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 vtx-positive enrichment cultures: the O104 antigen-associated gene \( \text{wzx}_{O104} \) and the gene encoding the H4 flagellar antigen, \( \text{fliC}_{H4} \). 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 µg/ml) and/or tetracycline (10 mg/ml), or onto Brilliance™ 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 \( \text{wzx}_{O104} \) in 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 \( \text{fliC}_{H4} \) RT-PCR that has been deployed at the EU-RL for \( E. \ co l i \) on the \( \text{fliC} \) gene sequence of the \( E. \ co l i \) 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.

This Real Time PCR protocol is used to test all the samples that give positive results for the presence of vtx genes by using the first step of the ISO TS 13136 – Horizontal method for the detection of Shiga toxin-producing \( Escherichia \ co l i \) (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

1. **Test portion 25 g or 25 ml**

2. 225 ml BPW- Homogenize
   - **5 min**

3. **Enrichment 18 h-24 hrs**
   - **37°C ± 1°C**

4. 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 conventional PCR (Annex 2)
     - **2-4 hrs**
Annex 1

Real-time PCR for the detection of \( wzx_{O104} \) and \( fliC_{H4} \) genes

1. Principle of the method

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

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 \( wzx_{O104} \) and \( fliC_{H4} \) 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 (MgCl₂ 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>
</tr>
</thead>
<tbody>
<tr>
<td><strong>wzx</strong>&lt;sub&gt;O104&lt;/sub&gt;(1)</td>
<td>TGGTCGCGAAAGAATTCAAC AAAATCCTTTAAAACATACGCC Probe- TTGGTTTTTTTGTATTAGCAATAAGTGGTGTC</td>
<td>100</td>
<td>2,333,750–2,333,730 2,333,673–2,333,651 2,333,724–2,333,693</td>
<td>CU928145</td>
</tr>
<tr>
<td><strong>fliC</strong>&lt;sub&gt;H4&lt;/sub&gt;(2)</td>
<td>GCTGGGGGTAAACAAGTCAA CCAGTGCTTTAACGGATCG Probe- TCTTACACTGACACCGCGTC</td>
<td>192</td>
<td>604-623 796-777 631-650</td>
<td>AY249989</td>
</tr>
</tbody>
</table>

(2) EU RL VTEC
Annex 2

Multiplex conventional PCR for the detection of vtx, TerD, wzxO104 and fliC_H4 genes (From the “Laborinfo Stand 01.06.2011” developed at the “Konsiliarlabor für Hämolytisch–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 -18ºC 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)
Primer sequences:

- vtx2a-F2: GCGATACTGRGACTGTGGCC
- vtx2a-R3: CCGKCAACCTTCACTGTAAATGTG
- vtx2a-R2: GGCCACCTTCACTGTGAATGTG

The thermal profile is:
95°C for 15 min (HotStart Taq activation)
35 cycles of 94°C for 50 sec, 64°C for 40 sec and 72°C for 60 sec, ending with 72°C 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.