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Microbiology of the food chain — Horizontal method for the detection of pathogenic *Yersinia enterocolitica*

Microbiologie de la chaîne alimentaire — Méthode horizontale pour la recherche de Yersinia enterocolitica *pathogènes*

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Foreword

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The procedures used to develop this document and those intended for its further maintenance are described in the ISO/IEC Directives, Part 1. In particular the different approval criteria needed for the different types of ISO documents should be noted. This document was drafted in accordance with the editorial rules of the ISO/IEC Directives, Part 2 (see www.iso.org/directives).

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Any trade name used in this document is information given for the convenience of users and does not constitute an endorsement.

For an explanation on the voluntary nature of standards, the meaning of ISO specific terms and expressions related to conformity assessment, as well as information about ISO's adherence to the World Trade Organization (WTO) principles in the Technical Barriers to Trade (TBT) see the following URL: www.iso.org/iso/foreword.html.

This document was prepared by the European Committee for Standardization (CEN) Technical Committee CEN/TC 275, *Food analysis — Horizontal methods*, in collaboration with ISO Technical Committee ISO/TC 34, *Food products*, Subcommittee SC 9, *Microbiology*, in accordance with the agreement on technical cooperation between ISO and CEN (Vienna Agreement).

This third edition cancels and replaces the second edition (ISO 10273:2003), which has been technically revised with the following main changes.

— In confirmation of pathogenic *Y. enterocolitica*, tests related to pathogenicity have been added or specified and relocated in the frontline. Accordingly, the word "presumptive" has been removed from the title wording (pathogenic *Y. enterocolitica*) since standard contains mandatory tests related to pathogenicity and allows separation of pathogenic and non-pathogenic *Y. enterocolitica*.

— Direct plating on cefsulodin, Irgasan™[1)] and novobiocin (CIN) agar has been added.

— Incubation time for peptone, sorbitol and bile salts (PSB) enrichment broth and CIN agar has been changed.

— Inoculation and incubation time for Irgasan™, ticarcillin and potassium chlorate (ITC) enrichment broth has been changed and specified.

— Salmonella/shigella agar with sodium desoxycholate and calcium chloride (SSDC) has been replaced by CIN agar and optional chromogenic medium.

— Inoculation of CIN agar without prior potassium hydroxide (KOH) treatment of enrichment broth has been changed to optional procedure (in parallel to mandatory KOH treatment).

— The preparation (shelf life) of KOH and ammonium iron(II) sulfate solutions has been specified.

1) Irgasan™ is an example of a suitable product available commercially. This information is given for the convenience of users of this document and does not constitute an endorsement by ISO of this product.

— Suspect colonies from primary culture are streaked (purified) on CIN agar and (optionally) on chromogenic agar to facilitate better selection of characteristic colonies that need further confirmation. The use of stereomicroscope in identification of characteristic colonies is emphasized.

— All biochemical confirmation tests, except for pyrazinamidase test, can be replaced by real-time polymerase chain reaction (PCR) detection of *ail*-gene in accordance with ISO/TS 18867.

— Five confirmation tests (indole, trehalose, xylose, citrate, tween-esterase) have become optional. Test for salicin has been added as an optional (biotyping) test. Test for calcium requirements at 37°C has been replaced by congo red magnesium-oxalate (CR-MOX) test. Three tests (oxidase, Kligler's agar and ornithine decarboxylase) have been deleted.

— The procedure for cold-enrichment of *Y. enterocolitica* has been added as Annex D;

— Performance characteristics have been added to Annex C.

— Performance testing for the quality assurance of the culture media has been added to Annex B and Annex D.

Introduction

This document specifies a horizontal method for the detection of *Yersinia enterocolitica* associated with human disease. Because of the large variety of food and feed products, this horizontal method may not be appropriate in every detail for certain products, and for some other products it may be necessary to use different methods. Nevertheless, it is hoped that in all cases every attempt will be made to apply this horizontal method as far as possible and that deviations from this will only be made if absolutely necessary for technical reasons.

The main changes, listed in the foreword, introduced in this document compared to ISO 10273:2003, are considered as major (see ISO 17468).

When this document 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 in the case of particular products.

