SSI TECHNICAL REPORT

External quality assessment scheme for typing of verocytotoxin-producing *E. coli* (VTEC), self-funded participants, 2015-2016

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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	CVD419. Plasmid encoded O157-enterohaemolysin.
EAEC	Enteroaggregative *E. coli*
ECDC	The European Centre for Disease Prevention and Control
EEA	European Economic Area
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*
EU	European Union
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 Typeable
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
VT	verocytoxin
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 the 2015-2016 external quality assessment (EQA) scheme for typing of verocytotoxin-producing *Escherichia coli* (VTEC) for self-funded participants. The EQA 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 spectrum beta lactamase (ESBL).

Twenty-three public health national reference laboratories from 16 countries participated in at least one of the EQA parts. Ten laboratories (52%) participated in the PFGE part, and among those, seven (64%) 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). Eight laboratories completed the gel analysis, and all performed in fair to good accordance with the guidelines.

Twenty-one laboratories performed O typing with some laboratories only typing a subset of the test strains. An average score of 77% (range 23–100%) 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 typing, while O156 was associated with a significantly poorer result (24%). The participation for H typing was lower (14 laboratories). An average score of 90% (range 79–93%) was obtained for the H type of the strains (some laboratories only typed a selection of the test strains). Notably, only two laboratories could determine the correct O:H serotype for all 10 strains.

Genotypic detection of *eae* was performed by 20 laboratories, correct detection (evaluated per strain) was in average 97%. Seventeen laboratories submitted results for *ehxA* and all of them did so correctly. Eleven laboratories participated in the detection of *aggR* and seven for *aaiC* from all 10 strains. All laboratories identified correctly the presence or absence of the genes the strains (100% score). Detection of *vtx1* and *vtx2* was performed by 22 laboratories with excellent outcomes (99% and 97% average scores). Subtyping of *vtx1* and *vtx2* performed by 18 and 20 laboratories, respectively, obtained scores of 99% and 81%. Most errors in the subtyping of *vtx2* were due to erroneous detection of multiple genotypes.

In comparison to the genotypic methods, the phenotypic methods were in general performed less frequently, however the performance was high. Only 5 laboratories participated in the phenotypic detection of VT using the Vero cell assay (VCA). However, 100% of the results were correct. Fifteen laboratories participated in fermentation of sorbitol and in average 97% of reported results were correct. Eight and seven laboratories participated in the analysis for β-glucuronidase and enterohaemolysin productions, with 95% and 94% correct results reported, respectively. Nine laboratories participated in the ESBL analysis, with 98% correct results reported.

In general, the performance within each analysis is high. However, full O:H serotyping and *vtx2* subtyping needs - for some participants – to be given extra attention. Currently, 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. It is therefore critical that laboratories pursue efforts to harmonise protocols and participate in EQA schemes.

1 Introduction

1.1 Surveillance of VTEC infections and laboratory characterisation

Verocytotoxin-producing *E. coli* (VTEC) are a group of *E. coli* characterised by the ability to produce toxins designated verocytotoxins (VTs). 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 2015, 5,901 confirmed cases of VTEC infections were reported in the EU [1]. The EU notification rate was 1.27 cases per 100,000 population, which was slightly lower than the notification rate in 2014 (1.32).

The state-of-the-art characterisation of VTEC includes O:H serotyping in combination with the detection of 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*tx2d* subtype [6-9]. However, the PstI site is also absent in six variants of *vtx2a*, in two variants of *vtx2c*, in *vtx2f* and in all four variants of subtype *vtx2g* [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.

1.2 Roles of External Quality Assessment (EQA) and objectives of this scheme

External quality assessment (EQA) is an important aspect of quality management systems. An EQA provides an objective evaluation of a laboratory's performance. By doing so, it has numerous roles of public health importance. It constitutes an early warning for systematic problems, provides objective evidence of testing quality, and identifies areas needing improvement and specific training needs amongst participants. An EQA also allows for comparison among different test sites. Standardised laboratory techniques and national and international comparison of results have many benefits for

public health. They include 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, identification of new risk factors, and aid in studying the characteristics of a particular pathogen and its behaviour in a community of hosts.

Since 2002, the International 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*. The laboratory has been arranging annual EQA rounds for the national reference laboratories in the EU/EEA and non-EU countries on serotyping and virulence typing and some years PFGE for VTEC. The overall aim of this VTEC EQA scheme is the harmonization of the typing methods used for VTEC, in order to produce comparable typing data for VTEC strains between laboratories, both at national and international level, and to inform public health authorities of the aetiology, risk factors and burden of food- and waterborne diseases and zoonoses. To achieve this aim, the EQA included:

Pulsed-field gel electrophoresis (PFGE) typing:

The exercise focused on the production of raw PFGE gels of high quality, normalisation of PFGE images using the BioNumerics (BN) software, and interpretation of the results, therefore assessing the quality of the standard PFGE molecular typing and comparability of the collected test results between participating laboratories and countries.

Serotyping:

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

Genotyping - Virulence determination:

The EQA included the following:

- Detection of virulence genes *eae*, *vtx1*, *vtx2* and *ehxA*.
- Subtyping of *vtx1* and *vtx2* genes, by conventional gel-based PCR using the recently published protocol [10].
- Detection of additional virulence genes

This choice was motivated by the list of virulence data currently collected at the EU level (participants had the possibility to report on optional genes).

Phenotypic tests – Virulence determination:

The EQA included phenotypic assays for the detection of production of verocytotoxin through VCA or enzyme immunoassay (EIA), fermentation of sorbitol, enterohaemolysin, β-glucuronidase, and extended-spectrum beta-lactamases (ESBL).

This EQA is the 12th of its kind. This EQA scheme was open to public health national reference laboratories worldwide. National reference laboratories in the EU were funded by ECDC and data is presented in an ECDC report (https://ecdc.europa.eu/sites/portal/files/media/en/publications/Publications/EQA-seventh-VTEC.pdf). This report only includes the results from the self-funded participants.

The EQA was conducted according to the International Standard ISO/IEC 17043:2010, entitled '*Conformity assessment – General requirements for proficiency testing'* (first edition, 1 February 2010) [11].

2 Study design

2.1 Organisation

This VTEC EQA was managed by SSI and conducted from November 2015 to June 2016.

Invitations were e-mailed to previously participating countries and laboratories from the international World Health Organisation (WHO) Global Foodborne Infections Network (GFN) by October 14th 2015 with a deadline for response of November 2nd 2015. Twenty-three laboratories accepted the invitation (Annex 1). Laboratories indicated to which components of the EQA they wanted to take part in (PFGE, serotyping, genotyping and/or phenotypic tests).

The EQA test strains were sent to the participating laboratories on the 12th of January to the 12th of February 2016.

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 and upload file to a ftp-site by the 18th of March 2016.

2.2 Selection of strains

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

Table 1. Characteristics of the ten strains for sero-, geno- and phenotyping

Method	Characterisation
O:H serotyping	O26:H11, O78:H2, O80:H2, O91:H14, O103:H2, O145:H34, O146:H21, O156:H4, O157:H-, O166:H15
Genotyping	*aaiC*, *aggR*, *eae*, *ehxA*, *vtx1*, *vtx1a*, *vtx1c*, *vtx2*, *vtx2a, vtx2b, vtx2c, vtx2d, vtx2f*
Phenotypic testing	VT, sorbitol, β-glucuronidase, enterohaemolysin, ESBL

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 14).

2.3 Carriage of strains

In January 2016, all test strains were blind-coded and shipped as UN 3373 Biological Substance, Category B. An individual letter stating the unique strain IDs was included in the packages, and distributed individually to the participants, together with the protocol for the EQA. An e-mail containing the same information was also sent to participants on the 19th of January. No participants reported damage to the shipment or errors in the specific strain IDs.

