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Microbiology of food and animal feeding stuffs — Horizontal method for the detection of *Escherichia coli* O157

Microbiologie des aliments — Méthode horizontale pour la recherche des Escherichia coli *O157*

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ISO copyright office
Case postale 56 • CH-1211 Geneva 20
Tel. + 41 22 749 01 11
Fax + 41 22 749 09 47
E-mail copyright@iso.ch
Web www.iso.ch

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Contents

Page

Foreword

ISO (the International Organization for Standardization) is a worldwide federation of national standards bodies (ISO member bodies). The work of preparing International Standards is normally carried out through ISO technical committees. Each member body interested in a subject for which a technical committee has been established has the right to be represented on that committee. International organizations, governmental and non-governmental, in liaison with ISO, also take part in the work. ISO collaborates closely with the International Electrotechnical Commission (IEC) on all matters of electrotechnical standardization.

International Standards are drafted in accordance with the rules given in the ISO/IEC Directives, Part 3.

Draft International Standards adopted by the technical committees are circulated to the member bodies for voting. Publication as an International Standard requires approval by at least 75 % of the member bodies casting a vote.

Attention is drawn to the possibility that some of the elements of this International Standard may be the subject of patent rights. ISO shall not be held responsible for identifying any or all such patent rights.

International Standard ISO 16654 was prepared by Technical Committee ISO/TC 34, *Food products*, Subcommittee SC 9, *Microbiology*.

Annex A forms a normative part of this International Standard.

Introduction

Because of the large variety of food and feed products, this horizontal method may not be appropriate in every detail for certain products. In this case, different methods specific to these products may be used if absolutely necessary for justified technical reasons. Nevertheless, every attempt should be made to apply this horizontal method as far as possible.

When this International Standard is next reviewed, account will be taken of all information then available regarding the extent to which this horizontal method has been followed and the reasons for deviations from this method in the case of particular products.

The harmonization of test methods cannot be immediate, and for certain groups of products International Standards and/or national standards may already exist that do not comply with this horizontal method. It is hoped that when such standards are reviewed they will be changed to comply with this International Standard so that eventually the only remaining departures from this horizontal method will be those necessary for well-established technical reasons.

Microbiology of food and animal feeding stuffs — Horizontal method for the detection of *Escherichia coli* O157

WARNING — *Escherichia coli* O157 can cause severe life-threatening illness and has a low infective dose. Laboratory-acquired infections have been reported.

In order to safeguard the health of laboratory personnel, it is essential that the whole of this method be carried out only by skilled personnel using good laboratory practices and preferably working in a containment facility. Relevant national Health and Safety Regulations relating to this organism must be adhered to.

Care must be taken in the disposal of all infectious materials.

1 Scope

This International Standard specifies a horizontal method for the detection of *Escherichia coli* serogroup O157.

Subject to the limitations discussed in the introduction, this International Standard is applicable to products intended for human consumption or for animal feeding stuffs.

2 Normative references

The following normative documents contain provisions which, through reference in this text, constitute provisions of this International Standard. For dated references, subsequent amendments to, or revisions of, any of these publications do not apply. However, parties to agreements based on this International Standard are encouraged to investigate the possibility of applying the most recent editions of the normative documents indicated below. For undated references, the latest edition of the normative document referred to applies. Members of ISO and IEC maintain registers of currently valid International Standards.

ISO 6887-1, *Microbiology of food and animal feeding stuffs — Preparation of test samples, initial suspension and decimal dilutions for microbiological examination — Part 1: General rules for the preparation of the initial suspension and decimal dilutions*.

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

3 Term and definition

For the purposes of this International Standard, the following term and definition applies.

3.1
***Escherichia coli* O157**
***E. coli* O157**
microorganisms which form typical colonies on the surface of the plating-out medium used in this International Standard, and which produce indole and agglutinate specifically with antiserum against the O157 antigen

NOTE 1 Sorbitol-positive *E. coli* O157 strains are not detected on CT-SMAC (5.2) media.

NOTE 2 Some indole-negative mutations have been found.

4 Principle

The detection of *Escherichia coli* O157 necessitates four successive stages (see annex A).

a) **Enrichment** of the test portion homogenized in modified tryptone soya broth containing novobiocin (mTSB + N) with incubation at 41,5 °C ± 1 °C for 6 h and subsequently for a further 12 h to 18h.

b) **Separation and concentration** of microorganisms by means of immunomagnetic particles coated with antibodies to *E. coli* O157.

c) **Isolation** by subculture of the immunomagnetic particles with adhering bacteria onto cefixime tellurite sorbitol MacConkey agar (CT-SMAC) and the user's choice of a second selective isolation agar.

d) **Confirmation** of sorbitol-negative colonies from CT-SMAC and colonies typical of *E. coli* O157 on the second isolation agar, by indole production and agglutination with *E. coli* O157 antiserum.

