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Microbiology of food and animal feed — Real-time polymerase chain reaction (PCR)-based method for the detection of food-borne pathogens — Horizontal method for the detection of Shiga toxin-producing *Escherichia coli* (STEC) and the determination of O157, O111, O26, O103 and O145 serogroups

Microbiologie des aliments — Méthode basée sur la réaction de polymérisation en chaîne (PCR) en temps réel pour la détection des micro-organismes pathogènes dans les aliments — Méthode horizontale pour la détection des Escherichia coli producteurs de Shigatoxines (STEC) et la détermination des sérogroupes O157, O111, O26, O103 et O145

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Foreword

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Introduction

Shiga toxin-producing *Escherichia coli* (STEC) are pathogenic *E. coli*, which can cause diarrhoea as well as more severe diseases in humans such as haemorrhagic colitis and haemolytic uremic syndrome (HUS). Although STEC may belong to a large number of serogroups, those that have been firmly associated with the most severe forms of the disease, in particular HUS, belong to O157, O26, O111, O103, and O145 (Reference [1]).

The following nomenclature has been adopted in this Technical Specification:

— *stx*: Shiga toxin genes (synonymous with *vtx)*;

— Stx: Shiga toxin (synonymous with Vtx: Verocytotoxin);

— STEC: Shiga toxin-producing *Escherichia coli (*synonymous with VTEC: Verocytotoxin-producing *Escherichia coli).*

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Microbiology of food and animal feed — Real-time polymerase chain reaction (PCR)-based method for the detection of food-borne pathogens — Horizontal method for the detection of Shiga toxin-producing *Escherichia coli* (STEC) and the determination of O157, O111, O26, O103 and O145 serogroups

IMPORTANT — It is necessary to consider any STEC as pathogenic to humans and potentially to cause severe disease depending on both the risk profile of the food commodity (ready-to-eat foods vs. foods intended to be consumed after technological treatment such as pasteurization, cooking etc. used to reduce any bacteria present in the food) and the health status of the subject ingesting the food.

Moreover, given the high genomic plasticity of this bacterial species, it is possible that novel arrangements of virulence features can give rise to novel sero-pathogroups such as the Shiga toxin-producing enteroaggregative *E. coli* O104 that caused the HUS outbreaks in Germany and France in 2011-05/06. Novel atypical *E. coli* sero-pathogroups can arise from the acquisition of an *stx*-converting bacteriophage by an *E. coli* strain belonging to pathogroups different from STEC.

Such atypical strains fall in the scope of this method and can be efficiently detected as they are positive for the presence of the *stx* genes.

1 Scope

This Technical Specification describes the identification of Shiga toxin-producing *Escherichia coli* (STEC) by means of the detection of the following genes:

a) the major virulence genes of STEC, *stx* and *eae* (References [2][3]);

b) the genes associated with the serogroups O157, O111, O26, O103, and O145 (References [3][4]).

In any case, when one or both of the *stx* genes is/are detected, the isolation of the strain is attempted.

The isolation of STEC from samples positive for the presence of the genes specifying the serogroups in the scope of this method can be facilitated by using serogroup-specific enrichment techniques (e.g. immunomagnetic separation, IMS).

The protocol uses real-time PCR as the reference technology for detection of the virulence and serogroup-associated genes.

This Technical Specification is applicable to:

1) products intended for human consumption and the feeding of animals;

2) environmental samples in the area of food production and food handling;

3) environmental samples in the area of primary production.

2 Normative references

The following referenced documents are indispensable for the application of this document. For dated references, only the edition cited applies. For undated references, the latest edition of the referenced document (including any amendments) applies.

ISO 7218, *Microbiology of food and animal feeding stuffs — General requirements and guidance for microbiological examinations*

ISO 20838, *Microbiology of food and animal feeding stuffs — Polymerase chain reaction (PCR) for the detection of food-borne pathogens — Requirements for amplification and detection for qualitative methods*

ISO 22174, *Microbiology of food and animal feeding stuffs — Polymerase chain reaction (PCR) for the detection of food-borne pathogens — General requirements and definitions*

3 Terms and definitions

For the purposes of this document, the following terms and definitions apply.

Definitions 3.1 to 3.3 have been compiled from the epidemiological data on disease caused by STEC managed by organizations such as the US Centers for Disease Control and, in the EU, by the European Centre for Disease Prevention and Control and the European Food Safety Authority.