The 21st January 2016, instructions to the submission of results procedure were e-mailed to the participants. This included links to the online uploading site and submission forms, preconfigured BN databases with correct experiment settings (PFGE part) and XML export file.

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, the participants were instructed to use the distributed preconfigured BN database and analyse the PFGE gel including normalization and band assignment. Submission of results included online uploading of PFGE images, as either TIFF file or XML export file including the BN analysis. Guidelines to correct image acquisition, setting up the BN database and export of XML files from BN were included in the EQA protocol.

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, vtx1c)* and *vtx2* (*vtx2a, vtx2b, vtx2c, vtx2d and vtx2f*) were assessed (suggested protocol [14]). Additionally two genes related to EAEC, the chromosomally encoded protein gene (*aaiC*) and enteroaggregative adherence transcription regulator gene (*aggR*), were included.

The phenotypic part of the EQA involved the detection of VT production by either Vero Cell Assay (VCA) or Enzyme Immunoassay (EIA), fermentation of sorbitol, enterohaemolysin, β-glucuronidase and production of ESBL.

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.

Individual evaluation reports and certificates of attendance were distributed to the participants in June 2016.

3 Results

3.1 Participation

Laboratories could choose to participate in the full scheme or a selection of the methods. Table 2 summarises participation rate for each method.

The participation rate in O group/H type depended 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. A laboratory participated if a result was provided for at least one strain.

Table 2. Summary of participation rate

Method	Comment	Number of laboratories	(%)
PFGE	Gel only	2	(9)
	Gel & analysis (BioNumerics)	8	(35)
Serotyping	Full O:H serotyping	14	(61)
	O typing	21	(91)
	H typing	14	(61)
Virulence determination	*aaiC*	8	(35)
	aggR	13	(57)
	eae	20	(87)
	ehxA	17	(74)
	vtx1	22	(96)
	vtx2	22	(96)
	vtx subtyping	20	(87)
Phenotypic tests	Vero cell assay	5	(22)
	Sorbitol	15	(65)
	β-glucuronidase	8	(35)
	Enterohaemolysin	7	(30)
	ESBL production	9	(39)

3.2 Pulsed-field gel electrophoresis (PFGE)

Twelve laboratories participated in the PFGE part, sending TIFF files (raw gel images). Two laboratories performed PFGE on the EQA strains intended for sero-, geno- and phenotyping and were not included in this report. Eight of the remaining ten laboratories also analysed their gels in BN and submitted data as XML files (Table 2).

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 are 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, at least an 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.

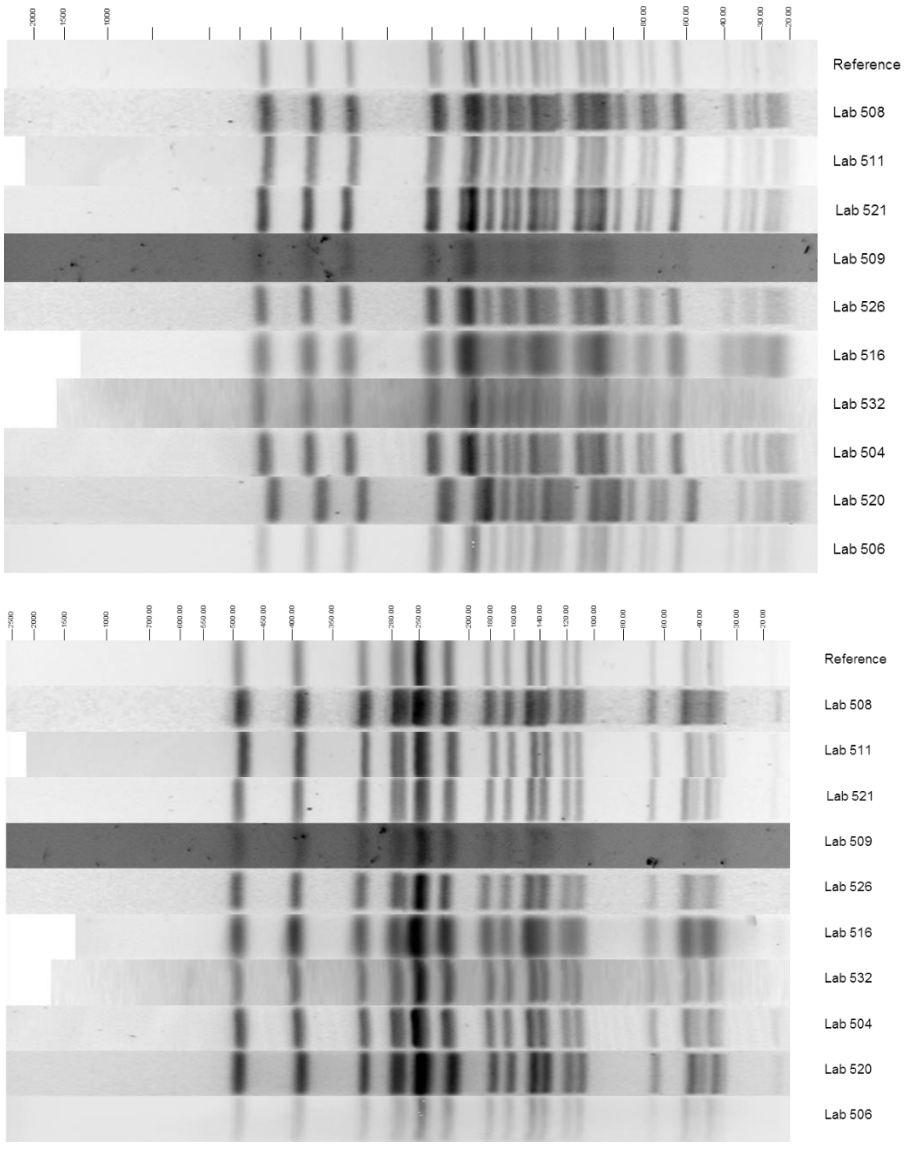

Figure 1. Example of PFGE profiles of two strains (EQA-7 strain 2 and 9) by EQA participants.

Some variation in the qualities of the gels submitted by the participating laboratories was observed (Table 3). Each laboratory could score a maximum of 28 points; two laboratories achieved 27 points and the average among the twelve laboratories was 24 points. For three parameters; cell suspension, lanes, and restriction, participants obtained a high average score (≥3.8), i.e. between good and excellent (Table 3). Participants obtained an average score of >3.0 in image acquisition and running conditions, gel background and DNA degradation while the score for parameter bands in average was 2.7.

Table 3. Results of PFGE gel quality for 10 participating laboratories

Parameters	1 – poor (%)	2 – fair (%)	3 – good (%)	4 – excellent (%)	Average score
Image acquisition and running conditions	10	10	40	40	3.1
Cell suspension	0	0	20	80	3.8
Bands	20	10	50	20	2.7
Lanes	0	0	20	80	3.8
Restriction	0	0	10	90	3.9
Gel background	0	30	20	50	3.2
DNA degradation	0	20	10	70	3.5

The distribution of scores (percentages of ten participating laboratories) in the seven TIFF Quality Grading parameters. Also shown is the average score.

Two laboratories (20%) were graded 1 (poor) in the parameter Bands. Both gels demonstrated very fuzzy bands. One laboratory was graded 1 in the parameter Image acquisition and running conditions due to the band spacing of standards did not match the global standard, thus affecting analysis. 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 2 in the parameters Gel Background and DNA Degradation.