NOTE Further characterization, by for example pathogenic markers, of the positive isolates can be obtained by forwarding them to an appropriate reference laboratory.

5 Culture media, reagents and antisera

For current laboratory practices, see ISO 7218.

5.1 Enrichment medium: Modified tryptone soya broth with novobiocin (mTSB + N)

See reference [1].

5.1.1 Modified tryptone soya broth (mTSB)

5.1.1.1 Composition

Enzymatic digest of casein	17,0 g
Enzymatic digest of soya	3,0 g
D(+)-glucose	2,5 g
Bile salts No. 3	1,5 g
Sodium chloride	5,0 g
Dipotassium hydrogen phosphate (K_2HPO_4)	4,0 g
Water	1000 ml

5.1.1.2 Preparation

Dissolve the components or the dehydrated complete medium in the water, by heating if necessary. Adjust the pH, using the pH-meter (6.6), if necessary, so that after sterilization it is 7,4 ± 0,2 at 25 °C.

Dispense the medium in appropriate amounts in flasks or bottles (6.7).

Sterilize for 15 min in the autoclave (6.1) set at 121 °C.

5.1.2 Novobiocin solution

5.1.2.1 Composition

Novobiocin	0,45 g
Water	100 ml

5.1.2.2 Preparation

Dissolve the novobiocin in the water and sterilize by membrane filtration.

Prepare on the day of use.

5.1.2.3 Preparation of the complete medium

Immediately before use, add 1 ml or 4 ml of novobiocin solution (5.1.2) to either 225 ml or 900 ml of cooled mTSB (5.1.1).

The final concentration of novobiocin is 20 mg per litre of mTSB.

5.2 First selective isolation medium: Cefixime tellurite sorbitol MacConkey agar (CT-SMAC)

See reference [2].

5.2.1 Base medium

5.2.1.1 Composition

Enzymatic digest of casein	17,0 g
Enzymatic digest of animal tissues	3,0 g
Sorbitol	10,0 g
Bile salts No. 3	1,5 g
Sodium chloride	5,0 g
Neutral Red	0,03 g
Crystal Violet	0,001 g
Agar	9 g to 18 g [a]
Water	1000 ml

[a] Depending on the gel strength of the agar.

5.2.1.2 Preparation

Dissolve the basic components or the complete dehydrated base in the water by boiling. Adjust the pH (6.6), if necessary, so that after sterilization it is 7,1 ± 0,2 at 25 °C.

Sterilize for 15 min in the autoclave (6.1) set at 121 °C.

5.2.2 Potassium tellurite solution

5.2.2.1 Composition

Potassium tellurite for bacteriological use	0,25 g
Water	100 ml

5.2.2.2 Preparation

Dissolve the potassium tellurite in the water and sterilize by membrane filtration.

This solution may be stored at room temperature for up to 1 month, but discard it if a white precipitate forms.

5.2.3 Cefixime solution

5.2.3.1 Composition

Cefixime	5,0 mg
Water	100,0 ml

5.2.3.2 Preparation

Dissolve the cefixime in the water and sterilize by membrane filtration.

NOTE Cefixime may need to be dissolved in ethanol.

This solution may be stored at 3 °C ± 2 °C for 1 week.

5.2.4 Complete medium

5.2.4.1 Composition

Base medium (5.2.1)	1 000 ml
Potassium tellurite solution (5.2.2)	1,0 ml
Cefixime solution (5.2.3)	1,0 ml

5.2.4.2 Preparation

Either cool the freshly sterilized base medium (5.2.1) to between 44 °C and 47 °C (6.5), or melt it by steaming the previously sterilized and solidified base medium, then cool to between 44 °C and 47 °C.

Add 1 ml of the tellurite solution and 1 ml of the cefixime solution to 1000 ml of the base medium. Mix and pour about 15 ml amounts into sterile Petri dishes (6.15). Allow to solidify.

The final concentration of tellurite is 2,5 mg/l and cefixime 0,05 mg/l.

Immediately before use, dry the agar plates, preferably with the lids removed and with the agar surfaces facing downwards, in an oven set at a temperature between 25 °C and 50 °C (6.2), until the droplets have disappeared from the surface of the medium. Do not dry them any further. The agar plates may also be dried in a laminar-flow safety cabinet for 30 min with half-open lids, or overnight with the lids in place.

