Detection and identification of Verocytotoxin-producing *Escherichia coli* (VTEC) O104:H4 in food by Real Time PCR

**Laboratory procedure**

**Aims and field of application:**

The ongoing large outbreak of VTEC infections in Germany is caused by a VTEC strain belonging to serotype O104:H4, a serotype (and serogroup), not comprised among those usually associated with severe infections in Europe and worldwide.

Some characteristics of the outbreak strain have been reported:

1. It produces VT2, and harbors the vtx2a gene subtype.
2. It lacks the gene coding for the adherence factor intimin (*eae* gene), which is considered as a hallmark of the pathogenic VTEC.
4. All but one strain tested so far posses the genetic markers of typical of Enteroaggregative *Escherichia coli* (EAggEC): the *aggR*, *aatA*, *aaiC* and *aap* genes.

These features have to be considered when defining a diagnostic strategy for the detection in food or environmental samples. The absence of the *eae* gene may pose problems, since the ISO TS 13136 - Horizontal method for the detection of Shiga toxin-producing *Escherichia coli* (STEC) belonging to O157, O111, O26, O103 and O145 serogroups - Qualitative Real-time polymerase chain reaction (PCR)-based Method – is based on the stepwise detection of the *eae* gene in *vtx*-positive samples. Serotyping is cumbersome and only few laboratories may possess antiserum specific to this particular serogroup.
Therefore, two genetic markers can be considered for the molecular screening of the \(\text{vtx}\)-positive enrichment cultures: the O104 antigen-associated gene \(\text{wzx}_{\text{O104}}\) and the gene encoding the H4 flagellar antigen, \(\text{fliC}_{\text{H4}}\). The markers associated with the enteroaggregative adhesion could also be considered as targets of the diagnostic procedure and such a test is currently under evaluation at the EU RL VTEC.

The antibiotic resistance characteristics of the VTEC O104 outbreak strain can be exploited for the isolation step, by plating PCR-positive enrichment cultures samples onto MacConkey agar supplemented with streptomycin (20 \(\mu\text{g/ml}\)) and/or tetracycline (10 mg/ml), or onto Brilliance™ \(\text{ESBL}\) Agar from Oxoid or ChromID ESBL from Biomerieux or similar media available in the commerce.

The proposed method aims at the identification of the presence of O104 antigen-associated gene \(\text{wzx}_{\text{O104}}\) in \(\text{vtx}\)-positive enrichment cultures. The molecular design of this Real Time PCR has been described in the literature (Bugarel et al., 2010). The same assay can be used to identify and confirm the O104:H4 serotype of isolated strains in combination with the \(\text{fliC}_{\text{H4}}\) RT-PCR that has been deployed at the EU-RL for \(E.\ coli\) on the \(\text{fliC}\) gene sequence of the \(E.\ coli\) strain U9-41 present in GenBank under the accession number AY249989.

**Food samples screening - Procedure**

Enrichment cultures are performed by adding a 25 gr test portion of food sample or 25 ml of milk sample to 225 ml of Buffered Peptone Water, and incubating for 18 -24 h at 37°C ± 1°C. One ml aliquot of such a culture is used for DNA extraction and purification. For testing of seeds used for production of sprouts to be consumed raw, refer to **Annex 4**.

This Real Time PCR protocol is used to test all the samples that give positive results for the presence of \(\text{vtx}\) genes by using the first step of the ISO TS
13136 – Horizontal method for the detection of Shiga toxin-producing *Escherichia coli* (STEC) belonging to O157, O111, O26, O103 and O145 serogroups - Qualitative Real-time polymerase chain reaction (PCR)-based Method.

The Real-time PCRs for *wzx* \(_{O104}\) and *fliC* \(_{H4}\) are performed using the primers and probes described in Annex 1. Amplification conditions to be applied will depend on the system used, and will refer to the instructions supplied with the instrument and the reagents’ kit of choice.

A standard two-step thermal profile has been used at EU RL VTEC with satisfactory results for both the reactions. The details were as follows:

95°C X 10’

35 cycles of

95°C X 15”

60°C X 1’

Enrichment cultures positive for presence of *wzx* \(_{O104}\) gene are streaked onto MacConkey agar or TBX plates or any other media suitable for *E. coli* isolation. A second more selective plate can be chosen among the antibiotic-containing media described in the previous paragraph.

