidase or ESBL)*
Note: Twenty-three laboratories participated in at least one method
**out of the 12 participants in the TIFF*

Table 3: Detailed participation table

Participants / method	O:H serotype group	O group[1]	H type[2]	Vero cell assay	ESBL	Haemolysin production	β-glucuronidase production	Sorbitol fermentation	*eae* gene	*ehxA* gene	*vtx1* gene	*vtx2* gen	*vtx* subtypes	*aggR*	*aaiC*
n_{max}	16	22	16	6	8	9	10	19	20	15	22	22	18	12	7
Percentage of participants	70%	96%	70%	26%	35%	39%	43%	83%	87%	65%	96%	96%	78%	52%	30%

n_{max}*: highest number of participants*
[1] *Participation in O grouping was 14–22 laboratories*
[2] *Participation in H typing was 15–16 laboratories*

3.2 Pulsed-field gel electrophoresis (PFGE)

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

3.2.1 Gel quality

All laboratories were able to produce profiles recognisable as the profile for the relevant EQA strain, examples of the profiles from two test strains from all laboratories is provided in Figure 1. The gels were graded according to the modified TIFF Quality Grading Guidelines, where seven parameters are used in the grading (Annex 2). In general, an acceptable quality should be obtained for each parameter since a low quality score in just one category can have a high impact on the ability to further analyse the image and compare to other profiles. In general, acceptable quality (fair – score of 2) should be achieved for each parameter. A score of 1 in just one category resulted in a non-acceptable gel, making inter–laboratory comparison impossible.

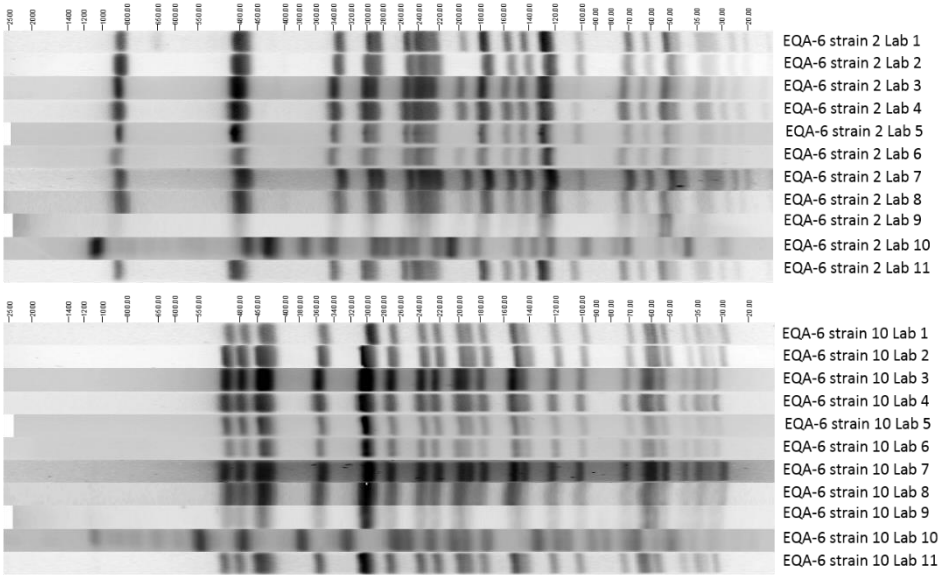

Figure 1: Example of PFGE profiles of two strains (EQA-6 strain 2 and 10) by EQA participants (Lab 1-11)*.
*one laboratory is missing from the figure as a different PFGE protocol was applied and the profiles could not be aligned with the other EQA participants.

Some variation in the qualities of the gels submitted by the participating laboratories was observed (Table 4). For three parameters; restriction, gel background and DNA degradation participants obtained a high average score (≥ 3.6), i.e. between good and excellent (Table 4). Participants obtained and average score of 3.3 in running conditions, cell suspension and lanes. For the last parameter 'Bands', participants had an average score below 3 (2.3), i.e. between fair and good.

Table 4: Results of PFGE gel quality for 12 participating laboratories

Parameters	1 – poor (%)	2 – fair (%)	3 – good (%)	4 – excellent (%)	Average score
Image acquisition and running conditions	8	0	50	42	3.3
Cell suspension	0	17	33	50	3.3
Bands	8	50	42	0	2.3
Lanes	0	8	50	42	3.3
Restriction	0	8	16	75	3.7
Gel background	0	8	0	92	3.8
DNA degradation	0	8	8	84	3.8

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

The laboratories obtained diverse scores for the parameter Image Acquisition and Running Conditions (Table 4). Ninety-two percent of participants were graded fair [2], good [3] or excellent [4] in for the parameter Image Acquisition and Running Conditions, one participant (8%) had a critical score [1]. In the parameter Bands, 92% of laboratories were graded a score of 2 or above (Table 4). Eight percent of participants obtained the score 1 in the parameter Bands, making further analysis of the gel impossible. One (8%) laboratory produced a gel graded 1 (poor) in two of the seven parameters, and was linked to the use of a running conditions that were divergent from the protocol, or through the use of a commercial ladder in the place of the PulseNet *S.* Braenderup ladder. Profiles from gels with poor quality in just one parameter are impossible to compare with profiles produced on other gels. All the participants Gel Quality scores are listed in Annex 4.

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

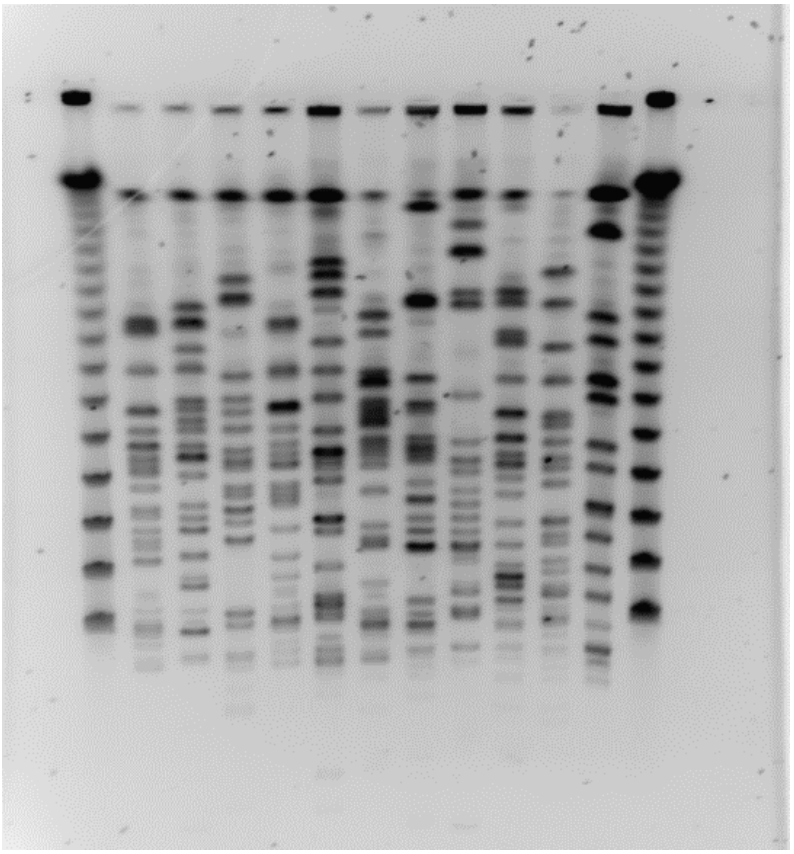

Figure 2: A gel graded 1 in parameter Bands and Image acquisition and running conditions, and a score of 2 in the parameters Cell Suspension, Lanes, Restriction and Gel background.

Note: The most critical score was in the parameters Bands and Image acquisition and running conditions

Figure 3 depicts a gel with low scores in the parameters Bands, cell suspension and DNA degradation.

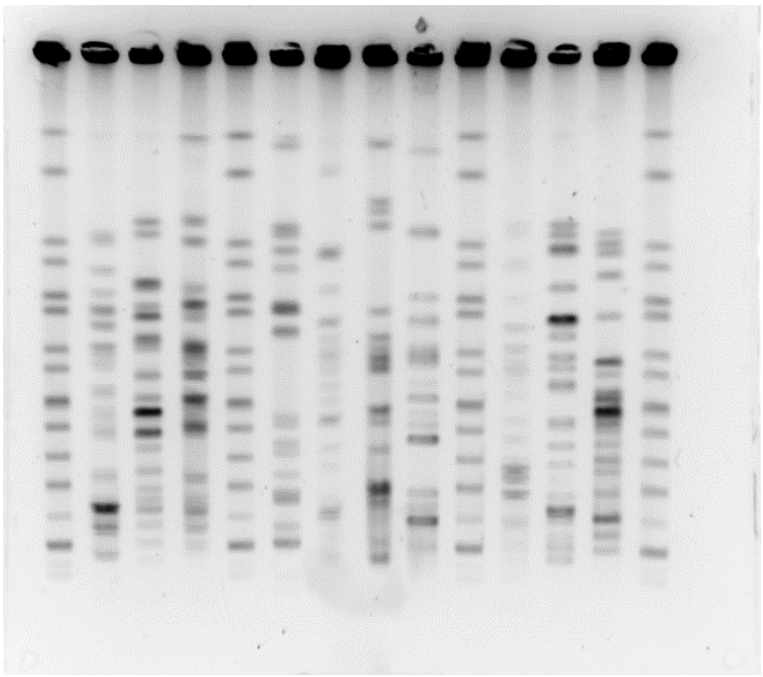

Figure 3: A gel graded 2 in Bands and DNA degradation

A gel with high scores in all seven parameters is shown in Figure 4. The image is captured and cropped correctly, there is an even distribution of DNA, the bands are clear, there is no debris, nor background or shadow bands.