The harmonization of test methods cannot be immediate and, for certain group 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 document so that eventually the only remaining departures from this horizontal method will be those necessary for well-established technical reasons.

Microbiology of the food chain — Horizontal method for the detection of pathogenic *Yersinia enterocolitica*

WARNING — In order to safeguard the health of laboratory personnel, it is essential that tests for detecting pathogenic *Yersinia enterocolitica* are only undertaken in properly equipped laboratories, under the control of a skilled microbiologist, and that great care is taken in the disposal of all incubated materials. Persons using this document should be familiar with normal laboratory practice. This document does not purport to address all of the safety aspects, if any, associated with its use. It is the responsibility of the user to establish appropriate safety and health practices and to ensure compliance with any national regulatory conditions.

1 Scope

This document specifies a horizontal method for the detection of *Y. enterocolitica* associated with human disease. It is applicable to

— products intended for human consumption and the feeding of animals, and

— environmental samples in the area of food production and food handling.

2 Normative references

The following documents are referred to in the text in such a way that some or all of their content constitutes requirements 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 6887 (all parts), *Microbiology of the food chain — Preparation of test samples, initial suspension and decimal dilutions for microbiological examination*

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

ISO 11133:2014, *Microbiology of food, animal feed and water — Preparation, production, storage and performance testing of culture media*

3 Terms and definitions

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

ISO and IEC maintain terminological databases for use in standardization at the following addresses:

— IEC Electropedia: available at http://www.electropedia.org/

— ISO Online browsing platform: available at http://www.iso.org/obp

3.1
pathogenic *Yersinia enterocolitica*
psychrotrophic bacteria forming characteristic colonies on solid selective media and possessing the biochemical and molecular properties meeting the pathogenicity criteria described when confirmation tests are carried out in accordance with this document

3.2
detection of pathogenic *Yersinia enterocolitica*
determination of the presence or absence of pathogenic *Yersinia enterocolitica* (3.1) in a given mass or volume of product or a specified surface, when the tests are carried out in accordance with this document

4 Abbreviated terms

For the purposes of this document, the following abbreviations apply.

CEB	cold enrichment broth
CIN	Cefsulodin, Irgasan™ and Novobiocin
CR-MOX	congo red magnesium-oxalate
ITC	Irgasan™, Ticarcillin and potassium chlorate
KOH	potassium hydroxide
MRB	modified rappaport broth
PCR:	polymerase chain reaction
PSB	peptone, sorbitol and bile salts
TSB	tryptic soy broth
WDCM	World Data Centre for Microorganisms

5 Principle

5.1 General

Detection of pathogenic *Y. enterocolitica* involves four successive stages (see Annex A for a diagram of procedure and confirmation). In addition to the general procedure, for example during outbreak investigations, optional cold enrichment procedure as described in Annex D may be used.

5.2 Direct plating from liquid enrichment medium

The sample is homogenized into a liquid enrichment medium (PSB broth), after which a specified amount is inoculated onto two to four CIN agar[15] plates. Inoculated plates are incubated at 30 °C for 24 h.

NOTE Additional plates like chromogenic agar medium for detection of pathogenic *Y. enterocolitica* can also be used.[9,13,18]

5.3 Enrichment in liquid enrichment medium and selective liquid enrichment medium

A specified amount of inoculated PSB enrichment medium (5.2) is transferred into a selective liquid enrichment medium ITC broth.[17] The ITC broth and initial PSB suspension are incubated at 25 °C for 44 h.

5.4 Plating out after enrichment and identification

Using the enrichments obtained in 5.3, surface plating on the CIN agar is performed by transferring first a specified amount of enrichment (5.3, see Clause 10 for the procedure) into 0,5 % KOH solution and, after mixing for specified amount of time (KOH treatment or alkaline treatment), inoculating onto a CIN plate. Inoculated plates are incubated at 30 °C for 24 h. Colonies typical of pathogenic *Y. enterocolitica* are

identified (see 10.5) and the colony morphology is verified as presumptive pathogenic *Y. enterocolitica* by successive culturing onto selective plates (see 10.5).