Up to 50 isolated colonies with typical *E. coli* morphology or growing on the media with antibiotics are collected and point-inoculated on Nutrient Agar (NA) (single colonies) and H\(_2\)O (5 pools by 10 colonies each). *vtx* gene detection is performed on the isolated colonies or pools by Real Time or conventional PCR (reference methods can be found at http://www.iss.it/vtec). Colonies positive for *vtx* genes will be tested for the O104 antigen-associated gene *wzx* \(_{O104}\) and the gene encoding the H4 flagellar antigen, *fliC* \(_{H4}\).

For strain characterization, the Real-time PCRs for *wzx* \(_{O104}\) and *fliC* \(_{H4}\) can be run as duplex PCR, labeling the two probes with compatible phluorophors (e.g. FAM and HEX).
Alternatively, confirmation of isolated colonies as possessing the virulence profile of the German outbreak strain can be accomplished by using the conventional PCR protocol developed by the “Konsiliarlabor für Hämolytisch–Urämisches Syndrom (HUS)” and described in the “Laborinfo Stand 01.06.2011” downloadable from the website www.ehec.org. The approach includes the detection of the vtx, TerD, rfbO104, and fliC H4 genes in a multiplex PCR reaction. A scheme of the procedure is in Annex 2.

As an optional step, the vtx2 gene sub-typing may be carried out. The vtx-genotype of the German outbreak strain has been reported to be vtx2a. A conventional PCR for vtx2-genes subtyping has been distributed for the 6th proficiency test organised jointly by the EU RL VTEC in the framework of the 2010 work program and the WHO Collaborating Centre for Reference and Research on Escherichia and Klebsiella, at the Statens Serum Institute, Copenhagen, upon mandate of the ECDC.

An extract of the laboratory procedure sent for the 6th ring test describing the protocol for the detection of the vtx2a subtype is included in Annex 3.
Flow-diagram of the screening procedure

Test portion 25 g or 25 ml

225 ml BPW- Homogenize
5 min

Enrichment 18 h-24 hrs
37°C ± 1°C

Draw 1 ml of the enrichment culture, DNA purification and Real Time PCR screening for vtx genes detection (first step of ISO TS 13136)
2-4 hrs

Positive result to vtx genes:
Test for wzxO104 gene
1-2 hrs

Negative result to vtx gene:
Reporting: Absence of VTEC

Negative result to wzxO104 gene:
Reporting: Absence of VTEC O104

If positive to stx and wzxO104 genes:
Presumptive presence of VTEC O104
Isolation onto Maconkey agar or TBX, and additional solid media with antibiotics
18-24 hrs

Test isolated colonies for stx wzxO104, fliC_H4 genes by Real Time or confirm by convetional PCR (Annex 2)
2-4 hrs
Annex 1

Real-time PCR for the detection of wzx\textsubscript{O104} and fliC\textsubscript{H4} genes

1. Principle of the method

This Real-time PCR protocol aims at the detection of the O104 serogroup-associated gene, wzx\textsubscript{O104}, coding for the O-antigen flippase Wzx and the gene encoding the flagellar antigen H4, fliC\textsubscript{H4}.

2. Operating procedure

The protocols are based on the 5’ nuclease PCR assay. Considering that Real-time PCR may use different instruments and probes labeling chemistry, the amplification conditions to be applied may vary depending on the system used. Refer to the instructions supplied with the instrument and reagents’ kit of choice.

A standard two-step thermal profile has been used at EU RL VTEC with satisfactory results for both the reactions. The details were as follows:

95°C X 10’
35 cycles of
95°C X 15”
60°C X 1’

The primers and probes to be used are listed in the table below. A typical Real Time PCR reaction is described below. The chemistry of the reporter and quencher phluorophores are not indicated being largely dependent on the Real-time PCR systems available in each laboratory.