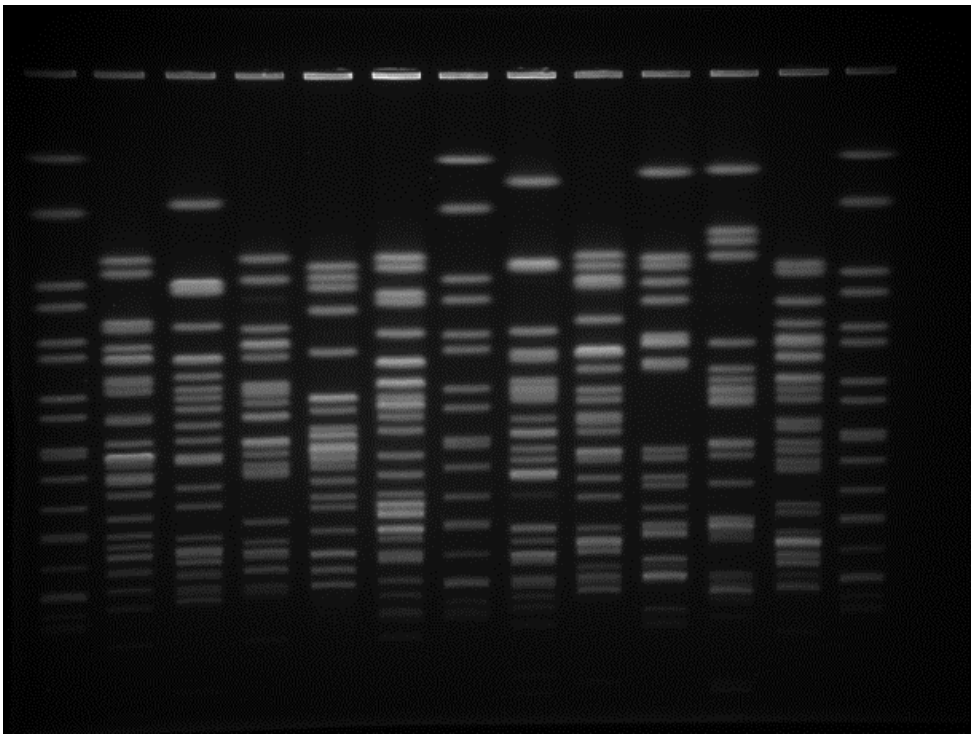

Figure 4: A gel with high scores in all 7 parameters

3.2.2 Gel analysis with BioNumerics

Nine laboratories analysed their gel in BN and were able to produce XML files according to the protocol attached to the invitation letter. The participants' ability to perform gel analysis was graded according to the modified BioNumerics Gel Analysis Quality Guidelines developed at SSI, including five parameters for the grading (Annex 3).

Table 5: Results of the BN analysis for 17 laboratories

Parameters	1 – poor	2 – fair	3 – Excellent	Average score
Position of the gel	0%	0%	100%	3.0
Strips	0%	11%	89%	2.9
Curves	0%	56%	44%	2.4
Normalisation	12%	44%	44%	2.9
Band assignment	0%	33%	66%	2.1

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

For three parameters, Position of the Gel, Strips and Normalisation, participants obtained a very high average score, of 2.7 or above (Table 5). The participants were graded a bit lower with an average of 2.4 in the parameter Curves. The average score for participants in Band assignment was 2.1.

An optimal Band assignment in BN is crucial, and this is very dependent on the overall quality of the gel and the score of the parameter Band from the TIFF quality grading guidelines (Annex 2). Very fuzzy and/ or thick bands make correct Band assignment an impossible task. In Figure 4, the comparison of three gels from two participants and the reference strain illustrates differences in band quality.

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

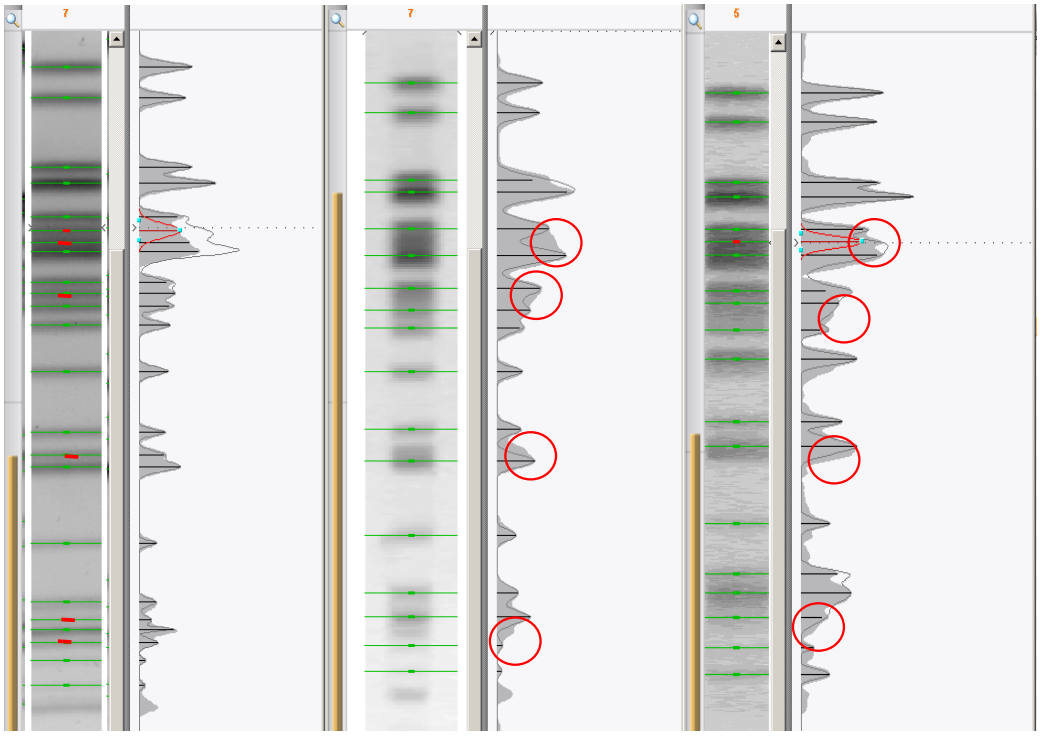

Figure 4: Comparison of Band assignment from two participants in BN analysis

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

3.3 Serotyping

On average for the 10 test strains 84% of the 22 participating laboratories reported the correct O grouping (Table 6). Results were lowest (59%) for serotype O174 (TT20) and highest (100%) for serotype O157 (QQ17 and RR18). Overall, 11 laboratories (50%) reported the correct O grouping for all 10 test strains.

H typing was correctly performed by an average of 93% of the 16 participants, which represents only 73% of the number of participants performing serotyping. Results were lowest (81% of laboratories correct) for the LL12 strain (H26), 87% for OO15 strain (H-/H8) and 88% for MM13 strain (H6) and highest (100% of laboratories correct) for NN14, QQ17 and RR18 (H7). The majority of incorrect H types were due to reporting a strain as not typable. Eleven laboratories reported the correct H type for all 10 test strains.

In all, 8 laboratories (36%) reported the correct O and H grouping for all the 10 test strains.

Table 6: Average scores for the O:H serotyping

Strain	Correct results	O:H serotyping	O group	Type of incorrect antigens	H type	Type of incorrect antigens
n_{max}			22		16	
KK11	O26:H11	64% (14)	95% (21)	NT (1)	94% (15)	NT (1)
LL12	O41:H26	59% (13)	73% (16)	NT (3), Rough (1) NON-O157 (1) O121 (1)	81% (13)	NT (1) H- (2)
MM13	O63:H6	59% (13)	68% (15)	NT (6), NON-O157 (1)	88% (14)	NT (1) H- (1)
NN14	O104:H7	68% (15)	77% (17)	NT (4), O157(1)	100% (16)	
OO15	O111:H-/H8	64% (14)	100% (22)		87% (14)	NT (1), H19 (1)
PP16	O121:H19	68% (15)	91% (20)	NT (1) , NON-O157 (1)	94% (15)	NT (1)
QQ17	O157:H7	73% (16)	100% (22)		100% (16)	
RR18	O157:H7	73% (16)	100% (22)		100% (16)	
SS19	O166:H15	68% (15)	73% (16)	NT (1), NON-O157 (1)	94% (15)	NT(1)
TT20	O174:H21	56% (12)	59% (13)	NT (1), NON-O157(1), O15 (1)	94% (14)	NT(1), H- (1)
Average		**65%**	**84%**		**93%**	

n_{max} = maximal number of participants. Percentages and no. of laboratories with correct reported results,(based on the results submitted by all participants Annexes 6 and 7). NT, not typeable; ND not determined.

An average of 65% (ranging from 56–73% depending on the strain) of laboratories could correctly identify O:H serotype in the 10 test strains. Correct O:H serotyping ranged from 73% for serotypes O157:H7 to 56% for serotype O174:H21 (Table 6). The majority of incorrect O:H-types were due to reporting a strain as not typable. This leads to the conclusion that it is more difficult for laboratories to correctly serotype a strain when the serotype is less common.

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 15 to 22 laboratories for all the 10 test strains, with high average scores (98–100% correct) (Table 7). With regard to the detection of *eae*, a perfect score was obtained for seven strains. Three strains were incorrectly identified by one or two laboratories, with two false negative and two false positive results. Detection of *vtx*1 genes had high average correct score of 99%. Similarly detection of *vtx*2 genes had an average correct score of 98%. Four laboratories missed the presence of *vtx2* in strain MM13 (O63:H6), which has the *vtx2f* gene. One false negative *vtx1* gene was submitted by one laboratory for strain LL12 (O41:H26), this laboratory also reported one of the false negatives for the *vtx2* gene. In total, *vtx1* and *vtx2* were misidentified five times; *vtx*1 (one false negative), *vtx2* (four false negatives). All laboratories correctly identified the presence or absence of the *ehxA* gene for all 10 strains. The complete results for all laboratories for all strains are presented in Annexes 13-16.

Table 7: Average scores for virulence determination

Strain	*eae* gene	*vtx*1 gene	*vtx*2 gene	*ehx*A gene
N	20	22	22	15
KK11	100%	100%	100%	100%
LL12	100%	95%	100%	100%
MM13	100%	100%	82%	100%
NN14	100%	100%	100%	100%
OO15	90%	100%	100%	100%
PP16	100%	100%	100%	100%
QQ17	100%	100%	100%	100%
RR18	100%	100%	100%	100%
SS19	95%	100%	100%	100%
TT20	95%	100%	100%	100%
Average	**98%**	**99%**	**98%**	**100%**

Percentages and no. (n) of laboratories with correct reported results (based on the results submitted by all participants Annexes 13-16).