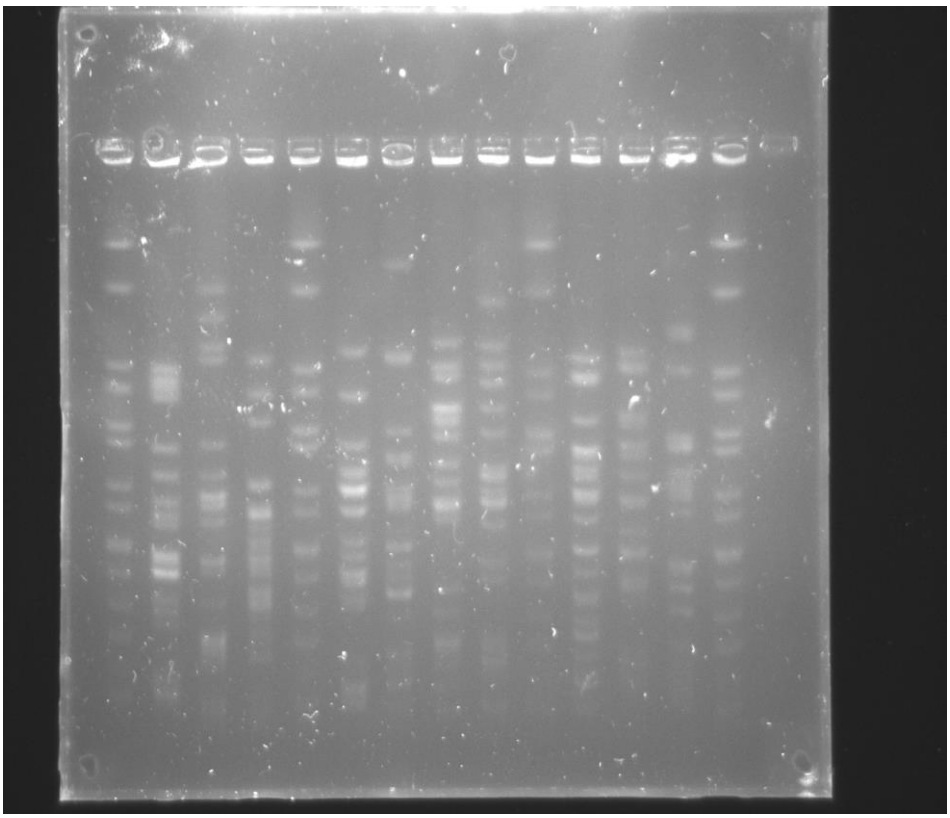

Figure 2. A gel graded 1 in parameters Bands and a score of 2 in the parameters Gel Background and DNA degradation

Figure 3 depicts a gel with a low score in the parameter Image Acquisition & Running Conditions (score of 1). Running time was too short which did not allow for adequate separation of the fragments.

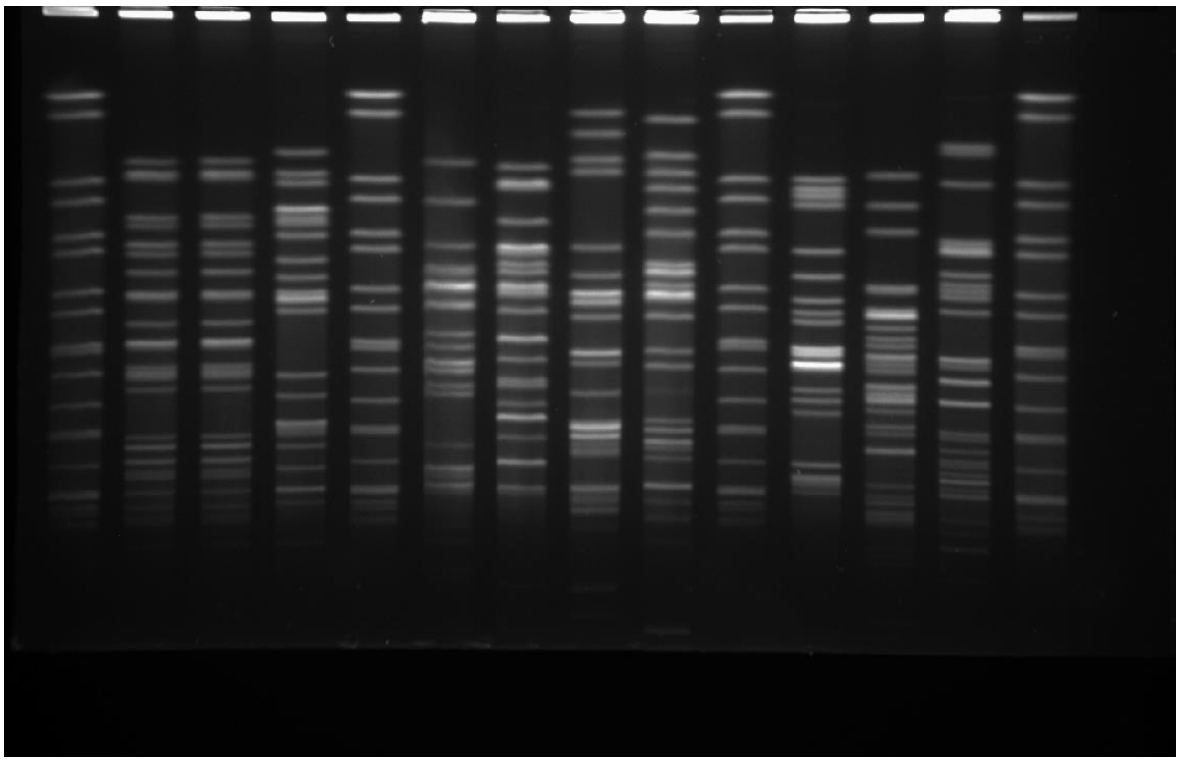

Figure 3. A gel graded 1 in Running Conditions.

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 with one or two faint shadow bands, there is no debris, nor background.

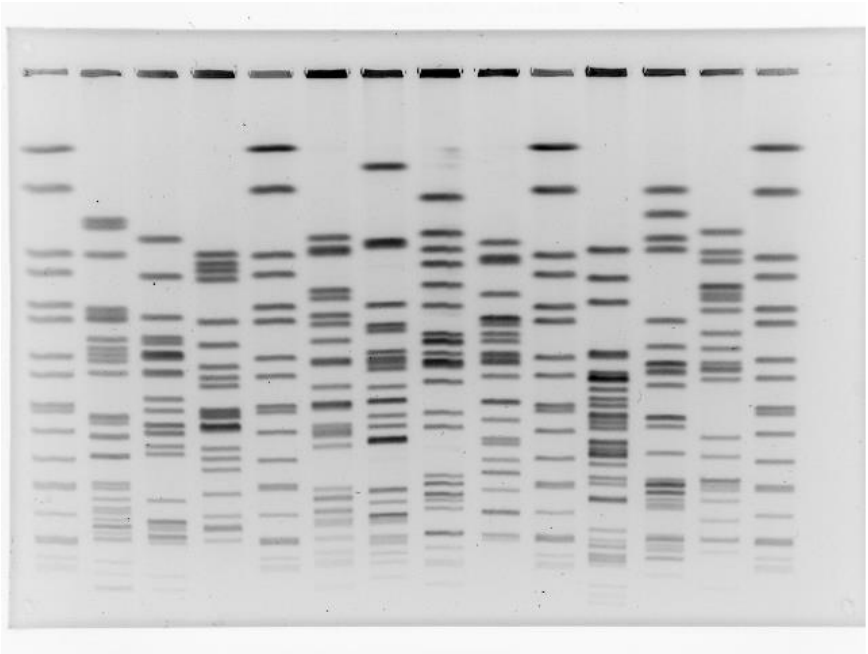

Figure 4. A gel with high scores in all 7 parameters

3.2.2 Gel analysis with BioNumerics

Eight laboratories analysed their gel in BN and submitted 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 4. Results of the BN analysis for 8 laboratories

Parameters	1 – poor (%)	2 – fair (%)	3 – Excellent (%)	Average score
Position of the gel	0	50	50	2.5
Strips	0	37.5	62.5	2.6
Curves	0	75	25	2.3
Normalisation	0	25	75	2.8
Band assignment	0	50	50	2.5

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

Parameter Normalisation was performed best by the participating laboratories with an average score of 2.8 (Table 4). Three parameters, Position of gel, Strips and Band assignment had an average score between 2.5 to 2.6. Parameter Curves was graded lowest, with an average score of 2.3.