NOTE Additional plates like chromogenic agar medium for detection of pathogenic *Y. enterocolitica* can also be used.[9,13,18]

5.5 Confirmation

On colonies identified as presumptive pathogenic *Y. enterocolitica* (5.2 and 5.4), confirmation of pathogenic *Y. enterocolitica* is carried out by appropriate biochemical or/and molecular confirmation tests (see 10.6 and Figure A.2).

6 Culture media and reagents

For current laboratory practice, see ISO 7218.

For performance testing of culture media see ISO 11133 and Annex B.

Composition of culture media and reagents and their preparation are described in Annex B. Alternatively, dehydrated complete media, diluents or ready-to-use media may be used; follow the manufacturer's instructions.

7 Equipment and consumables

Disposable equipment is an acceptable alternative to reusable glassware if it has suitable specifications. Usual microbiological laboratory equipment (see ISO 7218) and, in particular, the following.

7.1 Apparatus for dry sterilization (oven) **or wet sterilization** (autoclave).

As specified in ISO 7218.

7.2 Incubators, in accordance with ISO 7218, capable of operating at 4 °C ± 2 °C, 25 °C ± 1 °C, 30 °C ± 1 °C and 37 °C ± 1 °C.

7.3 Sterile blender bags, test tubes, **bottles and/or flasks**, of appropriate capacity.

7.4 Petri dishes, with a diameter of approximately 90 mm and (optional) large size (diameter approximately 140 mm).

7.5 Pipettes. Graduated pipettes or automatic pipettes, with a wide opening, and of nominal capacities 1 ml and 10 ml, graduated respectively in 0,1 ml and 0,5 ml divisions and Pasteur pipettes.

7.6 Loops and spreaders. Sterile loops, approximately 6 mm in diameter (10 µl volume), and inoculation needle or wire. L-shaped or T-shaped single-use spreaders. Cotton buds (see optional protocol in Annex D).

7.7 Stereomicroscope, equipped with dark field illumination or obliquely (45° angle) transmitted light.

7.8 Peristaltic blender.

8 Sampling

Sampling is not part of the method specified in this document. See the specific International Standard dealing with the product concerned. If there is no specific International Standard dealing with the

sampling of the product concerned, it is recommended that the parties concerned come to an agreement on this subject.

Recommended sampling techniques are given in the following documents:

— ISO/TS 17728 for food and animal feed;

— ISO 13307 for primary production stage;

— ISO 17604 for carcasses;

— ISO 18593 for environmental samples.

It is important that the laboratory receives a sample that is representative and the sample should not have been damaged or changed during transport or storage.

9 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 available, it is recommended that the parties concerned come to an agreement on this subject.

10 Procedure (as shown in Annex A)

10.1 Test portion and initial suspension

10.1.1 See the relevant document of ISO 6887 (all parts) or any specific International Standard appropriate to the product concerned.

10.1.2 For preparation of the initial suspension, in the general case, use as diluent the pre-enrichment medium specified in B.2 (PSB broth). Pre-warm the PSB broth to room temperature before use.

In general, an amount of test portion (mass or volume) is added to a quantity of PSB (mass or volume) to yield a tenfold dilution. For this, a 25 g test portion is mixed with 225 ml of PSB.

Homogenize the suspension, preferably by using a peristaltic blender (7.8) for 1 min.

This document has been validated for test portions of 25 g or ml. A smaller test portion may be used, without the need for additional validation/verification, providing that the same ratio between enrichment broth and test portion is maintained. A larger test portion than that initially validated may be used, if a validation/verification study has shown that there are no negative effects on the detection of pathogenic *Y. enterocolitica.*

NOTE Validation can be conducted in accordance with the appropriate documents of ISO 16140 (all parts). Verification for pooling samples can be conducted in accordance with the protocol described in ISO 6887-1:2017, Annex D (verification protocol for pooling samples for qualitative tests).

10.1.3 Prepare the selective enrichment ITC suspension by transferring 10 ml of PSB suspension (10.1.2) into 90 ml of ITC broth (B.3) and mix.

10.2 Direct plating on selective agar

Using the initial PSB suspension obtained (10.1.2), divide a total volume of 1 ml onto two to four CIN agar plates (B.6) and spread it over the plates with a spreader (7.6).