3.4.2 Subtyping of *vtx1* and *vtx2*

The number of laboratories participating in subtyping of *vtx* genes was between 18 and 20 (78-87% of the participants). The average subtyping results of *vtx* genes were calculated based on the number of participants, including laboratories, which reported false negatives for *vtx1* or *vtx2*. The results indicate that the participants followed our recommendation to perform subtyping on all test strains irrespective of the results of the detection of *vtx1* and *vtx2*; in general laboratories correctly subtyped strains despite a negative *vtx* detection result. For *vtx1a* the range was from 94% correct in RR18 to 100% for strain OO15. One false negatives were reported for subtype *vtx2*. One laboratory reported three false positives (the presence of *vtx1a* twice and *vtx1c* once in combination with the correct result). On average, *vtx2* was correctly typed in 87% of submitted results. The range by strain was from 60% for *vtx2b* + *vtx2d* in strain TT20 (O174:H21) to 100% for *vtx2a* in strain RR18 (O157:H7) and for the three *vtx2* negative samples. False positive and negative results are included in Table 8. Strain SS19 and QQ17, the strains with the lowest percentage of correct detection, had the highest false positive results, mainly *vtx2c* + *vtx2d*. The complete results are presented in Annex 17.

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

	***vtx1* subtyping**				***vtx2* subtyping**			
Strain	Original	Found *vtx1* gene	False positive	False negative	Original	Found *vtx2* gene	False positive	False negative
N		18				20		
KK11	-	100% (18)			*vtx2a*	90% (18)	1 *vtx2a + vtx2c*	
LL12	*vtx1d*	94% (17)	1 *vtx1a + vtx1d*		-	100% (20)		
MM13	-	100% (18)			*vtx2f*	85% (17)	1 *vtx2f + vtx2d*, 1 *vtx2f + vtx2e*, 1 *vtx2f + vtx2b*	
NN14	*vtx1c*	94% (17)	1 *vtx1a + vtx1c*		-	100% (20)		
OO15	*vtx1a*	100% (18)			-	100% (20)		
PP16	-	100% (18)			*vtx2a*	95% (19)		1
QQ17	-	100% (18)			*vtx2a + vtx2c*	75% (15)	4 *vtx2a* 1 *vtx2a*, vtx2c, *vtx2d*	
RR18	*vtx1a*	94% (17)	1 *vtx1a + vtx1c*		*vtx2a*	100% (20)		
SS19	-	100% (18)			*vtx2d*	65% (13)	1 *vtx2c*, *5 vtx2c + vtx2d* *1 vtx2b + vtx2d + vtx2c*	
TT20	-	100% (18)			*vtx2b + vtx2d*	60% (12)	5 *vtx2b* 1 *vtx2d* 1 *vtx2b + vtx2d + vtx2c* 1 *vtx2b + vtx2c + vtx2d + vtx2a*	
Average		**98%**				**87%**		

n = number of participants. Percentages are calculated based on the results submitted by the participants listed in Annex 17.

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

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

	vtx1a	***vtx1c***	***vtx1d***	***vtx2a***	***vtx2b***	***vtx2c***	***vtx2d***	***vtx2f***
Sensitivity	0.98	1.00	1.00	0.99	0.95	0.75	0.84	0.95
Specificity	0.99	0.99	1.00	0.99	0.99	0.94	0.99	1.00

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

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.

Twelve laboratories correctly reported none of the strains had an *aggR* gene. Seven laboratories reported false negative results for strain NN14 (*aaic* gene), which has been found to harbour a variant of the *aaiC* gene. However analysis have shown the variant is not covered by the conventional primers used to detect the *aaiC* gene.

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

		% of correct results (no.)	
Strain	**False negative results (n)**	***aggR***	***aaiC***
n		12	7
KK11		100% (12)	100% (7)
LL12		100% (12)	100% (7)
MM13		100% (12)	100% (7)
NN14	*aaiC* (7)	100% (12)	0% (0)
MM15		100% (12)	100% (7)
PP16		100% (12)	100% (7)
QQ17		100% (12)	100% (7)
RR18		100% (12)	100% (7)
SS19		100% (12)	100% (7)
TT20		100% (12)	100% (7)

n = number of participants. Percentages are calculated based on the results submitted by the participants listed in Annex 18.
No false positive results were submitted for any of the genes
Other additional virulence genes detected by the participating laboratories are shown in Table 11 and are only included for future reference. The EQA provider did not test these genes.

Table 11: Additional virulence genes in this EQA test strains

Strain no.	Positive gene results (n)	Suggested negative gene results
KK11	*eae-β* (1), *lpfAO113* (1), *iha*(1), tox-B(1), efa(1), hlyA(1)	*cdt-V, astA, IpfO141/O145, TEM, ctx-Mu, ctx-M-15, eltA, aatA, estA, IpaH, estAh, estAp, altA, eltI, EAF, bfpA, elt, est*
LL12	*lpfO141*/O154 (1), *estAh* (1)	*cdt-V, astA, iha, toxB, efa, IpfAO113, TEM, ctx-Mu, ctx-M-15, eltA, aatA, estA, IpaH, estAp, altA, eltI, EAF, bfpA,eltI, EAF, bfpA, hlyA, elt, est*
MM13	*eae*-α2 (1)	*cdt-V, astA, iha, toxB, efa,IpfO141/O145, IpfAO113, TEM, ctx-Mu, ctx-M-15, eltA, aatA, estA, IpaH, estAh, estAp, altA, eltI, EAF, bfpA, hlyA, elt, est*
NN14	*lpfO113* (1), *estAh* (1)	*cdt-V, astA, iha, toxB, efa,IpfO141/O145, TEM, ctx-Mu, ctx-M-15, eltA, aatA, estA, IpaH, estAp, altA, eltI, EAF, bfpA, hlyA, elt, est*
MM15	*eae*-θ (1), *lpfO113* (1), *iha* (1), *efa* (1)	*cdt-V, astA, toxB,IpfO141/O145, TEM, ctx-Mu, ctx-M-15, eltA, aatA, estA, IpaH, estAh, estAp, altA, eltI, EAF, bfpA, elt, est*
PP16	*eae*-γ (1), *iha* (1), *tox*-B (1), *efa* (1), *lpfO141*/O154 (1), *hlyA*(1)	*cdt-V, astA, IpfAO113, TEM, ctx-Mu, ctx-M-15, eltA, aatA, estA, IpaH, estAh, estAp, altA, eltI, EAF, bfpA, elt, est*
QQ17	*eae*-ε (1), *lpfO113* (1), *tox*-B (1), *efa* (1), *hlyA*(1)	*cdt-V, astA, IpfAO113, TEM, ctx-Mu, ctx-M-15, eltA, aatA, estA, IpaH, estAh, estAp, altA, eltI, EAF, bfpA, elt, est*
RR18	*eae*-γ (1), *iha* (1), *tox*-B (1), *efa* (1), *lpfO141*/O154 (1), *hlyA*(1)	*cdt-V, astA, IpfAO113, TEM, ctx-Mu, ctx-M-15, eltA, aatA, estA, IpaH, estAh, estAp, altA, , eltI, EAF, bfpA, elt, est*
SS19	*astA* (2), *BLEE* (1), *ctx*-M-15 (1), *eltA* (3), *eltI* (1), *hlyA*(1)	*cdt-V, iha, toxB, efa,IpfO141/O145, IpfAO113, TEM, ctx-Mu, aatA, estA, IpaH, estAh, estAp, altA, EAF, bfpA, elt, est*
TT20	*lpfO113* (1), *iha* (1), *estAh* (1), *altA* (1), *elt* (1), *est*(1)	*cdt-V, astA, toxB, efa,IpfO141/O145, TEM, ctx-Mu, ctx-M-15, eltA, aatA, estA, IpaH, estAh, estAp, eltI, EAF, bfpA*

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

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

3.4.4 Phenotypic test

Participation in phenotypic detection ranged lower than the genotypic detections from only 26% (VCA) to 82% (sorbitol fermentation). The proportions of correct results were, in average: 96% for detection of ESBL production, 97% for β-glucuronidase production and 98% for VCA, enterohaemolysin production and fermentation of sorbitol (Table 12).

Table 12: Average scores of the phenotypic tests

Strain/method	VCA	ESBL production	Enterohaemolysin production	β-glucuronidase production	Sorbitol fermentation
N	6	8	9	10	19
KK11	100% (6)	100% (8)	100% (9)	100% (10)	100% (19)
LL12	100% (6)	88% (7)	100% (9)	100% (10)	100% (19)
MM13	100% (6)	100% (8)	89% (8)	100% (10)	89% (17)
NN14	100% (6)	100% (8)	100% (9)	100% (10)	89% (17)
OO15	100% (6)	88% (7)	100% (9)	70% (7)	100% (19)
PP16	83% (5)	100% (8)	100% (9)	100% (10)	100% (19)
QQ17	100% (6)	100% (8)	100% (9)	100% (10)	100% (19)
RR18	100% (6)	100% (8)	100% (9)	100% (10)	100% (19)
SS19	100% (6)	88% (7)	100% (9)	100% (10)	100% (19)
TT20	100% (6)	100% (8)	100% (9)	100% (10)	100% (19)
Average	**98%**	**96%**	**98%**	**97%**	**98%**

n =Number of participants. The percentages are calculated based on the results of the participants presented in Annexes 8-12.

The errors in the detection of ESBL production, enterohaemolysin production and VCA were submitted by a single laboratory. For β-glucuronidase production and sorbitol fermentation three and four laboratory submitted one incorrect results each. Detailed results for all phenotypic tests can be found in Annexes 8 (VCA), 9 (ESBL), 10 (enterohaemolysin), 11 (β -glucuronidase), and 12 (sorbitol).

4 Conclusions

Twenty-four laboratories signed up for EQA on VTEC for self-funded participants in 2014-2015 (one did not submit results). The EQA included PFGE, and 12 laboratories participated in the PFGE exercise. Eleven (92%) of those laboratories were able to produce a PFGE gel of sufficiently high quality to allow comparison with profiles obtained by other laboratories. For the critical parameter 'Image acquisition and running conditions' only one of the participants scored poor (1). The production of distinct bands is another important gel quality assessment parameter; the laboratory also scored poor (1) due to either too faint, too thick or too fuzzy bands, the remaining laboratories scored either fair (six laboratories) or good (five laboratories). The BN software suite was used for the normalisation and interpretation of profiles. Nine (75%) laboratories analysed also the resulting gels and eight of these laboratories performed in good accordance with the guidelines.