An optimal Band assignment in BN is crucial, and 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 5, 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.

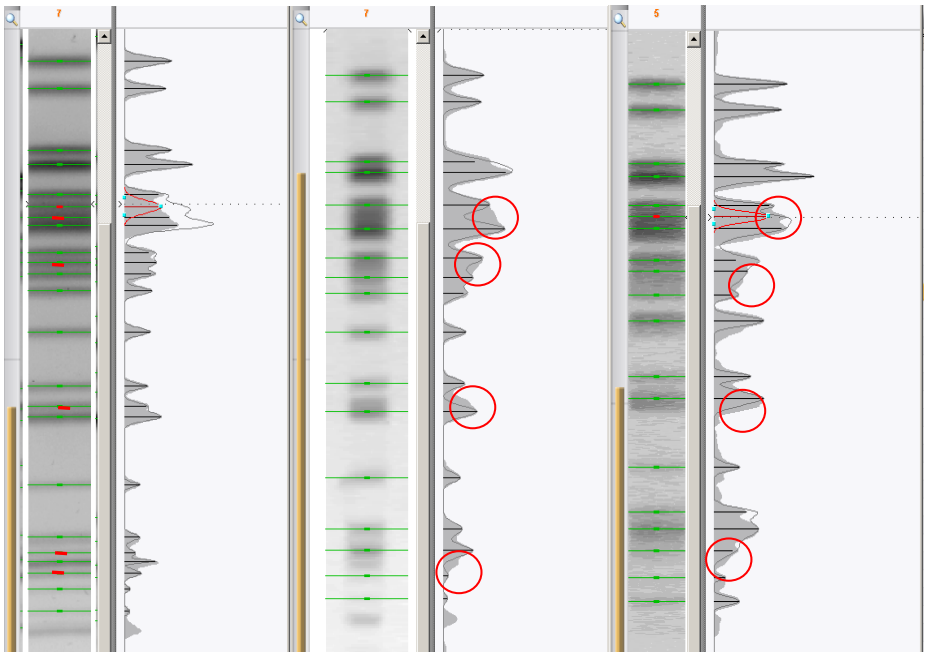

Figure 5. Comparison of Band assignment from two participants in BN analysis

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

Twenty-one laboratories performed O typing. On average, for the 10 test strains 77% of the reported results were correct for the O grouping (Figure 6). Results were lowest (24%) for O group O156 (FF16) and highest (100%) for O group O157 (HH18). Overall, 4 laboratories (19%) reported the correct O grouping for all 10 test strains.

Fourteen laboratories performed H typing. On average, for the 10 test strains 90% of the reported results were correct for the H typing (Figure 6). Results were lowest (79%) for H type H2 (AA11). Overall, 7 laboratories (50%) reported the correct H type for all 10 test strains.

In all, 2 laboratories (14%) reported the correct O:H serotype for all the 10 test strains.

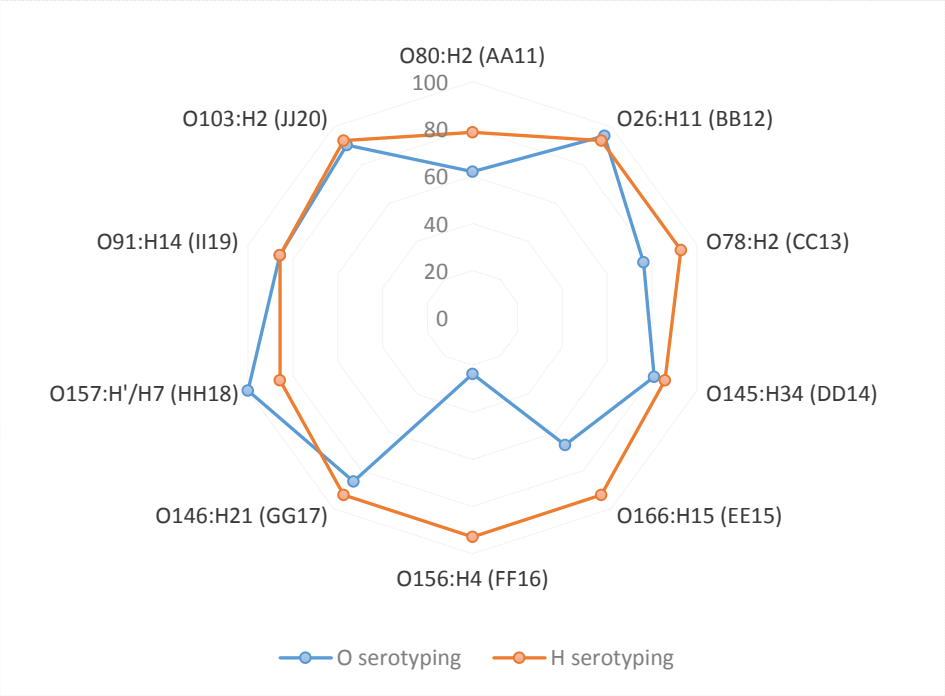

Figure 6. Average serotyping score among EQA participants (scale 0 to 100%)

The complete results for all laboratories for all strains are presented in Annexes 6 and 7.

3.4 Virulence determination

Twenty-two laboratories participated, at least partially, in the genotyping part of the EQA scheme, consisting of detection of EAEC genes (*aaiC* and *aggR*), virulence genes (*eae*, *ehxA*, *vtx1* and *vtx2*) and subtyping of *vtx1* and *vtx2* genes.

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 17 to 22 laboratories for all the 10 test strains, with high average result scores ranging from 97% to 100% (Table 5). The lowest individual laboratory score was observed for the detection of the gene *eae*, with one laboratory scoring at 70%. The discrepancies between expected results and results provided by the

participating laboratories are detailed in Table 6. The gene *vtx2* was missed 6 times, 5 of them for strain DD14 which was the only strain carrying subtype *vtx2f*. The gene *eae* was misidentified 5 times (3 false positive by one laboratory).

The complete results for all laboratories for all strains are presented in Annexes 8-11.

Table 5. Average scores for virulence determination

Gene	*Average score (%)*	*Range (%)*
***vtx1* (n=22)**	99	90-100
***vtx2* (n=22)**	97	80-100
***eae* (n=20)**	97	70-100
***ehxA* (n=17)**	100	-

n represents the number of laboratories that participated for this specific component of the EQA.

The range refers to the laboratory scores for each gene (average of the scores obtained for each strain).

Table 6. Description of the discrepancies observed for *vtx1, vtx2* and *eae* gene identification

Gene	*Strains affected*	*False negative*	*False positive*
***vtx1* (n=2)**	AA11	-	1
	GG17	1	-
***vtx2* (n=6)**	DD14	5	-
	HH18	1	-
***eae* (n=6)**	AA11	1	-
	CC13	-	2
	FF16	-	1
	GG17	-	1
	II19	-	1

3.4.2 Subtyping of *vtx1* and *vtx2*

The number of laboratories participating in subtyping of *vtx1* and *vtx2* genes was 18 and 20, respectively. 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.

Overall, *vtx1* and *vtx2* typing scores were 99% and 81%, respectively when including *vtx1* or *vtx2* negative strains.