For isolated strains characterization the Real-time PCR for wzx\textsubscript{O104} and fliC\textsubscript{H4} can be run as duplex PCR, labeling the two probes with compatible phluorophors (e.g. FAM and HEX).
RT PCR reaction assembly:

Buffer 10X to 1X (MgCl<sub>2</sub> 3mM)

Primer Fwd 500 nM

Primer Rev 500 nM

Probe 200nM

DNA 2 μl of DNA purified from 1 ml of culture and diluted 1:10 can be sufficient

Water to final volume

Please note that due to the urgent necessity of the present protocol the procedure described above has been tested by the EU-RL VTEC on a limited set of isolated strains only and has not been validated on the enrichment cultures yet.

3. Controls

A VTEC strain belonging to serotype O104:H4 should be used as positive control. DNA extracted by a VTEC strain belonging to serogroup O104 will be made available soon by the EU-RL VTEC to the NRLs which will require it.

The Real-time PCR procedure requires an inhibition/extraction control. Details on the possible systems to be used as inhibition/extraction control are given below. In particular, two different internal amplification controls (IACs) can alternatively be used:

- A commercially available TaqMan® Exogenous Internal Positive Control (Applied Biosystems, Foster City, CA, USA). The kit includes all reagents necessary (primers, a Vic™ probe, IAC target DNA and blocking solution). The IAC target DNA must be diluted 10 times to achieve a copy number of approximately 100 per PCR reaction. The PCR product length is not declared to the customer.

- The open formula pUC 19 based internal amplification control IAC developed by Fricker et al. (2007. Appl Environ Microbiol 73, 1892-1898).
Approximately 100 copies of target DNA (pUC 19) should be used per PCR reaction. The size of the IAC is 119 bp.

The latter may be also used as an extraction control by adding 100 copies of the pUC 19 plasmid to the sample aliquot prior to the DNA purification step.

Table 1. Primers and probes used in 5’ nuclease PCR assays

<table>
<thead>
<tr>
<th>Target gene (Ref.)</th>
<th>Forward primer, reverse primer and probe sequences (5’-3’) *</th>
<th>Amplicon size (bp)</th>
<th>Location within sequence</th>
<th>GenBank accession number</th>
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<tbody>
<tr>
<td>wzxO104(1)</td>
<td>TGTCGCACAAGAATTTCACAAAATCCTTTAAACTATACGCC</td>
<td>100</td>
<td>2,333,750–2,333,730</td>
<td>CU928145</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>2,333,673–2,333,651</td>
<td></td>
</tr>
<tr>
<td></td>
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<td></td>
<td>2,333,724–2,333,693</td>
<td></td>
</tr>
<tr>
<td></td>
<td>AAAATCCTTTAAACTATACGCC</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Probe-</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>TTGGTTTTTTTTGTATTAGCAATAAGTGGTGTC</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>fliC_H4(2)</td>
<td>GCTGGGGGTAACAAGTCAA</td>
<td>192</td>
<td>604-623</td>
<td>AY249989</td>
</tr>
<tr>
<td></td>
<td>CCAGTGCTTTTAACGGATCG</td>
<td></td>
<td>796-777</td>
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<tr>
<td></td>
<td>Probe-</td>
<td></td>
<td>631-650</td>
<td></td>
</tr>
<tr>
<td></td>
<td>TCTTACACTGACACCGCGTC</td>
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</tr>
</tbody>
</table>

(2) EU RL VTEC
Annex 2

**Multiplex conventional PCR for the detection of vtx, TerD, wzx_{O104} and fliC_{H4} genes** (From the “Laborinfo Stand 01.06.2011” developed at the "Konsiliarlabor für Hämolysitisch–Urämisches Syndrom (HUS)" - www.ehec.org).
Annex 3

Conventional PCR for the subtyping of the vtx2 gene

1 Principle of the method
The method is based on PCR amplification of specific DNA regions from a template DNA, with oligonucleotides triggering the start of the PCR reaction. The procedure concerns the detection of the vtx2a gene subtype and is performed by specific PCR reactions, using primers designed on the basis of analyses of existing vtx sequences (reported in Appendix 1).

The method is composed of the following steps:
- Template preparation
- Setting up the PCR reaction
- Determination of the PCR results by agarose gel electrophoresis.

2 Template preparation
Isolated strains are streaked onto solid media (e.g. TSA) and incubated over night.
A single bacterial colony is inoculated in TSB and incubated over night.
25 µl of the overnight culture are added to 975 µl Milli Q water in Eppendorf tube and boiled for 15 minutes. Centrifuge at 18.000 g 5 minutes. Upon transfer to a clean tube, the supernatant is used directly for PCR and stored at -18°C for further analyses.