Sixteen of the laboratories (69%) participated in the full O:H serotyping, with eight laboratories reporting correct O group and H type results for all 10 test strains. On average, throughout the test strains correct results were reported correctly for O-group by 84% of the laboratories, for the H group by 83% of laboratories. The overall full O:H typing were correctly reported by 65% of laboratories. However, not all laboratories reported the full scheme of all O groups and H types.

The participation rate was 87% for *eae*, 96% for both *vtx1* and *vtx2,* and 65% for *ehxA.* Gene detection of *eae, vtx1*, *vtx2* and *ehxA* by strain was 82–100% correct. The score of the vtx2 detection (82%) was mainly caused by one strain (MM13) positive for *vtx2f*. Subtyping for *vtx* was performed by 78-96% of the participants, with an average of 98% of correct results for *vtx1* and 87% for *vtx2.*

Phenotypic characterisation generally showed very good results: 98% correct results for VT, enterohaemolysin production and for fermentation of sorbitol, 97% for β-glucuronidase production, and 96% for detection of ESBL production. Phenotypic characterisation was not performed as often as genotypic characterisation: detection of VT production (26% of the participants), enterohaemolysin production (39%), β-glucuronidase production (43%), sorbitol (83%), and ESBL (35%).

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

For the laboratory with a poor PFGE results, additional trouble shooting and training activities should be considered; in particular the use of the *S.* Braenderup ladder and the recommended running conditions.

5 Discussion

Since 2002, the Reference and Research Centre on *Escherichia* and *Klebsiella*, 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*.

5.1 Pulsed-field gel electrophoresis (PFGE)

Twelve laboratories participated in the PFGE component of this 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 modified TIFF quality grading guidelines which evaluate seven parameters. Scores were given between 1 and 4 (poor, fair, good and excellent). The majority (92%) of laboratories were able to produce gels with sufficiently high quality (above a score of 1) in all seven parameters.

The main issue in this VTEC EQA was in the parameter Bands. A grading of fair and above was given for 92% of gels, while 8% of gels scored 1 in this parameter. In general, major improvements could be made when capturing the image and producing a TIFF image. However, none of the laboratories obtained a score of excellent in this category and many laboratories seemed to adapt the image settings in effort to alter the contrast for the image to enhance weak bands. Unfortunately, this manipulation of the image can result in thicker bands and blurry gels and therefore makes it difficult to distinguish between double bands.

It is important to use running conditions as described for the relevant organism as these varies significantly between species. The same protocol has to be implemented by a laboratory if they wish their gels to be compared to those from other laboratories. Nevertheless, in this EQA, 92% of all gels obtained a score of at least 2 in all parameters, and are therefore suitable for inter-laboratory comparison. Other common deviations from protocol was seen in Image Acquisition, where some laboratories forget to fill the whole image with the gel, include wells and leave 1 to 1.5 cm below the smallest band on the gel. This is less critical than using incorrect running conditions, but can still have major impact on the ability to assign bands correctly. The other parameters are not the most problematic in this EQA, but it is still desirable to improve the laboratories' capacity in these areas. In general, for a highly sensitive method such as PFGE, it is of high importance to follow the protocol. In order to improve the categories Gel Background and DNA Degradation, major improvements can be made by carefully following the instructions regarding the lysis step time of restriction for the relevant enzyme, washing plugs six times, and de-staining the gel adequately after dyeing.

Nine (75%) of those performing PFGE did the subsequent gel analysis, i.e. the normalisation and band assignment, producing 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, it is important to be able to perform national surveillance as well as submit profiles to some surveillance systems such as the ECDC system The European Surveillance System (TESSy). Eight out of the nine laboratories who submitted gel analysis data analysed PFGE gels in fair to excellent (2–3) in accordance with the guidelines. The *S.* Braenderup reference strain was only run only once and a lambda ladder was run twice, therefore Normalization could not be carried out.

5.2 Serotyping

This EQA had 22 participants for the serotyping part. An average of 84% of the 14–22 participating laboratories correctly performed O grouping of the 10 test strains.

The clear trend in this EQA was that the more common serotypes could be identified more reliably. Correct O:H serotyping ranged from 100% correct typing of both of the O157:H7 strain to 59% correct typing of serotype O174:H21. No systematic typing errors were observed. Only three O groups and five H types were mistyped. The remainder of incorrect typing was submitted as non typeable (NT).

In summary, 11 of the 22 (50%) laboratories were able to correctly determine the O group for all test strains, 11 (50%) laboratories correctly identified all H groups whilst 8 (30%) laboratories were able to correctly determine the full O:H group of all 10 test strains.

5.3 Virulence determination

5.3.1 Genotypic tests

Genotypic detection of virulence genes *eae*, *vtx1, vtx2* and *ehxA* was performed by 15–22 of laboratories for all the 10 test strains. The participation rate varied substantially between the different tests in this EQA, being highest for

the genotypic detection of the *vtx* genes (95%) and lowest for the detection of *ehxA* (65%). In general, the percentage of correct results was very high (98–100%). The incorrect results for the *eae* gene originated from errors by two laboratories.

Detection of *vtx1* and *vtx2* genes was achieved with a high percentage of correct results (99% and 98%). However, the majority of false negative results originated from testing the strain MM13 (*vtx2f*). Four laboratories did not detect the strain positive for *vtx2.* The importance of awareness of *vtx2f* has been described by Friesema *et al.*, 2014 [15], where cases of HUS caused by strains harbouring *vtx2f* have been discussed [16]

In previous EQAs, the major problem in subtyping the *vtx* genes was distinguishing between *vtx2a*, *vtx2b*, *vtx2c* and *vtx2d*. A revised protocol for subtyping of *vtx* genes was published in September 2012 [14]. This year, correct results ranged from 94–100% for subtyping *vtx1* and from 60–100% for subtyping *vtx2*. Correct subtyping of both *vtx1* was obtained at an average of 98% and *vtx2* an average of 87%.

All seven laboratories failed to detect *aaiC* in strain NN14 (O104:H7), which has been found to harbour a variant of the *aaiC* gene not covered by the conventional primers used to detect the *aaiC* gene [17].

5.3.2 Phenotypic tests

The participation in the phenotypic detection was between 26 and 83% on average (6–19 laboratories). The lowest participation was for VCA, where only six participants (26%) delivered results for 10 strains.

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 by 83% of the participating laboratories. The second highest participation was for β-glucuronidase (43%), followed by enterohaemolysin (39%), ESBL (34%) and VCA production (26%).

The errors in the detection of ESBL production were submitted by a single laboratory. In summary, the performance level for phenotypic characterisation was very high, but certain laboratories need to assess their protocols.

5.3 General remarks

The inconsistency in the number of performed tests per strain and per laboratory was notable in all VTEC EQAs so far. Laboratories never explained why a specific test was not performed on all 10 test strains. This was particularly evident for O grouping where laboratories submitted multiple instances of 'NT'. A similar situation was encountered for H typing. These inconsistencies reduce comparability between the tests and the laboratories and complicate the analyses.

6 Recommendations

6.1 Laboratories

By evaluating the results from this EQA a number of technical issues were identified, which have an impact on the quality of typing results. For each method, improvements of the performance could be achieved by following a range of measures.

The quality of PFGE profiles is dependent on the use of standardised and controlled laboratory procedures. Therefore, laboratories can improve their performance by applying and strictly adhering to the standardised protocols. Such protocols detail, for example, temperatures, times, and the number of repeated washing steps. Deviations from the protocol should be avoided unless such deviations have been thoroughly evaluated. Certain elements to the protocol cannot be modified, in particular 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.

Laboratories might have produced a high quality gel, but failed to document this due to sub-optimal staining, destaining and issues with 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. Errors can be avoided if laboratory personnel would carefully follow the instructions on how to produce and submit TIFF and XML files of the PFGE results. In addition, some laboratories had difficulties creating and sending TIFF and XML files of the PFGE results, however laboratories seem to proofread the results before submission.

In this EQA the majority of laboratories participated in O:H serotyping. Serotyping is essential for the characterisation of *E. coli* and VTEC. However, obvious challenges with the serotyping of non-common strains were observed.

For both genotypic and phenotypic tests 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 Participation

The PFGE part of this VTEC EQA had 12 participants; 75% of the participating laboratories performed the BN gel analysis. Eighty-three percent of the gels produced were of sufficiently high quality for inter-laboratory comparison, and almost all completed the BN analyses were at an acceptable level. However, there is still a need to improve laboratory procedures, gel analysis, and interpretation with BN software. Training to get familiar with the electrophoresis equipment and image acquisition equipment.

The relatively low levels of participation for the phenotypic tests for VT production, ESBL, enterohaemolysin β-glucuronidase production are only performed by a small number of laboratories.

7 References

1. EFSA (European Food Safety Authority) and ECDC (European Centre for Disease Prevention and Control). 4-9-2015. The European Union Summary Report on Trends and Sources of Zoonoses, Zoonotic Agents and Food-borne Outbreaks in 2014. EFSA Journal 13(12):4329: 1-191. Parma, Italy.

2. Bielaszewska M, Friedrich AW, Aldick T, Schürk-Bulgrin R, Karch H. Shiga toxin activatable by intestinal mucus in *Escherichia coli* isolated from humans: predictor for a severe clinical outcome. Clin Infect Dis. 2006 Nov 1;43(9):1160-7.

3. Friedrich AW, Bielaszewska M, Zhang WL, Pulz M, Kuczius T, Ammon A, et al. 2002. *Escherichia coli* harboring Shiga toxin 2 gene variants: frequency and association with clinical symptoms. J Infect Dis. 2002 Jan 1;185(1):74-84.

4. Persson S, Olsen KE, Ethelberg S, Scheutz F. Subtyping method for Escherichia coli Shiga toxin (verocytotoxin) 2 variants and correlations to clinical manifestations. J Clin Microbiol. 2007 Jun;45(6):2020-4.

5. Scheutz F, Ethelberg S. Nordic meeting on detection and surveillance of VTEC infections in humans. Copenhagen: Statens Serum Institut; 2007.