Among the *vtx1* positive strains (GG17, HH18, II19 and JJ20), the average score obtained by all the laboratories was 100%. The only discrepancy in *vtx1* subtyping occurred in strain AA11 (*vtx1* negative) subtyped as *vtx1c* by one laboratory.

Among the *vtx2* positive strains (AA11, BB12, DD14, EE15, FF16, GG17, HH18, II19), the average score obtained by all the laboratories was 78%. The details of the discrepancies observed for each strain are provided in Table 7.

The complete results for all laboratories for all strains are presented in Annexes 12-13.

Table 7. Description of the discrepancies observed for *vtx2* subtyping.

		***vtx2* subtyping**		
Strains affected	**Original**	**Number of laboratories reporting identification of a discrepant genotype (genotypes identified) ***	**False negative**	**Not done**
AA11	*vtx2a*	*1 (vtx2f)*	1	2
BB12	*vtx2a*	2 (1 *vtx2b,* 1 *vtx2d)*	1	2
CC13	-	-	-	2
DD14	*vtx2f*	1 (*vtx2a*)	1	-
EE15	*vtx2d*	4 (*1 vtx2a*, 4 *vtx2c*)	-	2
FF16	*vtx2d*	4 (*1 vtx2a, 1 vtx2b,* 3 *vtx2c*)	-	2
		1 (*vtx2a)*	-	2
GG17	*vtx2b*			
HH18	*vtx2c*	4 (*1 vtx2a*, 4 *vtx2d*)	-	2
II19	*vtx2b*	1 (*vtx2a*)		2
JJ20	-	-	-	2

* in some cases a mixed result was reported, with either the original genotype plus a discrepant genotype, either a mixture of discrepant genotypes.

Details on the strains used for the EQA are provided in Annex 14.

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

Genotypic detection of virulence genes, *aggR* and *aaiC,* was performed by 13 and 8 laboratories, respectively. Most laboratories (11/13 for *aggR* and 7/8 for *aaiC*) identified correctly the presence or absence of the genes in all 10 strains (100% score). Two and one laboratories only determined the presence or absence of *aggR* and *aaiC*, respectively, in strain 13, which led them to obtain a 10% score for the identification of these genes among the 10 strains included in the EQA. Missing results were classified as incorrect.

The complete results for all laboratories for all strains are presented in Annex 15.

3.4.4 Detection of additional virulence genes – not evaluated in this EQA

Participants had the possibility to perform additional genotyping on the 10 strains provided. However, these results were not evaluated as part of the EQA.

Four additional virulence genes were present in strains CC13, EE15 and II19, namely *aatA*, *astA*, *eltA* and *saa*. Table 8 lists the original genes present and those reported.

Table 8. Description of the additional virulence genes reported.

Strains	Original	Reported
AA11	-	
BB12	-	
CC13	*aatA, astA*	3 *aatA*
DD14	-	
EE15	*eltA*	6 *eltA* 2 *eltA, astA* A *astA*
FF16	-	2 *astA*
GG17	-	
HH18	-	
II19	*saa*	1 *saa*
JJ20	-	1 *aatA*

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

3.4.5 Phenotypic test

Two laboratories participated fully in the phenotypic test component of the EQA, performing the five tests evaluated (VCA, ESBL production, Enterohaemolysin production, β-glucuronidase production and sorbitol fermentation). An additional 15 laboratories participated partially by performing one to four tests. The proportion of correct results was high ranging from 95% to 100% (Table 9). The discrepancies are detailed in Table 10.

Table 9. Average scores for the performance of the phenotypic tests.

Phenotypic tests	*Average score (%)*	*Range (%)*
VCA (n=5)	100	-
ESBL production (n=9)	98	80-100
Enterohaemolysin production (n=7)	94	80-100
β-glucuronidase production (n=8)	95	70-100
Sorbitol fermentation (n=15)	97	90-100

n represents the number of laboratories that participated for this specific component of the EQA.

The range refers to the laboratory scores for each gene (average of the scores obtained for each strain).

Table 10. Discrepancies of the phenotypic tests.

Phenotypic tests	*Strains affected*	*False negative*	*False positive*
ESBL production	CC13	-	1
	EE15	1	-
Enterohaemolysin production	GG17	2	-
	HH18	1	-
	II19	1	-
β-glucuronidase production	AA11	1	-
	CC13	1	-
	JJ20	2	-
Sorbitol fermentation	DD14	-	4

Detailed results for all phenotypic tests can be found in Annexes 16 (VCA), 17 (ESBL), 18 (enterohaemolysin), 19 (β -glucuronidase), and 20 (sorbitol).

4 Discussion

4.1 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). Two laboratories performed PFGE on the test strains intended for sero-, pheno- and genotypic testing and was excluded from further analysis. 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). Seven of the ten laboratories were able to produce gels with sufficiently high quality (above a score of 1) in all seven parameters.

The main issues in this VTEC EQA were in the parameters Bands and Image Acquisition & Running Conditions. In general, improvements could be made when capturing the image and producing a TIFF image. Only two of the laboratories obtained a score of excellent in the parameter Band and many laboratories seemed to adapt the image settings in an 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 thereby making it difficult to distinguish between double bands. Running conditions could also be improved in order to separate the bands of the standards and test strains. 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.

In this EQA, only 70% of all gels obtained a score of at least 2 in all parameters, and were therefore suitable for inter-laboratory comparison. Other common deviations from protocol was seen in Image Acquisition, where some laboratories forgot 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.

Eight (80%) of the laboratories 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 relevant surveillance systems such as the ECDC system The European Surveillance System (TESSy). All of the eight laboratories who submitted gel analysis data analysed PFGE gels in fair to excellent (2–3) in accordance with the guidelines. Parameter Curves had the lowest average (2.3). Emphasis should be given to define curves neither too narrow nor too broad.

4.2 Serotyping

This EQA had 21 participants for the serotyping part. On average, 77% of results generated by the laboratories were correct. Most laboratories (77%) failed to accurately identify O156.

The clear trend in this EQA was that the more common serotypes could be identified more reliably. No systematic typing errors were observed. Only three O groups and two H types were mistyped, excluding performance of one laboratory that mistyped most strains. The remainder of incorrect typing was submitted as non typeable (NT). The performance of serotyping (O group/H type) is highly affected by the range of available antisera. Laboratories using a limited panel of antisera were encouraged to report serotype results as NT for strains they were unable to type.

In summary, four of the 21 (19%) laboratories were able to correctly determine the O group for all test strains, 7 (50%) laboratories correctly identified all H types whilst 2 (14%) laboratories were able to correctly determine the full O:H serotype of all 10 test strains.

In addition to O grouping, H typing is crucial for outbreak detection, epidemiological surveillance, taxonomic differentiation of *E. coli*, and detection of pathogenic serotypes. Thus, it remains a main challenge to enable more of the NPHRL to perform complete and reliable O:H serotyping, particular H typing.

4.3 Virulence determination

4.3.1 Genotypic tests

Genotypic detection of virulence genes *eae*, *vtx1, vtx2* and *ehxA* was performed by 17 to 22 laboratories for all the 10 test strains. In general, the percentage of correct results was very high (97–100%).

Detection of *vtx1* and *vtx2* genes was achieved with a high percentage of correct results (99% and 97%). However, the majority of false negative results originated from testing the strain DD14 (*vtx2f*). Five laboratories did not detect the strain positive for *vtx2.* However, three of these five laboratories were, independently of this first result, able to correctly type *vtx2* as *vtx2f*. 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, 17].

This year, correct results, among vtx1 and *vtx2* positive strains, was 100% and 78% for subtyping *vtx1* and *vtx2,* respectively. Performances varied across laboratories, with 6 laboratories unable to correctly genotype *vtx2* in 2 or more of the 10 strains. The incorrect *vtx2* results were mainly due to reporting two or more *vtx2* subtypes for strains harbouring one type only. Another 2 laboratories only had the capacity to genotype *vtx2f*.