If prepared in advance, the undried plates may be stored in the dark in plastic bags or other moisture-retentive containers, in a refrigerator at 3 °C ± 2 °C for up to 2 weeks.

5.3 Second selective isolation medium

Use any other solid selective medium, at the choice of the laboratory, complementary to CT-SMAC agar and especially appropriate for the isolation of *Escherichia coli* O157.

Immediately before use, dry the agar plates, preferably with the lids removed and with the agar surfaces facing downwards, in an oven set at a temperature between 25 °C and 50 °C (6.2), until the droplets have disappeared

from the surface of the medium. Do not dry them any further. The agar plates may also be dried in a laminar-flow safety cabinet for 30 min with half-open lids, or overnight with the lids in place.

If prepared in advance, the undried plates may be stored in the dark in plastic bags or other moisture-retentive containers, in a refrigerator at 3 °C ± 2 °C for a time that causes no change to its performance.

5.4 Nutrient agar

5.4.1 Composition

Meat extract	3,0 g
Peptone	5,0 g
Agar	9 g to 18 g [a]
Water	1 000 ml

[a] Depending on the gel strength of the agar.

5.4.2 Preparation

Dissolve the components or the dehydrated complete medium in the water, by heating if necessary. Adjust the pH, if necessary, so that after sterilization it is 7,0 ± 0,2 at 25 °C.

Transfer the medium into flasks or bottles (6.7) of appropriate capacity.

Sterilize for 15 min in the autoclave (6.1) set at 121 °C.

5.4.3 Preparation of nutrient agar plates

Transfer about 15 ml of the molten, cooled medium (5.4.2) at between 44 °C and 47 °C (6.5) to Petri dishes and allow to solidify.

Immediately before use, dry the agar plates, preferably with the lids removed and with the agar surfaces facing downwards, in an oven set at a temperature between 25 °C and 50 °C (6.2), until the droplets have disappeared from the surface of the medium. Do not dry them any further. The agar plates may also be dried in a laminar-flow safety cabinet for 30 min with half-open lids, or overnight with the lids in place.

If prepared in advance, the undried plates may be stored in the dark, in plastic bags or other moisture-retentive containers, in a refrigerator at 3 °C ± 2 °C for up to 2 weeks.

5.5 Tryptone/tryptophan medium

5.5.1 Composition

Tryptone	10,0 g
Sodium chloride	5,0 g
DL-Tryptophan	1,0 g
Water	1 000 ml

5.5.2 Preparation

Dissolve the components in the water by boiling if necessary. Adjust the pH (6.6) so that after sterilization it is 7,5 ± 0,2 at 25 °C.

Dispense in 5 ml amounts into test tubes or bottles (6.7) of appropriate capacity.

Sterilize for 15 min in the autoclave (6.1) set at 121 °C.

5.6 Kovac's indole reagent

5.6.1 Composition

4-Dimethylaminobenzaldehyde	5,0 g
2-Methylbutan-1-ol or pentan-1-ol	75,0 ml
Hydrochloric acid (ρ_{20} 1,18 g/ml to 1,19 g/ml)	25,0 ml

5.6.2 Preparation

Dissolve the 4-dimethylaminobenzaldehyde in the alcohol, by warming if necessary in a water bath (6.5) maintained at between 44 °C and 47 °C. Cool to room temperature and add the hydrochloric acid.

Protect from light in a brown glass bottle and store at 3 °C ± 2 °C.

The reagent shall be light yellow to light brown and free of precipitate.

5.7 Anti-*Escherichia coli* O157 immunomagnetic particles

These are immunomagnetic particles coated with specific antibodies against *E. coli* O157 for concentration and separation of these microorganisms.

NOTE They are available from commercial sources. The manufacturer's instructions should be followed precisely regarding their preparation for use.

5.8 Wash buffer: Modified phosphate buffer, 0,01mol/l, of pH 7,2

5.8.1 Composition

Sodium chloride	8,0 g
Potassium chloride	0,2 g
Disodium hydrogen phosphate (anhydrous)	1,44 g
Potassium dihydrogen phosphate (anhydrous)	0,24 g
Polyoxyethylene sorbitan monolaurate (Tween 20 syrup)	0,2 ml
Water	1 000 ml

5.8.2 Preparation

Dissolve the components in water. Adjust the pH (6.6), if necessary, to 7,2 ± 0,2 at 25 °C.

Dispense in bottles or flasks (6.7) in appropriate volumes for use

Sterilize for 15 min in the autoclave (6.1) set at 121 °C. The solution may appear cloudy but becomes clear on standing.