3.1
Shiga toxin-producing *Escherichia coli*
STEC
E. coli strains possessing the Stx-coding genes

3.2
Shiga toxin-producing *Escherichia coli* causing the attaching and effacing lesion
STEC causing the attaching and effacing lesion
E. coli strains possessing the Stx-coding genes and the intimin-coding gene *eae*

NOTE This combination of virulence genes is often associated with the most severe forms of STEC-induced disease.

3.3
Shiga toxin-producing *Escherichia coli* belonging to highly pathogenic serogroups
STEC belonging to highly pathogenic serogroups
E. coli strains possessing the Stx-coding genes, the intimin-coding gene *eae* and belonging to one of the serogroups O157, O111, O26, O103, and O145

4 Principle

4.1 General

The method specified comprises the following sequential steps:

a) microbial enrichment;

b) nucleic acid extraction;

c) detection of virulence genes;

d) detection of serogroup-associated genes;

e) isolation from positive samples.

Figure A.1 is a flow diagram of the screening procedure.

4.2 Microbial enrichment

The number of STEC cells to be detected is increased by incubating the test portion in a non-selective liquid nutrient medium chosen from:

a) modified tryptone-soy broth (tryptone soy broth supplemented with 1,5 g/l bile salts No.3, mTSB) supplemented with 16 mg/l of novobiocin (mTSB+N);

b) buffered peptone water (BPW);

c) modified tryptone-soy broth (tryptone-soy broth supplemented with 1,5 g/l bile salts No.3, mTSB) supplemented with 12 mg/l of acriflavin (mTSB+A) for analysis of milk and dairy products.

The mTSB shall be used when analysing matrices suspected to contain high levels of contaminating microflora. Novobiocin and acriflavin inhibit the growth of Gram-positive bacteria and promote the growth of Gram-negative cells, including STEC. The BPW shall be used to analyse samples which are assumed to contain stressed target bacteria (such as frozen products), to resuscitate stressed STEC cells, and expected lower levels of contaminating microflora than in fresh samples.

NOTE The addition of novobiocin is controversial and has been investigated by several authors. It has been observed that the minimum inhibitory concentration of the antibiotic for non-O157 STEC is lower than for O157 strains (Reference [5]). The addition of novobiocin in the enrichment mTSB at the usual concentration of 20 mg/l, as specified in ISO 16654,[19] seems to inhibit the growth of about one-third of non-O157 strains (Reference [6]) increasing the risk of false-negative results.

4.3 Nucleic acid extraction

The nucleic acid is extracted according to the requirements of the adopted detection system.

4.4 Target genes

The purified nucleic acid is used for the detection of the following target genes:

— the main virulence genes for STEC: *stx* genes, encoding the Shiga toxins and the *eae* gene, encoding a 90 kDa protein, the intimin, involved in the attaching and effacing mechanism of adhesion, a typical feature of the STEC strains causing severe disease. The *stx* genes encode a family of toxins including two main types: *stx*1 and *stx*2. The latter comprises seven recognized variants (from *stx*2*a* to *stx*2*g*) (Reference [22]). Only the variants *stx*2*a*, *stx*2*b*, and *stx*2c have been found to be produced by the STEC strains included in Clause 1, and therefore constitute the target Stx-coding genes of this Technical Specification. The GenBank accession numbers corresponding to the *stx*2 variants-coding genes are:

  — *stx*2a: X07865

  — *stx*2*b*: L11078

  — *stx*2*c*: M59432

— the intimin-coding *eae* gene

— the *rfbE(O157), wbdI(O111), wzx(O26), ihp1(O145)* and *wzx(O103)* genes, to identify the corresponding serogroups.

4.5 Detection

The detection of the target genes is performed according to the adopted detection system.

4.6 Isolation

If the presence of a STEC is suspected, the isolation is attempted. If one of the serogroups specified in the scope of this Technical Specification is detected, a serogroup-specific enrichment (e.g. IMS) can be performed followed by plating on to tryptone–bile–glucuronic agar (TBX) or a specific selective medium if available (see Annex F, Notes 2 and 3) in order to facilitate the isolation of the STEC from the background flora.

5 Diluents, culture media and reagents

During the analysis, unless otherwise stated, use only reagents of recognized analytical grade and sterile distilled or demineralized water or water of equivalent purity.

5.1 Culture media

5.1.1 Modified tryptone- soy broth (mTSB)

5.1.1.1 Basic medium

Composition and pH

Enzymatic digest of casein	17 g
Enzymatic digest of soy	3 g
D(+)-Glucose	2,5 g
Sodium chloride	5 g
Dipotassium hydrogenphosphate (K_2HPO_4)	4 g
Bile salts No. 3	1,5 g
Water	to 1 000 ml
pH 7,4 ± 0,2	

Preparation

Dissolve the components or the dehydrated medium in water. Adjust pH with a pH-meter to pH 7,4 ± 0,2 at 25 °C and sterilize by autoclaving at 121 °C for 15 min.