All laboratories performed very well in identifying *aggR* and *aaiC* (100% score).

4.3.2 Phenotypic tests

The participation in the phenotypic detection was between 23% and 68% (5–15 laboratories). Similarly to last year, the lowest participation was for VCA, where only five participants delivered results for 10 strains. Overall, performance of the participating laboratories was excellent, with average scores of 97% to 100%.

In general, the most important phenotypic test is the sorbitol fermentation, which is used to screen for the highly virulent SF O157:H7 clone. The fermentation of sorbitol was performed by 68% of the

participating laboratories. Laboratories' performance was excellent for all but one strain (DD14) for which 4 laboratories provided a false positive result.

4.4 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.

5 Conclusions and Recommendations

This EQA identified needs to support and train laboratories to improve their capacity in PFGE, sero- and genotyping. Laboratories are always welcome to contact the International Reference and Research Centre on *Escherichia* and *Klebsiella* at Statens Serum Institut, Copenhagen, Denmark for troubleshooting and advice on the performance of the laboratory procedures.

Specific recommendations for PFGE include strict adherence 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.

The overall performance of laboratories in O:H serotyping was affected by incomplete participation, mainly due to the range of antisera available in each laboratory. While budget restrictions might be the reason underlying the use of limited panels of antisera, we can only recommend capacity building and expansion of those panels in order to improve serotyping capacity, which is critical in outbreak investigations and for epidemiological surveillance. We also recommend for the laboratories to maintain their capacity to perform phenotypic tests.

Laboratory performance for identification of virulence genes was excellent. We recommend those who misidentified *vtx2* subtypes in particular to reassess their genotyping protocols. The erroneous simultaneous detection of multiple subtypes is likely related to the different PCR cycler equipment and DNA polymerases used, which might require protocol optimisations (for example, increasing the annealing temperature to improve specificity of the reaction).

A final conclusion is that the EQA fulfils its objectives, allowing the participating laboratories to assess their performance and to identify areas needing improvement and specific training needs by receiving an objective evaluation. All participating laboratories are encouraged to participate again in any of the subsequent EQAs in order to improve and document the quality of their performance and maintain good laboratory practices.

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Annex 1. List of participants

Country	Institution	Laboratory
Argentina	Instituto Nacional de Enfermedades Infecciosas	Servicio Fisiopatogenia
Australia	CSIRO	Food and Nutrition Department
Australia	Department of Health, Forensic and Scientific Services	Public Health Microbiology
Australia	Peter Doherty Institute	Public Health laboratory, Microbiological Diagnostic Unit
Brazil	Instituto Adolfo Lutz - Secretaria de Estado da Saúde	Laboratório de Referência Nacional para Sindrome Hemolítica Urêmica
Canada	Ottawa Laboratory (Carling)	Research and development section
Canada	Canadian Science Centre for Human and Animal Health	Enteric Diseases Program, National Microbiology laboratory
Canada	Public Health Agency of Canada	*E. coli* Laboratory
Canada	Université de Montréal	OIE Reference Laboratory for Escherichia coli
Chile	Facultad de Medicina, Universidad de Chile	Programa de Microbiología, Enteropathogens laboratory
China	Shanghai Changning Center For Disease Control And Prevention	Department of microbial test
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
Norway	Norwegian Veterinary Institute	National reference laboratory for E. coli in food and animals
Paraguay	Ministerio De Salud Pública Y Bienestar Social	Laboratorio Central De Salud Pública
Scotland	Royal Infirmary of Edinburgh	Scottish E. Coli O157/VTEC Reference Laboratory
South Africa	National Institute for Communicable Diseases	Center for Enteric Diseases -Bacteriology
USA	Center for Disease Control and Prevention	Enteric Diseases Laboratory Branch
USA	US FDA	Microbiology
USA	The Pennsylvania State University	*E. coli* Reference Center

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	504	506	508	509	511	516	520	521	526	532
Running Conditions	3	1	3	3	4	4	3	4	2	4
Cell Suspension	4	4	4	3	4	3	4	4	4	4
Bands	3	3	3	1	3	1	4	4	3	2
Lanes	4	4	4	4	4	3	4	4	3	4
Restriction	4	4	4	4	4	4	4	3	4	4
Gel Background	4	3	2	2	4	4	3	4	4	2
DNA Degradation	4	3	4	2	4	4	4	4	4	2

Scored according to Annex 2 (TIFF quality grading guidelines)

BN analysis

Parameters/Laboratory	504	506	508	509	511	516	520	521	526	532
Position of Gel Frame	3	N/A	3	N/A	2	2	3	3	2	2
Strips	3	N/A	2	N/A	2	3	3	3	2	3
Curves	3	N/A	3	N/A	2	2	2	2	2	2
Normalization	3	N/A	3	N/A	3	3	3	3	2	2
Band Assignment	2	N/A	3	N/A	3	2	2	3	3	2

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)

Strain no.	O group	H type	Vero cell assay	ESBL production	Haemolysin production	Beta-glucuronidase production	Sorbitol fermentation	*eae* gene	*ehxA* gene	*vtx1* gene	*vtx2* gene	*vtx1* subtypes	*vtx2* subtypes	*aggR* gene	*aaiC* gene	Additional virulence genes	Pathotype
AA11	O80	H2	+	-	+	+	+	+	+	-	+	-	*vtx2a*	-	-		VTEC
BB12	O26	H11	+	-	+	+	+	+	+	-	+	-	*vtx2a*	-	-		VTEC
CC13	O78	H2	-	-	-	+	+	-	-	-	-	-	-	+	+	*aatA, astA*	EAEC
DD14	O145	H34	+	-	-	+	-	+	-	-	+	-	*vtx2f*	-	-		VTEC
EE15	O166	H15	+	+	-	+	+	-	-	-	+	-	*vtx2d*	-	-	*eltA*	VTEC-ETEC
FF16	O156	H4	+	-	-	+	+	-	-	-	+	-	*vtx2d*	-	-		VTEC
GG17	O146	H21	+	-	+	+	+	-	+	+	+	*vtx1c*	*vtx2b*	-	-		VTEC
HH18	O157	H-/H7	+	-	+	-	-	+	+	+	+	*vtx1a*	*vtx2c*	-	-		VTEC
II19	O91	H14	+	-	+	+	+	-	+	+	+	*vtx1a*	*vtx2b*	-	-	*saa*	VTEC
JJ20	O103	H2	+	-	+	+	+	+	+	+	-	*vtx1a*	-	-	-		VTEC

EAEC, enteroaggregative *E. coli*; *ETEC*, enterotoxigenic *E. coli*; *VTEC*, vero cytotoxin-producing *E. coli*; -, negative; +, positive