6. Bielaszewska M, Friedrich AW, Aldick T, Schürk-Bulgrin R, Karch H. Production of mucus-activatable Shiga toxin (Stx) is a risk factor for a severe clinical outcome of infections caused by Stx-producing *Escherichia coli*. Int J Med Microbiol. 2006. 296:89.

7. de Sablet T, Bertin Y, Vareille M, Girardeau JP, Garrivier A, Gobert AP, et al. Differential expression of *stx_2* variants in Shiga toxin-producing *Escherichia coli* belonging to seropathotypes A and C. Microbiology. 2008 Jan;154(Pt 1):176-86.

8. Gobius KS, Higgs GM, Desmarchelier PM. Presence of activatable Shiga toxin genotype (stx(2d)) in Shiga toxigenic *Escherichia coli* from livestock sources. J Clin Microbiol. 2003 Aug;41(8):3777-83.

9. Jelacic JK, Damrow T, Chen GS, Jelacic S, Bielaszewska M, Ciol M, et al. Shiga toxin-producing *Escherichia coli* in Montana: bacterial genotypes and clinical profiles. J Infect Dis. 2003 Sep 1;188(5):719-29.

10. Scheutz F, Teel LD, Beutin L, Piérard D, Buvens G, Karch H, et al. Multicenter evaluation of a sequence-based protocol for subtyping Shiga toxins and standardizing Stx nomenclature. J Clin Microbiol. 2012 Sep;50(9):2951-63.

11. International Organization for Standardization. ISO/IEC 17043:2010 Conformity assessment – General requirements for proficiency testing. Geneva: International Organization for Standardization; 2010.

12. Ribot EM, Fair MA, Gautom R, Cameron DN, Hunter SB, Swaminathan B, et al. Standardization of pulsed-field gel electrophoresis protocols for the subtyping of *Escherichia coli* O157:H7, Salmonella, and Shigella for PulseNet. Foodborne Pathog Dis. 2006 Spring;3(1):59-67.

13. Scheutz F, Fruth A, Cheasty T, Tschäpe H. O Grouping and H typing: standard operation procedures (O SOP an H SOP) – *Escherichia coli* O antigen grouping and H antigen determination. Copenhagen: Statens Serum Institut; 2002.

14. Scheutz F, Morabito S, Tozzoli R, Caprioli A. Identification of three *vtx1* and seven *vtx2* subtypes of verocytotoxin encoding genes of *Escherichia coli* by conventional PCR amplification. Copenhagen: Statens Serum Institut; 2002.

15. Friesema, I., K. van der Zwaluw, T. Schuurman, M. Kooistra-Smid, E. Franz, D. Y. van, and P. W. van. 2014. Emergence of Escherichia coli encoding Shiga toxin 2f in human Shiga toxin-producing E. coli (STEC) infections in the Netherlands, January 2008 to December 2011. Euro.Surveill. 19:20787.

16. Friesema, I. H., M. G. Keijzer-Veen, M. Koppejan, H. S. Schipper, A. J. van Griethuysen, M. E. Heck, and P. W. van. 2015. Hemolytic uremic syndrome associated with Escherichia coli O8:H19 and Shiga toxin 2f gene. Emerg.Infect.Dis. 21:168-169. doi:10.3201/eid2101.140515 [doi]].

17. European Centre for Disease Prevention and Control. Sixth external quality assessment scheme for typing of verocytotoxin-producing E. coli (VTEC). Stockholm: ECDC; 2015.

Annex 1. List of participants

Country	Institution	Laboratory
Argentina	Instituto Nacional de Enfermedades Infecciosas	Servicio Fisiopatogenia
Australia	CSIRO	Food and Nutrition Flagship
Australia	The University of Melbourne	Microbiological Diagnostic Unit Public Health Laboratory
Australia	Peter Doherty Intsitute	Public Health Microbiology
Brasil	Instituto Adolfo Lutz - Secretaria de Estado da Saúde	Laboratório de Referência Nacional para Sindrome Hemolítica Urêmica
Canada	Ottawa Laboratory (Carling)	OLC
Canada	Canadian Science Centre for Human and Animal Health	Enteric Diseases Program
Canada	Public Health Agency of Canada	*E. coli* Laboratory
Canada	Université de Montréal	OIE Reference Laboratory for Escherichia coli
Chile	Instituto de Salud Publica de Chile	Bacteriologia
China	Country Shanghai Changning Center For Disease Control And Prevention	Department of microbial test
China	National Center for Food Safety Risk Assessment,	Microbiology Laboratory
France	Hôpital Robert Debré	Service de Microbiologie, CRN associé *E. coli*
Germany	Institut für Hygiene und Umwelt	Feintypisierung
Japan	National Institute of Infectious Disease	Department of Bacteriology I
Mexico	Facultad De Medicina	laboratorio de salud pública
New Zealand	ESR - NCBID	Enteric Reference Laboratory
South Africa	National Institute for Comminicable Diseases	CED-Bacteriology
Spain	Centro Tecnológico Agroalimentario (CETAL) and Universidade de Santiago de Compostela (USC)	Unidad Mixta de Microbiología Especializada en E. coli (UMMEC) del Laboratorio de Seguridad Alimentaria
Switzerland*	University of Zürich	Institute food Safety
UK	PHE colindale	Scottish VTEC Reference Laboratory
USA	Centers for Disease Control and Prevention	Enteric Diseases Laboratory Branch
USA	US FDA	Microbiology
USA	The Pennsylvania State University	*E. coli* Reference Center

*no submission of results

Annex 2. TIFF quality grading guidelines[1]

Parameter	TIFF Quality Grading Guidelines			
	Excellent	**Good**	**Fair**	**Poor**
Image acquisition and running conditions	By protocol, for example: - Gel fills whole TIFF - Wells included on TIFF - Bottom band of standard 1-1.5 cm from bottom of gel	Gel does not fill whole TIFF but band finding is not affected. Bottom band of standard not 1-1.5 cm from bottom of gel but analysis is not affected	- Gel does not fill whole TIFF and band finding slightly affected - Wells not included on TIFF - Bottom band of standard not 1-1.5 cm from bottom of gel and analysis is slightly affected. - Band spacing of standards does not match global standard and analysis is slightly affected.	- Gel does not fill whole TIFF and band finding is highly affected. - Bottom band of standard not 1-1.5 cm from bottom of gel and analysis is highly affected. - Band spacing of standards does not match global standard and analysis is highly affected.
Cell suspensions	The cell concentration is approximately the same in each lane	Up to two lanes contain darker or lighter bands than the other lanes.	More than two lanes contain darker or lighter bands than the other lanes, or at least one lane is much darker or lighter than the other lanes, making the gel difficult to analyse	The cell concentrations are uneven from lane to lane, making it impossible to analyse the gel.
Bands	Clear and distinct all the way to the bottom of the gel	- Slight band distortion in one lane but this does not interfere with analysis - Bands are slightly fuzzy and/or slanted - A few bands (three or less) are difficult to see clearly (i.e. DNA overload) especially at the bottom of the gel.	Some band distortion (i.e. nicks) in two to three lanes but can still be analysed. Fuzzy bands Some bands (four or five) are too thick Bands at the bottom of the gel are light but analysable.	- Band distortion that makes analysis difficult - Very fuzzy bands - Many bands too thick to distinguish - Bands at the bottom of the gel too light to distinguish
Lanes	Straight	- Slight 'smiling' (higher bands in outside lanes than inside) - Lanes gradually run longer towards the right or left (can still be analysed)	- Significant 'smiling' - Slight curves on the outside lanes - Can still be analysed	'Smiling' or curving that interferes with analysis
Restriction	Complete restriction in all lanes	One or two faint shadow bands on the gel	- One lane with many shadow bands - A few shadow bands spread out over several lanes	- More than one lane with several shadow bands - Lots of shadow bands over the whole gel.
Gel background	Clear	- Mostly clear background - Minor debris present that does not affect analysis	- Some debris present that may or may not make analysis difficult (e.g. auto band search finds too many bands) - Background caused by photographing a gel with very light bands (image contrast was 'brought up' in photographing gel (makes image look grainy).	Lots of debris present that make the analysis impossible.
DNA degradation (smearing in the lanes)	Not present	Minor background (smearing) in a few lanes but bands are clear.	Significant smearing in one to two lanes that may or may not make analysis difficult. Minor background (smearing) in many lanes.	- Smearing so that several lanes are not analysable (except of untypeable thiourea required).

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

Annex 3. BioNumerics (BN) gel analysis quality guidelines

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

Annex 4. Scores of the PFGE results

Gel quality

Parameters\Laboratory	506	508	540	521	511	515	526	516	520	550	504	530
Image and Running Conditions	3	3	1	4	4	3	3	4	3	4	4	3
Cell Suspension	4	4	2	3	3	3	4	2	4	3	4	4
Bands	3	2	1	2	3	2	3	2	3	2	3	2
Lanes	3	3	2	3	4	4	3	3	3	4	4	4
Restriction	4	4	2	4	4	4	4	3	4	4	4	3
Gel Background	4	4	2	4	4	4	4	4	4	4	4	4
DNA Degradation	4	4	4	4	4	4	4	2	4	3	4	4

Scored according to Annex 2 (TIFF quality grading guidelines)

BN analysis

Parameters\Laboratory	506	508	540	521	511	515	526	516	520	550	506	530
Position of Gel	N/A	3	N/A	3	3	3	3	N/A	3	3	3	3
Strips	N/A	3	N/A	3	3	3	2	N/A	3	3	3	3
Curves	N/A	2	N/A	3	2	2	3	N/A	3	2	3	2
Normalization	N/A	3	N/A	3	3	1	2	N/A	3	2	2	2
Band Assignment	N/A	3	N/A	2	2	3	3	N/A	3	2	3	3

Scores according to Annex 3 (BN gel analysis quality guidelines)

N/A Did not participate in the BN of the PFGE part

Annex 5. Original data (serotyping, genotyping and phenotyping)