5.1.1.2 Novobiocin solution

Composition

Novobiocin	0,16 g
Water	10 ml

Preparation

Dissolve the novobiocin in the water and sterilize by membrane filtration using 0,22 µm or 0,45 µm filters.

Prepare on the day of use.

5.1.1.3 Acriflavin solution

Composition

Acriflavin	0,12 g
Water	10 ml

Preparation

Dissolve the acriflavin in the water and sterilize by membrane filtration using 0,22 µm or 0,45 µm filters.

Prepare on the day of use.

5.1.1.4 Preparation of the complete medium

Immediately before use, add 1 ml of novobiocin (5.1.1.2) or acriflavin solution (5.1.1.3) to 1 000 ml of cooled mTSB (5.1.1.1).

The final concentration of novobiocin shall be 16 mg/l of mTSB.

The final concentration of acriflavin shall be 12 mg/l of mTSB.

5.1.2 Buffered peptone water (BPW)

Composition and pH

Peptone	10 g
Sodium chloride	5,0 g
Disodium phosphate (Na_2HPO_4)	3,5 g
Potassium dihydrogenphosphate (KH_2PO_4)	1,5 g
Water	to 1 000 ml
pH 7,0 ± 0,2	

Preparation

Dissolve the components or the dehydrated powder in the water. Adjust pH with a pH-meter to pH 7,0 ± 0,2 at 25 °C and sterilize by autoclaving at 121 °C for 15 min.

5.2 Reagents for nucleic acid extraction

The reagents to be used for nucleic acid extraction are not listed, being dependent on the method adopted (9.3).

5.3 Reagents for PCR

See ISO 20838.

5.3.1 Oligonucleotides (primers) and detection probes

Primers and probes for specific detection of the target gene sequences by standard and real-time PCR are listed in Annexes C and E.

6 Equipment

Usual microbiological laboratory equipment (see ISO 7218) and, in particular, the following.

6.1 **Water bath** or **heating block** capable of being maintained at temperatures up to 100 °C.

6.2 **Incubator** according to ISO 7218, capable of being maintained at 37 °C± 1 °C.

6.3 **Nucleic acid extraction apparatus**.

Appropriate equipment according to the method adopted (if needed).

6.4 **Pipettes** of capacities between 1 µl and 100 µl, ISO 7550.[16]

6.5 Thin walled real-time PCR microtubes (0,2 ml/0,5 ml reaction tubes), multi-well PCR microplates or other suitable light transparent disposable plasticware.

6.6 Thermal cycler. Several brands of apparatus are available and can be chosen according to the laboratory policies.

6.7 PCR product detection apparatus.

Light emission following 5' nuclease PCR assay is detected by the real-time PCR apparatus.

6.8 Peristaltic blender with sterile bags, possibly with a device for adjusting speed and time.

7 Sampling

Sampling is not part of the method specified in this Technical Specification. See the specific International Standard dealing with the product concerned or specific regulations. If there is no specific International Standard dealing with sampling of the product concerned, it is recommended that the parties concerned come to an agreement on this subject.

It is important that the laboratory receive a truly representative sample which has not been damaged or changed during transport or storage.

8 Preparation of test sample

Prepare the test sample in accordance with the specific International Standard dealing with the product concerned. If there is no specific International Standard, it is recommended that the parties concerned come to an agreement on this subject.

9 Procedure

9.1 Test portion and initial suspension

9.1.1 General

Use the quantity of enrichment medium necessary to give a final dilution of 10^{-1} of the original test portion.

9.1.2 For matrix samples assumed to contain a high level of background flora

For solid matrices, aseptically transfer a test portion (x g) of sample to a peristaltic blender bag containing $9x$ ml of mTSB to which novobiocin or acriflavin has been added (5.1.1.4). Bags with filters are preferred.

Homogenize in a peristaltic blender (see ISO 7218) (6.8).

For liquid matrices, transfer the test portion (x ml) of liquid sample, using a sterile pipette, into the tube or bottle containing $9x$ ml of the enrichment mTSB to which novobiocin or acriflavin has been added (5.1.1.4).

9.1.3 For matrix sample assumed to contain stressed target bacteria

Allow frozen products to thaw at room temperature, and then transfer the test portion (x g or x ml) to a peristaltic blender bag or tube containing $9x$ ml of BPW (5.1.2) and proceed as above.