Gene abbreviations

aaiC: Chromosomal gene marker for Enteroaggregative *E. coli*

aatA: PCR fragment, encodes the dispersin (aap) transporter, good plasmid marker for Enteroaggregative *E. coli*

aggR: Encoding the master regulator in Enteroaggregative *E. coli*

astA: Encoding heat-stable enterotoxin 1 (EAST1)

eae: CVD434. *E. coli* attaching and effacing gene probe

ehxA CVD419. Plasmid encoding O157-enterohaemolysin

eltA: G119. Encoding heat-labile enterotoxin A, almost identical to cholera toxin

saa: Encoding autoagglutinating adhesion

vtx1: NTP705. Encoding verotoxin1, almost identical with the Shiga toxin

vtx2: DEP28. Encoding verotoxin2, variants exist. Approx. 60% homology to vtx1

Annex 6. O group typing results

Strain	O group	Participating laboratory																				
		403	405	501	504	506	508	509	511	514	516	518	519	520	521	523	528	530	531	532	533	534
AA11	**O80**	O80	**NT**	**ND**	O80	O80	**NT**	**O128**	O80	O80	O80	O80	O80	O80	O80	O80	O80	O80	**ND**	**NT**	**O128**	**NT**
BB12	**O26**	O26	O26	O26	O26	O26	O26	**O18**	O26	O26	O26	O26	O26	O26	O26	O26	O26	O26	O26	O26	O26	O26
CC13	**O78**	O78	**NT**	**ND**	O78	O78	**NT**	O78	O78	O78	O78	O78	O78	O78	O78	O78	**NT**	O78	**ND**	O78	O78	O78
DD14	**O145**	O145	**NT**	O145	O145	O145	O145	**NT**	O145	O145	O145	O145	**ND**	O145	O145	O145	O145	O145	O145	O145	**NT**	O145
EE15	**O166**	O166	**NT**	**ND**	O166	O166	O166	**NT**	O166	O166	O166	O166	O166	O166	O166	O166	**NT**	O166	**ND**	**NT**	**NT**	O166
FF16	**O156**	O156	**NT**	**ND**	**O141**	**NT**	O156	**O126**	O156	**O36**	O156	**Rough**	**ND**	**NT**	**O36**	O156	**NT**	NT	**ND**	**NT**	**O145**	**NT**
GG17	**O146**	O146	**NT**	**ND**	O146	O146	O146	O146	O146	O146	O146	O146	O146	O146	O146	O146	O146	O146	**ND**	O146	O146	O146
HH18	**O157**	O157	O157	O157	O157	O157	O157	O157	O157	O157	O157	O157	O157	O157	O157	O157	O157	O157	O157	O157	O157	O157
II19	**O91**	O91	**NT**	**ND**	O91	O91	O91	O91	O91	O91	O91	O91	O91	O91	O91	O91	O91	O91	**ND**	O91	O91	O91
JJ20	**O103**	O103	O103	O103	O103	O103	O103	O103	O103	O103	O103	O103	O103	O103	O103	O103	O103	O103	**ND**	O103	**NT**	O103

NT: not typeable

ND: Not done

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

Incorrect result

Annex 7. H typing results

Strain	H type	Participating laboratory													
		403	504	508	511	514	516	518	519	520	521	523	530	533	534
AA11	**H2**	H2	H2	H2	H2	H2	**H-**	H2	H2	H2	H2	H2	**H-**	**H7**	H2
BB12	**H11**	H11	H11	H11	H11	H11	H11	H11	H11	H11	H11	H11	H11	**H7**	H11
CC13	**H2**	H2	H2	H2	H2	H2	H2	H2	H2	H2	H2	H2	H2	**H7**	H2
DD14	**H34**	H34	H34	H34	H34	H34	H34	H34	**ND**	H34	H34	H34	H34	**NT**	H34
EE15	**H15**	H15	H15	H15	H15	H15	H15	H15	H15	H15	H15	H15	H15	**NT**	H15
FF16	**H4**	H4	H4	H4	H4	H4	H4	H4	H4	H4	H4	H4	H4	**H7**	H4
GG17	**H21**	H21	H21	H21	H21	H21	H21	H21	H21	H21	H21	H21	H21	**NT**	H21
HH18	**H-/H7**	H-	H7	H7	**NT**	H-	H-	H-	H7	H-	**NT**	H7	H-	H7	H7
II19	**H14**	H14	H14	H14	H14	**H-**	H14	H14	H14	H14	H14	H14	H14	**H7**	H14
JJ20	**H2**	H2	H2	H2	H2	H2	H2	H2	H2	H2	H2	H2	H2	**H7**	H2

NT: not typeable

ND: Not done

In this EQA the laboratories H type detection results 'Non typeable' or 'Not done' were considered as a negative result and scored accordingly.

Incorrect result

Annex 8. *eae* gene detection results

Strain	*eae* gene	Participating laboratory																			
		501	504	506	508	509	511	514	516	518	519	520	521	523	526	528	530	531	532	533	534
AA11	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
BB12	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
CC13	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	+	-
DD14	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
EE15	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
FF16	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-
GG17	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-
HH18	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
II19	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-
JJ20	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+

+= Positive, -= Negative

Incorrect result

Annex 9. *ehx*A gene detection results

Strain	*ehxA* gene	Participating laboratory																
		501	504	508	511	516	518	519	520	521	523	526	528	530	531	532	533	534
AA11	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
BB12	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
CC13	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
DD14	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
EE15	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
FF16	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
GG17	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
HH18	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
II19	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
JJ20	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+

+= Positive, -= Negative

Annex 10. *vtx*1 gene detection results

Strain	*vtx1* gene	Participating laboratory																					
		405	501	504	506	508	509	511	514	516	518	519	520	521	522	523	526	528	530	531	532	533	534
AA11	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-
BB12	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
CC13	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
DD14	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
EE15	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
FF16	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
GG17	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+
HH18	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
II19	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
JJ20	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+

+= Positive, -= Negative

Incorrect result

Annex 11. *vtx*2 gene detection results

Strain	*vtx2* gene	Participating laboratory																					
		405	501	504	506	508	509	511	514	516	518	519	520	521	522	523	526	528	530	531	532	533	534
AA11	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
BB12	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
CC13	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
DD14	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	+	-	-	+
EE15	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
FF16	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
GG17	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
HH18	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+
II19	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
JJ20	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

+= Positive, -= Negative

Incorrect result

Annex 12. *vtx1* subtyping results

Strain	*vtx1* subtype	Participating laboratory																	
		501	506	508	509	511	514	516	518	519	520	521	522	523	526	528	531	532	534
AA11	**-**	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	vtx1c	-	-
BB12	**-**	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
CC13	**-**	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
DD14	**-**	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
EE15	**-**	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
FF16	**-**	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
GG17	***vtx1c***	vtx1c	vtx1c	vtx1c	vtx1c	vtx1c	vtx1c	vtx1c	vtx1c	vtx1c	vtx1c	vtx1c	vtx1c	vtx1c	vtx1c	vtx1c	vtx1c	vtx1c	vtx1c
HH18	***vtx1a***	vtx1a	vtx1a	vtx1a	vtx1a	vtx1a	vtx1a	vtx1a	vtx1a	vtx1a	vtx1a	vtx1a	vtx1a	vtx1a	vtx1a	vtx1a	vtx1a	vtx1a	vtx1a
II19	***vtx1a***	vtx1a	vtx1a	vtx1a	vtx1a	vtx1a	vtx1a	vtx1a	vtx1a	vtx1a	vtx1a	vtx1a	vtx1a	vtx1a	vtx1a	vtx1a	vtx1a	vtx1a	vtx1a
JJ20	***vtx1a***	vtx1a	vtx1a	vtx1a	vtx1a	vtx1a	vtx1a	vtx1a	vtx1a	vtx1a	vtx1a	vtx1a	vtx1a	vtx1a	vtx1a	vtx1a	vtx1a	vtx1a	vtx1a