	O group	H type	Vero Cell assay	ESBL prod.	Haemolysin prod.	Beta-glucuronidase prod.	Sorbitol ferm.	*eae* gene	*ehxA* gene	*vtx1* gene	*vtx2* gene	*vtx* Subtypes			*aggR*	*aaiC*	Additional virulence genes	Group
KK11	O26	H11	+	-	+	+	+	+	+	-	+	-	*vtx2a*		-	-		VTEC
LL12	O41	H26	+	-	-	+	+	-	-	+	-	*vtx1d*	-		-	-		VTEC
MM13	O63	H6	+	-	-	+	-	+	-	-	+	-	*vtx2f*		-	-	*astA*	VTEC
NN14	O104	H7	+	-	-	+	+	-	-	+	-	*vtx1c*	-		-	+		EAEC-VTEC
OO15	O111	H- / H8	+	-	+	+	+	+	+	+	-	*vtx1a*	-		-	-		VTEC
PP16	O121	H19	+	-	+	+	+	+	+	-	+	-	*vtx2a*		-	-	*astA*	VTEC
QQ17	O157	H7	+	-	+	-	+	+	+	-	+	-	*vtx2a*	*vtx2c*	-	-		VTEC
RR18	O157	H7	+	-	+	-	-	+	+	+	+	*vtx1a*	*vtx2a*		-	-	*astA*	VTEC
SS19	O166	H15	+	+	-	+	+	-	-	-	+	-	*vtx2d*		-	-	*eltA*	VTEC
TT20	O174	H21	+	-	-	+	+	-	-	-	+	-	*vtx2b*	*vtx2d*	-	-		VTEC

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

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

Annex 6. O group serotyping results

Lab/Strain	O group	508	522	525	501	527	518	519	511	521	509	515	526	403	530	540	514	516	504	405	520	523	506	550
KK11	O26	O26	ND	O26	O26	O26	O26	NT	O26	O26	O26	O26	O26	O26	O26	O26	O26	O26	O26	O26	O26	O26	O26	O26
LL12	O41	O41	ND	O41	NT	O41	O41	NT	O41	O41	Rough	O41	NON-O157	O41	O41	O121	O41	O41	O41	NT	O41	O41	O41	NT
MM13	O63	O63	ND	NT	NT	O63	O63	NT	O63	O63	NT	O63	NON-O157	O63	O63	NT	O63	O63	O63	NT	O63	O63	O63	O63
NN14	O104	O104	ND	O104	NT	O104	O104	NT	O104	O104	NT	O104	O157	O104	O104	O104	O104	O104	O104	NT	O104	O104	O104	O104
OO15	O111	O111	ND	O111	O111	O111	O111	NT	O111	O111	O111	O111	O111	O111	O111	O111	O111	O111	O111	O111	O111	O111	O111	O111
PP16	O121	O121	ND	O121	O121	O121	O121	NT	O121	O121	O121	O121	NON-O157	O121	O121	O121	O121	O121	O121	NT	O121	O121	O121	O121
QQ17	O157	O157	ND	O157	O157	O157	O157	O157	O157	O157	O157	O157	O157	O157	O157	O157	O157	O157	O157	O157	O157	O157	O157	O157
RR18	O157	O157	ND	O157	O157	O157	O157	O157	O157	O157	O157	O157	O157	O157	O157	O157	O157	O157	O157	O157	O157	O157	O157	O157
SS19	O166	O166	ND	O166	NT	O166	O166	NT	O166	O166	NT	O166	NON-O157	O166	O166	NT	O166	O166	O166	NT	O166	O166	O166	O166
TT20	O174	NT	ND	O174	NT	ND	O174	NT	O174	O174	NT	O174	NON-O157	O174	O174	NT	O174	O174	O15	NT	O174	O174	O174	O174

NT: not typable

ND: Not done

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

Incorrect result

Annex 7. H type serotyping results

Lab/Strain	H type	508	522	525	501	527	518	519	511	521	509	515	526	403	530	540	514	516	504	405	520	523	506	550
KK11	H11	H11	ND	H11	ND	H11	H11	NT	H11	H11	ND	H11	ND	H11	H11	ND	H11	H11	H11	ND	H11	H11	ND	H11
LL12	H26	H26	ND	H26	ND	H-	H26	NT	H26	H26	ND	H26	ND	H26	H26	ND	H26	H26	H-	ND	H26	H26	ND	H26
MM13	H6	H6	ND	H6	ND	H6	H6	NT	H6	H6	ND	H6	ND	H6	H6	ND	H6	H6	H-	ND	H6	H6	ND	H6
NN14	H7	H7	ND	H7	ND	H7	H7	H7	H7	H7	ND	H7	ND	H7	H7	ND	H7	H7	H7	ND	H7	H7	ND	H7
OO15	H-/H8*	H8	ND	H-	ND	H-	H8	NT	H-	H-	ND	H-	ND	H-	H19	ND	H-	H-	H-	ND	H-	H8	ND	H-
PP16	H19	H19	ND	H19	ND	H19	H19	NT	H19	H19	ND	H19	ND	H19	H19	ND	H19	H19	H19	ND	H19	H19	ND	H19
QQ17	H7	H7	ND	H7	ND	H7	H7	H7	H7	H7	ND	H7	ND	H7	H7	ND	H7	H7	H7	ND	H7	H7	ND	H7
RR18	H7	H7	ND	H7	ND	H7	H7	H7	H7	H7	ND	H7	ND	H7	H7	ND	H7	H7	H7	ND	H7	H7	ND	H7
SS19	H15	H15	ND	H15	ND	H15	H15	NT	H15	H15	ND	H15	ND	H15	H15	ND	H15	H15	H15	ND	H15	H15	ND	H15
TT20	H21	H21	ND	H21	ND	H21	H21	NT	H21	H21	ND	H21	ND	H21	H21	ND	H21	H21	H21	ND	H21	H-	ND	H21

**H- was accepted as a correct result.*

Incorrect result

Annex 8. VCA results

Lab/Strain	VCA	508	522	525	501	527	518	519	511	521	509	515	526	403	530	540	514	516	504	405	520	523	506	550
KK11	+	+	ND	ND	ND	ND	+	ND	+	ND	ND	+	+	ND	ND	ND	ND	ND	+	ND	ND	ND	ND	ND
LL12	+	+	ND	ND	ND	ND	+	ND	+	ND	ND	+	+	ND	ND	ND	ND	ND	+	ND	ND	ND	ND	ND
MM13	+	+	ND	ND	ND	ND	+	ND	+	ND	ND	+	+	ND	ND	ND	ND	ND	+	ND	ND	ND	ND	ND
NN14	+	+	ND	ND	ND	ND	+	ND	+	ND	ND	+	+	ND	ND	ND	ND	ND	+	ND	ND	ND	ND	ND
OO15	+	+	ND	ND	ND	ND	+	ND	+	ND	ND	+	+	ND	ND	ND	ND	ND	+	ND	ND	ND	ND	ND
PP16	+	+	ND	ND	ND	ND	-	ND	+	ND	ND	+	+	ND	ND	ND	ND	ND	+	ND	ND	ND	ND	ND
QQ17	+	+	ND	ND	ND	ND	+	ND	+	ND	ND	+	+	ND	ND	ND	ND	ND	+	ND	ND	ND	ND	ND
RR18	+	+	ND	ND	ND	ND	+	ND	+	ND	ND	+	+	ND	ND	ND	ND	ND	+	ND	ND	ND	ND	ND
SS19	+	+	ND	ND	ND	ND	+	ND	+	ND	ND	+	+	ND	ND	ND	ND	ND	+	ND	ND	ND	ND	ND
TT2ND	+	+	ND	ND	ND	ND	+	ND	+	ND	ND	+	+	ND	ND	ND	ND	ND	+	ND	ND	ND	ND	ND

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

+= Positive, - = Negative

Incorrect result

Annex 9. ESBL production results

Lab/Strain	ESBL	508	522	525	501	527	518	519	511	521	509	515	526	403	530	540	514	516	504	405	520	523	506	550
KK11	-	-	ND	-	ND	-	-	ND	ND	ND	-	-	-	ND	-	ND	ND	ND	ND	ND	ND	ND	ND	ND
LL12	-	-	ND	-	ND	-	-	ND	ND	ND	-	-	+	ND	-	ND	ND	ND	ND	ND	ND	ND	ND	ND
MM13	-	-	ND	-	ND	-	-	ND	ND	ND	-	-	-	ND	-	ND	ND	ND	ND	ND	ND	ND	ND	ND
NN14	-	-	ND	-	ND	-	-	ND	ND	ND	-	-	-	ND	-	ND	ND	ND	ND	ND	ND	ND	ND	ND
OO15	-	-	ND	-	ND	-	-	ND	ND	ND	-	-	+	ND	-	ND	ND	ND	ND	ND	ND	ND	ND	ND
PP16	-	-	ND	-	ND	-	-	ND	ND	ND	-	-	-	ND	-	ND	ND	ND	ND	ND	ND	ND	ND	ND
QQ17	-	-	ND	-	ND	-	-	ND	ND	ND	-	-	-	ND	-	ND	ND	ND	ND	ND	ND	ND	ND	ND
RR18	-	-	ND	-	ND	-	-	ND	ND	ND	-	-	-	ND	-	ND	ND	ND	ND	ND	ND	ND	ND	ND
SS19	+	+	ND	+	ND	+	+	ND	ND	ND	+	+	-	ND	+	ND	ND	ND	ND	ND	ND	ND	ND	ND
TT2ND	-	-	ND	-	ND	-	-	ND	ND	ND	-	-	-	ND	-	ND	ND	ND	ND	ND	ND	ND	ND	ND

+ = Positive,- -. = Negative

Neg. = Negative

Annex 10. Enterohaemolysin production results

Lab/Strain	Haemolysin	508	522	525	501	527	518	519	511	521	509	515	526	403	530	540	514	516	504	405	520	523	506	550
KK11	+	+	ND	+	ND	ND	+	ND	ND	ND	ND	+	+	ND	ND	ND	+	+	+	ND	+	ND	ND	ND
LL12	-	-	ND	-	ND	ND	-	ND	ND	ND	ND	-	-	ND	ND	ND	-	-	-	ND	-	ND	ND	ND
MM13	-	-	ND	+	ND	ND	-	ND	ND	ND	ND	-	-	ND	ND	ND	-	-	-	ND	-	ND	ND	ND
NN14	-	-	ND	-	ND	ND	-	ND	ND	ND	ND	-	-	ND	ND	ND	-	-	-	ND	-	ND	ND	ND
OO15	+	+	ND	+	ND	ND	+	ND	ND	ND	ND	+	+	ND	ND	ND	+	+	Alfa	ND	+	ND	ND	ND
PP16	+	+	ND	+	ND	ND	+	ND	ND	ND	ND	+	+	ND	ND	ND	+	+	Alfa	ND	+	ND	ND	ND
QQ17	+	+	ND	+	ND	ND	+	ND	ND	ND	ND	+	+	ND	ND	ND	+	+	Alfa	ND	+	ND	ND	ND
RR18	+	+	ND	+	ND	ND	+	ND	ND	ND	ND	+	+	ND	ND	ND	+	+	+	ND	+	ND	ND	ND
SS19	-	-	ND	-	ND	ND	-	ND	ND	ND	ND	-	-	ND	ND	ND	-	-	-	ND	-	ND	ND	ND
TT2ND	-	-	ND	-	ND	ND	-	ND	ND	ND	ND	-	-	ND	ND	ND	-	-	-	ND	-	ND	ND	ND