Commercially available phosphate buffer with the same composition and the same performance may be used.

5.9 Normal saline solution

5.9.1 Composition

Sodium chloride	8,5 g
Water	1 000 ml

5.9.2 Preparation

Dissolve the sodium chloride in the water. Dispense in bottles or flasks (6.7) in appropriate volumes for use.

Sterilize for 15 min in the autoclave (6.1) set at 121 °C.

5.10 ***Escherichia coli* O157 antiserum,** available either from specialist laboratories or from commercial sources as separate somatic "O" 157.

The antiserum shall be tested with positive and negative controls prior to use on unknown isolates.

6 Apparatus and glassware

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

6.1 Apparatus for dry sterilization (oven) and/or wet sterilization (autoclave)

See ISO 7218.

6.2 **Drying cabinet** or **incubator,** capable of being maintained between 25 °C and 50 °C.

6.3 **Incubator**, capable of being maintained at 37 °C ± 1 °C.

6.4 **Incubator**, capable of being maintained at 41,5 °C ± 1 °C.

6.5 **Water bath,** capable of being maintained at between 44 °C and 47 °C.

6.6 **pH-meter**, capable of being read to the nearest 0,01 pH unit at 25 °C, enabling measurements to be made which are accurate to ± 0,1 pH unit.

6.7 **Test tubes, flasks** or **bottles,** of appropriate capacity, for sterilization and storage of culture media and incubation of liquid media.

6.8 **Measuring cylinders,** of appropriate capacity, for preparation of dilutions and complete media.

6.9 **Total-delivery graduated pipettes**, of nominal capacities 1 ml and 10 ml, graduated in 0,1 ml and 0,5 ml divisions, respectively.

6.10 **Loops and wires,** made of platinum/iridium or nickel/chrome or **Pasteur pipettes** or **single-use loops**.

6.11 **Mechanical air-displaced pipettors,** sterile, with an operating range from 20 µl to 200 µl with 10 µl divisions, or similar.

6.12 **Magnetic separator** with **magnetic rack,** for concentration of immunomagnetic particles, for use with Eppendorf-type plastic tubes (6.13).

6.13 **Eppendorf-type plastic tubes**, with screw caps, sterile, disposable, centrifuge type, of 1,5 ml capacity to fit the magnetic rack.

Avoid the creation of aerosols when opening.

6.14 Rotary mixer (windmill type, blood sample mixer), capable of rotating at 15 r/min to 20 r/min.

6.15 Petri dishes, of diameter 90 mm and 140 mm.

6.16 Vortex mixer

7 Sampling

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

It is recommended to cool the sample quickly before storage.

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

8 Preparation of test sample

Prepare the test sample in accordance with the specific International Standard appropriate to 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 (see annex A)

9.1 Test portion and initial suspension

See ISO 6887-1 and any specific International Standard appropriate to the product concerned.

NOTE Further parts of ISO 6887 are in preparation, see bibliography.

In general, to prepare the initial suspension, add a test portion of x g or x ml to 9x ml or 9x g of modified tryptone soya broth with novobiocin (mTSB + N) (5.1), pre-warmed in the incubator (6.4) to 41,5 °C to obtain a ratio of test portion to mTSB + N of 1/10 (mass to volume, or volume to volume).

It is recommended to use stomacher bags with mesh inserts to reduce the interference of food particles with immunocapture kits (9.3).

9.2 Enrichment

Incubate (6.4) the initial suspension, prepared in accordance with 9.1, at 41,5 °C for 6 h, and subsequently for a further 12 h to 18 h (i.e. to a total elapsed time of 18 h to 24 h).

A 6-h incubation followed by immunomagnetic separation and plating onto selective agars can yield a presumptive positive result which can become negative after a further 18-h incubation.

9.3 Immunomagnetic separation (IMS)

9.3.1 General

IMS should be carried out after 6 h and again, if necessary, after 12 h to 18 h of incubation.

The instructions below are for general guidance and may not be complete in all details. Therefore the manufacturer's instructions should be followed concerning the procedure and method for the use of immunocapture kits and the equipment needed.

9.3.2 Immunocapture

WARNING — Use aseptic techniques to avoid any external contamination and the creation of aerosols. Perform this protocol in a containment safety cabinet, if available. Wear gloves.

Using the magnetic separator (6.12) and antibody-coated immunomagnetic particles (5.7), carry out the following capture/separation procedure.