-= Negative

Incorrect result

Annex 13. *vtx2* subtyping results

Strain	*vtx2* subtype	Participating laboratory																		
		501	504	506	508	509	511	514	516	518	519	520	521	522	523	526	528	530	531	532	534
AA11	*vtx2a*	vtx2a	ND	vtx2a	vtx2a	vtx2a and vtx2f	vtx2a	vtx2a	vtx2a	vtx2a	vtx2a	vtx2a	vtx2a	vtx2a	-	vtx2a	vtx2a	ND	vtx2a	vtx2a	vtx2a
BB12	*vtx2a*	vtx2a	ND	vtx2a	vtx2a	vtx2a and vtx2d	vtx2a	vtx2a	vtx2a	vtx2a	vtx2a	vtx2a	vtx2a	vtx2a	-	vtx2a	vtx2a	ND	vtx2b	vtx2a	vtx2a
CC13	-	-	ND	-	-	-	-	-	-	-	-	-	-	-	-	-	-	ND	-	-	-
DD14	*vtx2f*	vtx2f	vtx2f	vtx2f	vtx2f	vtx2a and vtx2f	vtx2f	vtx2f	vtx2f	vtx2f	vtx2f	vtx2f	vtx2f	vtx2f	vtx2f	vtx2f	-	vtx2f	vtx2f	vtx2f	vtx2f
EE15	*vtx2d*	vtx2d	ND	vtx2d	vtx2d	vtx2c and vtx2d	vtx2d	vtx2d	vtx2c and vtx2d	vtx2d	vtx2d	vtx2d	vtx2d	vtx2d	vtx2d	vtx2d	vtx2c and vtx2d	ND	vtx2d	vtx2d	vtx2a and vtx2c and vtx2d
FF16	*vtx2d*	vtx2d	ND	vtx2d	vtx2d	vtx2b and vtx2d	vtx2d	vtx2d	vtx2c and vtx2d	vtx2d	vtx2d	vtx2d	vtx2d	vtx2d	vtx2d	vtx2d	vtx2d	ND	vtx2c	vtx2d	vtx2a and vtx2c and vtx2d
GG17	*vtx2b*	vtx2b	ND	vtx2b	vtx2b	vtx2b	vtx2b	vtx2b	vtx2b	vtx2b	vtx2b	vtx2b	vtx2b	vtx2b	vtx2a	vtx2b	vtx2b	ND	vtx2b	vtx2b	vtx2b
HH18	*vtx2c*	vtx2c	ND	vtx2c	vtx2c	vtx2c	vtx2c	vtx2c	vtx2c and vtx2d	vtx2c	vtx2c	vtx2c	vtx2c	vtx2c	vtx2c and vtx2d	vtx2c	vtx2c and vtx2d	ND	vtx2c	vtx2c	vtx2a and vtx2c and vtx2d
II19	*vtx2b*	vtx2b	ND	vtx2b	vtx2b	vtx2b	vtx2b	vtx2b	vtx2b	vtx2b	vtx2b	vtx2b	vtx2b	vtx2b	vtx2a	vtx2b	vtx2b	ND	vtx2b	vtx2b	vtx2b
JJ20	-	-	ND	-	-	-	-	-	-	-	-	-	-	-	-	-	-	ND	-	-	-

-= Negative

Incorrect result

Annex 14. Reference strains of *vtx* subtypes

SSI collection D number	Strain	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*
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	
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*

ND, Not Done

Annex 15. Virulence genes *aggR* and *aaiC*

aggR

Strain	*aggR* gene	Participating laboratory												
		501	508	514	516	518	519	520	523	526	528	530	532	534
AA11	-	-	-	-	-	ND	-	-	-	ND	-	-	-	-
BB12	-	-	-	-	-	ND	-	-	-	ND	-	-	-	-
CC13	+	+	+	+	+	+	+	+	+	+	+	+	+	+
DD14	-	-	-	-	-	ND	-	-	-	ND	-	-	-	-
EE15	-	-	-	-	-	ND	-	-	-	ND	-	-	-	-
FF16	-	-	-	-	-	ND	-	-	-	ND	-	-	-	-
GG17	-	-	-	-	-	ND	-	-	-	ND	-	-	-	-
HH18	-	-	-	-	-	ND	-	-	-	ND	-	-	-	-
II19	-	-	-	-	-	ND	-	-	-	ND	-	-	-	-
JJ20	-	-	-	-	-	ND	-	-	-	ND	-	-	-	-

aaiC

Strain	*aaiC* gene	Participating laboratory							
		508	516	519	523	526	528	532	534
AA11	-	-	-	-	-	ND	-	-	-
BB12	-	-	-	-	-	ND	-	-	-
CC13	+	+	+	+	+	+	+	+	+
DD14	-	-	-	-	-	ND	-	-	-
EE15	-	-	-	-	-	ND	-	-	-
FF16	-	-	-	-	-	ND	-	-	-
GG17	-	-	-	-	-	ND	-	-	-
HH18	-	-	-	-	-	ND	-	-	-
II19	-	-	-	-	-	ND	-	-	-
JJ20	-	-	-	-	-	ND	-	-	-

+= Positive, -= Negative

Incorrect result

Annex 16. VCA results

Strain	VCA result	Participating laboratory				
		504	508	511	518	526
AA11	+	+	+	+	+	+
BB12	+	+	+	+	+	+
CC13	-	-	-	-	-	-
DD14	+	+	+	+	+	+
EE15	+	+	+	+	+	+
FF16	+	+	+	+	+	+
GG17	+	+	+	+	+	+
HH18	+	+	+	+	+	+
II19	+	+	+	+	+	+
JJ20	+	+	+	+	+	+

+= Positive, - = Negative

Annex 17. ESBL production results

Strain	ESBL result	Participating laboratory								
		405	508	509	516	526	528	530	532	533
AA11	-	-	-	-	-	-	-	-	-	-
BB12	-	-	-	-	-	-	-	-	-	-
CC13	-	-	-	-	-	+	-	-	-	-
DD14	-	-	-	-	-	-	-	-	-	-
EE15	+	+	+	+	+	-	+	+	+	+
FF16	-	-	-	-	-	-	-	-	-	-
GG17	-	-	-	-	-	-	-	-	-	-
HH18	-	-	-	-	-	-	-	-	-	-
II19	-	-	-	-	-	-	-	-	-	-
JJ20	-	-	-	-	-	-	-	-	-	-

+ = Positive, - = Negative

Incorrect result

Annex 18. Enterohaemolysin production results

Strain	Entero-haemolysin result	Participating laboratory						
		504	508	514	516	518	526	530
AA11	+	+	+	+	+	+	+	+
BB12	+	+	+	+	+	+	+	+
CC13	-	-	-	-	-	-	-	-
DD14	-	-	-	-	-	-	-	-
EE15	-	-	-	-	-	-	-	-
FF16	-	-	-	-	-	-	-	-
GG17	+	-	+	+	+	+	+	-
HH18	+	+	+	+	+	+	+	-
II19	+	-	+	+	+	+	+	+
JJ20	+	+	+	+	+	+	+	+

+= Positive, -= Negative

Incorrect result

Annex 19. β-glucuronidase production results

Strain	β-glucuronidase	Participating laboratory							
		403	504	508	514	516	520	526	533
AA11	**+**	+	+	+	+	+	+	+	-
BB12	**+**	+	+	+	+	+	+	+	+
CC13	**+**	+	+	+	+	+	+	+	-
DD14	**+**	+	+	+	+	+	+	+	+
EE15	**+**	+	+	+	+	+	+	+	+
FF16	**+**	+	+	+	+	+	+	+	+
GG17	**+**	+	+	+	+	+	+	+	+
HH18	**-**	-	-	-	-	-	-	-	-
II19	**+**	+	+	+	+	+	+	+	+
JJ20	**+**	+	+	+	+	+	+	-	-

+= Positive, -= Negative

Incorrect result

Annex 20. Sorbitol fermentation results

Strain	Sorbitol fermentation result	Participating laboratory														
		403	405	501	504	506	508	509	514	516	518	520	526	528	530	533
AA11	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
BB12	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
CC13	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
DD14	-	+	-	-	-	-	-	-	+	-	+	-	-	+	-	-
EE15	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
FF16	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
GG17	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
HH18	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
II19	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
JJ20	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+

+= Positive, -= Negative

Incorrect result