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

+= Positive, -= Negative

Incorrect result

Annex 11. β-glucuronidase production results

Lab/Strain	β-glucuronidase production	508	522	525	501	527	518	519	511	521	509	515	526	403	530	540	514	516	504	405	520	523	506	550
KK11	+	+	ND	ND	ND	+	ND	ND	ND	ND	ND	+	+	+	+	ND	+	+	+	ND	+	ND	ND	ND
LL12	+	+	ND	ND	ND	+	ND	ND	ND	ND	ND	+	+	+	+	ND	+	+	+	ND	+	ND	ND	ND
MM13	+	+	ND	ND	ND	+	ND	ND	ND	ND	ND	+	+	+	+	ND	+	+	+	ND	+	ND	ND	ND
NN14	+	+	ND	ND	ND	+	ND	ND	ND	ND	ND	+	+	+	+	ND	+	+	+	ND	+	ND	ND	ND
OO15	+	+	ND	ND	ND	-	ND	ND	ND	ND	ND	+	-	-	+	ND	+	+	+	ND	+	ND	ND	ND
PP16	+	+	ND	ND	ND	+	ND	ND	ND	ND	ND	+	+	+	+	ND	+	+	+	ND	+	ND	ND	ND
QQ17	-	-	ND	ND	ND	-	ND	ND	ND	ND	ND	-	-	-	-	ND	-	-	-	ND	-	ND	ND	ND
RR18	-	-	ND	ND	ND	-	ND	ND	ND	ND	ND	-	-	-	-	ND	-	-	-	ND	-	ND	ND	ND
SS19	+	+	ND	ND	ND	+	ND	ND	ND	ND	ND	+	+	+	+	ND	+	+	+	ND	+	ND	ND	ND
TT2ND	+	+	ND	ND	ND	+	ND	ND	ND	ND	ND	+	+	+	+	ND	+	+	+	ND	+	ND	ND	ND

+= Positive, -= Negative

Incorrect result

Annex 12. Sorbitol fermentation results

Lab/Strain	Sorbitol fermentation	508	522	525	501	527	518	519	511	521	509	515	526	403	530	540	514	516	504	405	520	523	506	550
KK11	+	+	ND	+	+	+	+	ND	+	ND	+	+	+	+	+	+	+	+	+	+	+	ND	+	ND
LL12	+	+	ND	+	+	+	+	ND	+	ND	+	+	+	+	+	+	+	+	+	+	+	ND	+	ND
MM13	-	-	ND	-	-	-	-	ND	-	ND	-	-	-	-	-	-	+	+	-	-	-	ND	-	ND
NN14	+	+	ND	-	-	+	+	ND	+	ND	+	+	+	+	+	+	+	+	+	+	+	ND	+	ND
OO15	+	+	ND	+	+	+	+	ND	+	ND	+	+	+	+	+	+	+	+	+	+	+	ND	+	ND
PP16	+	+	ND	+	+	+	+	ND	+	ND	+	+	+	+	+	+	+	+	+	+	+	ND	+	ND
QQ17	-	-	ND	-	-	-	-	ND	-	ND	-	-	-	-	-	-	-	-	-	-	-	ND	-	ND
RR18	-	-	ND	-	-	-	-	ND	-	ND	-	-	-	-	-	-	-	-	-	-	-	ND	-	ND
SS19	+	+	ND	+	+	+	+	ND	+	ND	+	+	+	+	+	+	+	+	+	+	+	ND	+	ND
TT2ND	+	+	ND	+	+	+	+	ND	+	ND	+	+	+	+	+	+	+	+	+	+	+	ND	+	ND

+= Positive, -= Negative

Incorrect result

Annex 13. *eae* gene detection results

Lab/Strain	*eae*	508	522	525	501	527	518	519	511	521	509	515	526	403	530	540	514	516	504	405	520	523	506	550
KK11	+	+	ND	+	+	+	+	+	+	+	+	+	+	ND	+	+	+	+	+	ND	+	+	+	+
LL12	-	-	ND	-	-	-	-	-	-	-	-	-	-	ND	-	-	-	-	-	ND	-	-	-	-
MM13	+	+	ND	+	+	+	+	+	+	+	+	+	+	ND	+	+	+	+	+	ND	+	+	+	+
NN14	-	-	ND	-	-	-	-	-	-	-	-	-	-	ND	-	-	-	-	-	ND	-	-	-	-
OO15	+	+	ND	+	+	-	+	+	+	+	+	+	+	ND	+	+	+	+	+	ND	+	+	-	+
PP16	+	+	ND	+	+	+	+	+	+	+	+	+	+	ND	+	+	+	+	+	ND	+	+	+	+
QQ17	+	+	ND	+	+	+	+	+	+	+	+	+	+	ND	+	+	+	+	+	ND	+	+	+	+
RR18	+	+	ND	+	+	+	+	+	+	+	+	+	+	ND	+	+	+	+	+	ND	+	+	+	+
SS19	-	-	ND	-	-	-	-	-	-	-	-	-	-	ND	-	-	-	-	-	ND	-	-	+	-
TT20	-	-	ND	-	-	-	-	-	-	-	-	-	-	ND	-	-	-	-	-	ND	-	-	+	-

+= Positive, -= Negative

Incorrect result

Annex 14. *ehx*A gene detection results

Lab/Strain	ehxA	508	522	525	501	527	518	519	511	521	509	515	526	403	530	540	514	516	504	405	520	523	506	550
KK11	+	+	ND	+	+	ND	+	ND	+	+	ND	+	+	ND	+	ND	+	+	+	ND	+	+	ND	+
LL12	-	-	ND	-	-	ND	-	ND	-	-	ND	-	-	ND	-	ND	-	-	-	ND	-	-	ND	-
MM13	-	-	ND	-	-	ND	-	ND	-	-	ND	-	-	ND	-	ND	-	-	-	ND	-	-	ND	-
NN14	-	-	ND	-	-	ND	-	ND	-	-	ND	-	-	ND	-	ND	-	-	-	ND	-	-	ND	-
OO15	+	+	ND	+	+	ND	+	ND	+	+	ND	+	+	ND	+	ND	+	+	+	ND	+	+	ND	+
PP16	+	+	ND	+	+	ND	+	ND	+	+	ND	+	+	ND	+	ND	+	+	+	ND	+	+	ND	+
QQ17	+	+	ND	+	+	ND	+	ND	+	+	ND	+	+	ND	+	ND	+	+	+	ND	+	+	ND	+
RR18	+	+	ND	+	+	ND	+	ND	+	+	ND	+	+	ND	+	ND	+	+	+	ND	+	+	ND	+
SS19	-	-	ND	-	-	ND	-	ND	-	-	ND	-	-	ND	-	ND	-	-	-	ND	-	-	ND	-
TT2ND	-	-	ND	-	-	ND	-	ND	-	-	ND	-	-	ND	-	ND	-	-	-	ND	-	-	ND	-

+= Positive, -= Negative

Incorrect result

Annex 15. *vtx*1 gene detection results

Lab/Strain	VT1	508	522	525	501	527	518	519	511	521	509	515	526	403	530	540	514	516	504	405	520	523	506	550
KK11	-	-	-	-	-	-	-	-	-	-	-	-	-	ND	-	-	-	-	-	-	-	-	-	-
LL12	+	+	+	+	+	+	+	+	+	+	+	+	+	ND	+	+	+	+	+	+	+	+	-	+
MM13	-	-	-	-	-	-	-	-	-	-	-	-	-	ND	-	-	-	-	-	-	-	-	-	-
NN14	+	+	+	+	+	+	+	+	+	+	+	+	+	ND	+	+	+	+	+	+	+	+	+	+
OO15	+	+	+	+	+	+	+	+	+	+	+	+	+	ND	+	+	+	+	+	+	+	+	+	+
PP16	-	-	-	-	-	-	-	-	-	-	-	-	-	ND	-	-	-	-	-	-	-	-	-	-
QQ17	-	-	-	-	-	-	-	-	-	-	-	-	-	ND	-	-	-	-	-	-	-	-	-	-
RR18	+	+	+	+	+	+	+	+	+	+	+	+	+	ND	+	+	+	+	+	+	+	+	+	+
SS19	-	-	-	-	-	-	-	-	-	-	-	-	-	ND	-	-	-	-	-	-	-	-	-	-
TT2ND	-	-	-	-	-	-	-	-	-	-	-	-	-	ND	-	-	-	-	-	-	-	-	-	-

+= Positive, -= Negative

Incorrect result

Annex 16. *vtx*2 gene detection results

Lab/Strain	VT2	508	522	525	501	527	518	519	511	521	509	515	526	403	530	540	514	516	504	405	520	523	506	550
KK11	+	+	+	+	+	+	+	+	+	+	+	+	+	ND	+	+	+	+	+	+	+	+	+	+
LL12	-	-	-	-	-	-	-	-	-	-	-	-	-	ND	-	-	-	-	-	-	-	-	-	-
MM13	+	+	+	-	+	+	+	-	+	+	+	+	+	ND	+	+	+	+	-	+	+	+	-	+
NN14	-	-	-	-	-	-	-	-	-	-	-	-	-	ND	-	-	-	-	-	-	-	-	-	-
OO15	-	-	-	-	-	-	-	-	-	-	-	-	-	ND	-	-	-	-	-	-	-	-	-	-
PP16	+	+	+	+	+	+	+	+	+	+	+	+	+	ND	+	+	+	+	+	+	+	+	+	+
QQ17	+	+	+	+	+	+	+	+	+	+	+	+	+	ND	+	+	+	+	+	+	+	+	+	+
RR18	+	+	+	+	+	+	+	+	+	+	+	+	+	ND	+	+	+	+	+	+	+	+	+	+
SS19	+	+	+	+	+	+	+	+	+	+	+	+	+	ND	+	+	+	+	+	+	+	+	+	+
TT2ND	+	+	+	+	+	+	+	+	+	+	+	+	+	ND	+	+	+	+	+	+	+	+	+	+