3 Setting up the PCR reaction
PCR assays are set up in a total volume of 20 µl for standard PCR as follows:

1.25 µl H₂O
10 µl Mastermix (HotStart, Qiagen),
1.25 µl of each of three primers (STOCK solution of primers is 5 µM) §
5 µl supernatant of boiled lysate (STOCK)
Primers’ sequences:
vtx2a-F2  GCGATACTGRGBACTGTGGCC
vtx2a-R3  CCGKCAACCTTCACGTGAAATGTG
vtx2a-R2  GGCCACCTTCACGTGAATGTG

The thermal profile is:
95ºC for 15 min (HotStart Taq activation)
35 cycles of 94ºC for 50 sec, 64ºC for 40 sec and 72ºC for 60 sec, ending
with 72ºC for 3 min. PCR amplicons can be stored at 4°C until loading on
agarose gel.

In each PCR assay, a positive and a negative control must be included. The
DNA template to be used as positive control is available from the EU RL
VTEC upon request. The negative control is constituted by a sample without
template added.

4 Agarose gel electrophoresis
Prepare agarose gel in 1X Tris/Borate/EDTA (TBE) or Tris/Acetate/EDTA
(TAE). Each well of the gel is loaded with 10 µl of each reaction added with
loading dye at 1X final concentration. Run the samples in 1X running buffer
(TBE or TAE) in constant voltage (100 V). Use a molecular weight marker
suitable for assignment of the correct molecular weights to the amplicons
produced (refer to Appendix 1). Consider that a correct band assignment is a
crucial point in the assessment of the presence of the target genes. Make
sure that the bands produced by the reference strains match exactly the
expected molecular weight.
Agarose gels should be added of ethidium bromide to allow the visualisation
of DNA. This reagent is a DNA intercalating agent commonly used as a
nucleic acid stain in molecular biology laboratories. When exposed to
ultraviolet light, it will fluoresce with a red-orange colour. Ethidium bromide
should be added to a final concentration of 0.5 µg/ml before pouring the
agarose gel in the electrophoresis gel cast. Alternatively the agarose gel can be stained after electrophoresis in a 0.5 μg/ml ethidium bromide aqueous solution.

The expected amplicons size is about 350 bp.
Annex 4

Testing of seeds used for production of sprouts to be consumed raw

When the matrix to be analyzed is constituted by seeds the following considerations have to be made:

1. The seeds are generally contaminated at very low levels (0.1 to 1.8 cfu/gr as assessed for Salmonella. See reference below). Nonetheless, the sprouting process is characterized by conditions (humidity, heat) favoring the pathogen’s enrichment.

2. The seeds are generally dried. Therefore, the contaminating pathogens are supposed to be stressed.

3. The contamination may occur on the surface of the seed as well as being internal. The latter case occurs when the seeds are contaminated during the growth of the plants used for their production (primary contamination of crops). Contamination of the seeds’ surface can occur during all the phases of preparation, storage and general handling of the seeds (secondary contamination).

4. The enrichment cultures of seeds may contain inhibitors of the DNA polymerase used for the PCR screening of the samples.

In order to increase the analytical power of the proposed method, the following steps are carried out when dealing with seeds:

1. 50 gr of seeds are analyzed instead of the usual 25 gr of food items in order to increase the sensitivity of the assay.

2. The seeds are smashed in a sterile container (e.g. a stomacher bag) using a mortar with pestel or other similar tools before adding the enrichment broth.

3. The smashed seeds are transferred to a sterile container (flask or a new stomacher bag) added with 450 ml BPW and incubated for 24 hrs at 37°C (either static or in agitation).

4. A 5 ml aliquot of the enrichment culture is taken, mixed by vortexing (in order to detach any possible Enteroaggregative bacteria adhering to seeds), centrifuged at 500 X g 1 min to sediment the seeds’ debris.
One ml aliquot of the supernatant is taken at this stage and used for DNA preparation.

5. The DNA is diluted 1:10 before using it. In case of absence of amplification in the reactions containing the IAC, the DNA template is used at the dilution of 1:30.

Reference.