Mix the enrichment culture (9.2) and allow any coarse food materials to settle out. To an Eppendorf-type plastic tube (6.13), add 20 µl of the prepared immunomagnetic particles (5.7) at room temperature. Take 1 ml of the upper liquid from the enrichment culture, avoiding if possible the transfer of any food particles or fatty materials, and transfer to the Eppendorf-type plastic tube.

Mix the suspension on the rotary mixer (6.14) set at about 12 r/min to 20 r/min for 10 min.

9.3.3 Separation

Place each Eppendorf-type plastic tube (see 9.3.2) in the magnetic rack (6.12) and allow the magnetic particles to congregate against the magnet by gently rocking the rack through 180°. Open the cap carefully without disturbing the particles on the wall of the tube. Using a new sterile Pasteur pipette (6.10) for each sample and with the tube still in the magnetic rack, remove the liquid by sucking slowly from the bottom of the tube. Add 1 ml of sterile wash buffer (5.8) and replace the cap. Remove the magnet from the rack. Mix the contents of the tubes by gentle inversion of the rack through 180° and then return the magnet to the rack.

Take care to avoid cross contamination when adding fresh buffer.

Proceed as above to remove the wash liquid with a new Pasteur pipette for each sample. Repeat the washing procedure several times.

Remove from the magnetic separator and add 100 µl of sterile wash buffer (5.8) to the tube and re-suspend the magnetic particles.

NOTE This procedure could be difficult to apply to fat products or fresh cheese.

9.4 Plating out onto selective agars and identification of *E. coli* O157 colonies

9.4.1 Plating out

Using a mechanical-type pipettor (6.11), transfer 50 µl of the washed and re-suspended magnetic particles (9.3.3) to a pre-dried plate of cefixime tellurite sorbitol MacConkey agar (5.2) and also 50 µl to a pre-dried plate of the second isolation medium (5.3).

Streak out the particles using a sterile loop (6.10) to obtain many well-isolated colonies over the agar.

Incubate (6.3) the CT-SMAC (5.2) at 37 °C for 18 h to 24 h, and incubate the second selective agar at its recommended temperature and specified time.

Depending on the type of food sample and its microbial flora, incubation of the enrichment broth for 20 h to 24 h may give rise to a heavy growth of other bacteria on the selective agar plates so that colonies of *E. coli* O157 are difficult to find. Inoculation of selective agars with dilutions of the IMS preparation or volumes less than 50 µl per plate can increase the chance of gaining separated colonies of *E. coli* O157 but note that this can increase the detection limit as well.

9.4.2 Recognition of typical *E. coli* O157 colonies

On CT-SMAC agar, typical colonies are transparent and almost colourless with a pale yellowish-brown appearance and a diameter of approximately 1 mm.

Examine the second selective isolation agar for typical colonies of *E. coli* O157 following the manufacturer's instructions.

9.5 Confirmation

NOTE Commercially available miniaturized biochemical identification kits that permit the identification of sorbitol-negative and indole-positive *E. coli* and latex agglutination kits for *E. coli* O157 may be used, provided appropriate tests with known positive and negative strains are carried out to confirm performance.

9.5.1 Selection of colonies

Take five typical colonies from each plate, as selected in 9.4. If an agar plate contains less than 5 typical colonies, all the colonies shall be examined.

Streak each selected colony onto a plate of nutrient agar (5.4) to allow well-separated colonies to develop.

Incubate (6.3) the plates for 18 h to 24 h at 37 °C.

Use only pure cultures from the nutrient agar plate for the tests described in 9.5.2 and 9.5.3.

9.5.2 Biochemical confirmation: Indole formation

Inoculate one colony from the pure culture on nutrient agar (9.5.1) into a tube of tryptone/tryptophan medium (5.5).

Incubate (6.3) at 37 °C for 24 h.

Add 1 ml of Kovac's reagent (5.6) and allow to stand at room temperature for 10 min.

The formation of a red colour indicates a positive reaction. A yellow/brown colour indicates a negative reaction.

9.5.3 Serological identification

9.5.3.1 General

Only examine indole-positive colonies for their serological reaction with antiserum to *E. coli* O157.

9.5.3.2 Elimination of auto-agglutinating isolates

Place a drop of saline solution (5.9) onto a cleaned glass slide.

Using a loop (6.10), mix into this drop one colony from the nutrient agar plate (9.5.1) so as to obtain a homogeneous and turbid suspension.

Rock the slide gently for 30 s to 60 s. Observe the result against a dark background and, if necessary, with the aid of a magnifying lens.

If the suspension has formed visible clumps, the strain is considered to auto-agglutinate and shall not be tested further, as the reaction with the specific antiserum is not possible.