+= Positive, -= Negative

Incorrect result

Annex 17. *vtx* subtyping results

Lab/Strain	vtx1 subtype	508	522	525	501	527	518	519	511	521	509	515	526	403	530	540	514	516	504	405	520	523	506	550
KK11	-	-	-	-	-	ND	-	-	ND	-	-	-	-	ND	-	-	-	-	ND	ND	-	-	-	-
LL12	vtx1d	vtx1d	vtx1d	vtx1d	vtx1d	ND	vtx1d	vtx1d	ND	vtx1d	vtx1d	vtx1d	vtx1d	ND	vtx1d	vtx1d	vtx1d	vtx1a, vtx1d	ND	ND	vtx1d	vtx1d	vtx1d	vtx1d
MM13	-	-	-	-	-	ND	-	-	ND	-	-	-	-	ND	-	-	-	-	ND	ND	-	-	-	-
NN14	vtx1c	vtx1c	vtx1c	vtx1c	vtx1c	ND	vtx1c	vtx1c	ND	vtx1c	vtx1c	vtx1c	vtx1c	ND	vtx1c	vtx1c	vtx1c	vtx1a, vtx1c	ND	ND	vtx1c	vtx1c	vtx1c	vtx1c
OO15	vtx1a	vtx1a	vtx1a	vtx1a	vtx1a	ND	vtx1a	vtx1a	ND	vtx1a	vtx1a	vtx1a	vtx1a	ND	vtx1a	vtx1a	vtx1a	vtx1a	ND	ND	vtx1a	vtx1a	vtx1a	vtx1a
PP16	-	-	-	-	-	ND	-	-	ND	-	-	-	-	ND	-	-	-	-	ND	ND	-	-	-	-
QQ17	-	-	-	-	-	ND	-	-	ND	-	-	-	-	ND	-	-	-	-	ND	ND	-	-	-	-
RR18	vtx1a	vtx1a	vtx1a	vtx1a	vtx1a	ND	vtx1a	vtx1a	ND	vtx1a	vtx1a	vtx1a	vtx1a	ND	vtx1a	vtx1a	vtx1a	vtx1a, vtx1c	ND	ND	vtx1a	vtx1a	vtx1a	vtx1a
SS19	-	-	-	-	-	ND	-	-	ND	-	-	-	-	ND	-	-	-	-	ND	ND	-	-	-	-
TT20	-	-	-	-	-	ND	-	-	ND	-	-	-	-	ND	-	-	-	-	ND	ND	-	-	-	-

Lab/Strain	vtx2 subtype	508	522	525	501	527	518	519	511	521	509	515	526	403	530	540	514	516	504	405	520	523	506	550
KK11	vtx2a	vtx2a	vtx2a	vtx2a	vtx2a	vtx2a + vtx2c	vtx2a	vtx2a	vtx2a	vtx2a	vtx2a	vtx2a	vtx2a	ND	vtx2a	vtx2a	vtx2a	vtx2a	ND	ND	vtx2a	vtx2a	vtx2a	vtx2a
LL12	-	-	-	-	-	-	-	-	-	-	-	-	-	ND	-	-	-	-	ND	ND	-	-	-	-
MM13	vtx2f	vtx2f	vtx2f	-	vtx2f	vtx2f	vtx2f + vtx2d	vtx2b + vtx2f	vtx2f	vtx2f	vtx2f	vtx2f	vtx2f	ND	vtx2f	vtx2f	vtx2f	vtx2e + vtx2f	(vtx2f)	ND	vtx2f	vtx2f	vtx2f	vtx2f
NN14	-	-	-	-	-	-	-	-	-	-	-	-	-	ND	-	-	-	-	ND	ND	-	-	-	-
OO15	-	-	-	-	-	-	-	-	-	-	-	-	-	ND	-	-	-	-	ND	ND	-	-	-	-
PP16	vtx2a	vtx2a	vtx2a	vtx2a	vtx2a	vtx2a	-	vtx2a	vtx2a	vtx2a	vtx2a	vtx2a	vtx2a	ND	vtx2a	vtx2a	vtx2a	vtx2a	ND	ND	vtx2a	vtx2a	vtx2a	vtx2a
QQ17	vtx2a + vtx2c	vtx2a + vtx2c	vtx2a + vtx2c	vtx2a + vtx2c	vtx2a + vtx2c	vtx2a	vtx2a + vtx2c	vtx2a	vtx2a + vtx2c	vtx2a + vtx2c	vtx2a	vtx2a + vtx2c	vtx2a + vtx2c	ND	vtx2a + vtx2c	vtx2a + vtx2c	vtx2a	vtx2a	ND	ND	vtx2c	vtx2a + vtx2c	vtx2a + vtx2c	vtx2a, vtx2c, vtx2d
RR18	vtx2a	vtx2a	vtx2a	vtx2a	vtx2a	vtx2a	vtx2a	vtx2a	vtx2a	vtx2a	vtx2a	vtx2a	vtx2a	ND	vtx2a	vtx2a	vtx2a	vtx2a	ND	ND	vtx2a	vtx2a	vtx2a	vtx2a
SS19	vtx2d	vtx2d	vtx2d	vtx2d	vtx2d	vtx2c	vtx2c + vtx2d	vtx2c + vtx2d	vtx2d	vtx2d	vtx2c + vtx2d	vtx2d	vtx2d	ND	vtx2d	vtx2d + vtx2c	vtx2d	vtx2c + vtx2d	ND	ND	vtx2d	vtx2d	vtx2d	vtx2b + vtx2d + vtx2c
TT20	vtx2b + vtx2d	vtx2d + vtx2b	vtx2b + vtx2d	vtx2b + vtx2d	vtx2b + vtx2d	vtx2b	vtx2b + vtx2d	vtx2d	vtx2b + vtx2d	vtx2b + vtx2d	vtx2b + vtx2d	vtx2b	vtx2b + vtx2d	ND	vtx2b + vtx2d	vtx2b + vtx2d	vtx2b	vtx2b + vtx2d + vtx2c	ND	ND	vtx2b	vtx2b + vtx2d	vtx2b	vtx2b + vtx2c + vtx2d + vtx2a

Results from participant 504 were excluded, because they only tested for *vtx2f*

Incorrect result

Annex 18. Virulence genes *aggR* and *aaiC*

aggR

Lab/Strain	aagR	508	522	525	501	527	518	519	511	521	509	515	526	403	530	540	514	516	504	405	520	523	506	550
KK11	-	-	ND	ND	-	-	-	ND	ND	ND	ND	-	-	ND	-	-	-	-	ND	ND	-	ND	ND	-
LL12	-	-	ND	ND	-	-	-	ND	ND	ND	ND	-	-	ND	-	-	-	-	ND	ND	-	ND	ND	-
MM13	-	-	ND	ND	-	-	-	ND	ND	ND	ND	-	-	ND	-	-	-	-	ND	ND	-	ND	ND	-
NN14	-	-	ND	ND	-	-	-	ND	ND	ND	ND	-	-	ND	-	-	-	-	ND	ND	-	ND	ND	-
OO15	-	-	ND	ND	-	-	-	ND	ND	ND	ND	-	-	ND	-	-	-	-	ND	ND	-	ND	ND	-
PP16	-	-	ND	ND	-	-	-	ND	ND	ND	ND	-	-	ND	-	-	-	-	ND	ND	-	ND	ND	-
QQ17	-	-	ND	ND	-	-	-	ND	ND	ND	ND	-	-	ND	-	-	-	-	ND	ND	-	ND	ND	-
RR18	-	-	ND	ND	-	-	-	ND	ND	ND	ND	-	-	ND	-	-	-	-	ND	ND	-	ND	ND	-
SS19	-	-	ND	ND	-	-	-	ND	ND	ND	ND	-	-	ND	-	-	-	-	ND	ND	-	ND	ND	-
TT2ND	-	-	ND	ND	-	-	-	ND	ND	ND	ND	-	-	ND	-	-	-	-	ND	ND	-	ND	ND	-

aaiC

Lab/Strain	aaiC	508	522	525	501	527	518	519	511	521	509	515	526	403	530	540	514	516	504	405	520	523	506	550
KK11	-	-	ND	ND	ND	ND	ND	ND	ND	ND	ND	-	-	ND	-	-	ND	-	ND	ND	ND	ND	ND	-
LL12	-	-	ND	ND	ND	ND	ND	ND	ND	ND	ND	-	-	ND	-	-	ND	-	ND	ND	ND	ND	ND	-
MM13	-	-	ND	ND	ND	ND	ND	ND	ND	ND	ND	-	-	ND	-	-	ND	-	ND	ND	ND	ND	ND	-
NN14	+	-	ND	ND	ND	ND	ND	ND	ND	ND	ND	-	-	ND	-	-	ND	-	ND	ND	ND	ND	ND	-
OO15	-	-	ND	ND	ND	ND	ND	ND	ND	ND	ND	-	-	ND	-	-	ND	-	ND	ND	ND	ND	ND	-
PP16	-	-	ND	ND	ND	ND	ND	ND	ND	ND	ND	-	-	ND	-	-	ND	-	ND	ND	ND	ND	ND	-
QQ17	-	-	ND	ND	ND	ND	ND	ND	ND	ND	ND	-	-	ND	-	-	ND	-	ND	ND	ND	ND	ND	-
RR18	-	-	ND	ND	ND	ND	ND	ND	ND	ND	ND	-	-	ND	-	-	ND	-	ND	ND	ND	ND	ND	-
SS19	-	-	ND	ND	ND	ND	ND	ND	ND	ND	ND	-	-	ND	-	-	ND	-	ND	ND	ND	ND	ND	-
TT2ND	-	-	ND	ND	ND	ND	ND	ND	ND	ND	ND	-	-	ND	-	-	ND	-	ND	ND	ND	ND	ND	-

+= Positive, -= Negative

Incorrect result

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	