Invert the CIN plates and place them in the incubator set at 30 °C (7.2) for 24 h ± 2 h.

NOTE 1 Drying of CIN agar plates (e.g. in laminar airflow cabinet) before inoculation for half an hour can be required for complete absorption of the inoculum in the agar.

NOTE 2 The number of CIN agar plates to use depends on the expected level of background microflora of the samples.

10.3 Enrichment

Incubate the initial suspension in PSB (10.1.2) and selective enrichment broth ITC (10.1.3) at 25 °C (7.2) for 44 h ± 4 h (without agitation).

10.4 Plating out and incubation of plates

10.4.1 Plating from PSB and ITC by KOH treatment on CIN agar

Using a sterile pipette (7.5), transfer 0,5 ml of the PSB enrichment (10.3) into 4,5 ml of KOH solution (B.5) (prepared the day before use) and mix.[7] After 20 s ± 5 s of the addition of the PSB enrichment to the KOH solution, streak by means of a loop (7.6), the surface of a CIN agar plate (B.6) to obtain well-separated colonies. Repeat the procedure for ITC enrichment (10.3).

NOTE 1 It is crucial for method performance to prepare KOH on the day before use, see Annexes B and C.

Invert the CIN plates and place them in the incubator set at 30 °C (7.2) for 24 h ± 2 h.

NOTE 2 Additionally, it can be advantageous to inoculate [by means of a loop (7.6)] CIN agar plates with untreated (no KOH treatment) PSB and ITC.

NOTE 3 During KOH treatment the enrichment is diluted tenfold. Furthermore, this treatment can reduce the number of pathogenic *Y. enterocolitica* in the solution. Consequently, it can be advantageous, in some cases, to inoculate an additional CIN plate with 0,1 ml of inoculum.

10.4.2 Plating from PSB and ITC by KOH treatment on chromogenic agar (optional)

Repeat the procedure in 10.4.1 and inoculate, after KOH treatment, by means of a loop (7.6), the surface of a chromogenic agar plate[9,13,18] to obtain well-separated colonies.

Incubate the chromogenic plates according to the instructions of the manufacturer.

10.5 Identification of characteristic colonies

After incubation for 24 h ± 2 h, examine the CIN plates in order to detect the presence of characteristic colonies of *Y. enterocolitica*. This should be done with the help of a stereomicroscope (7.7) equipped with dark field illumination or obliquely transmitted light (45° angle).

On CIN agar, pathogenic *Y. enterocolitica* appears as small (approximately 1 mm or under), circular, smooth colonies with entire edge. The colonies have a small, deep red sharp bordered centre ("bull's eye"). The surrounding rim is translucent or transparent and, when examined with obliquely transmitted light, non-iridescent and finely granular.

NOTE 1 Dark field illumination or obliquely transmitted light helps to distinguish characteristic colonies of *Yersinia enterocolitica* from very similar colonies of other *Yersinia* species[12] and some non-*Yersinia* species.

NOTE 2 In case of dense growth of background flora on the CIN plates, the colony size of pathogenic *Y. enterocolitica* can be smaller and the typical red centre can be unclear or absent.

10.6 Confirmation

10.6.1 General

The use of control strains of *Yersinia* species is required especially in helping to distinguish between pathogenic *Y. enterocolitica* from other *Yersinia* species on CIN agar. Appropriate positive and negative control strains for each of the confirmation tests shall be used. Examples of suitable control strains are given in chapters dealing with these tests. A flow-diagram of the confirmation is given in Figure A.2.

10.6.2 Selection of colonies for confirmation

For confirmation, take from each plate of each selective medium (see 10.3) five colonies considered to be typical for pathogenic *Y. enterocolitica* if available (see 10.5).

Streak the selected colonies onto the surface of CIN agar plates (B.6) in order to allow well separated colonies to develop. Streak also control strains of *Y. enterocolitica* bioserotype 4/O:3, 2/O:9, and biotype 1A and other *Yersinia* species for comparison of the colony morphology.

Additionally, it is advantageous to streak typical colonies for confirmation and appropriate control strains on chromogenic agar, in parallel to CIN agar plating. For identification of characteristic colonies on chromogenic agar, follow the manufacturer's instructions on evaluation of typical morphology of the colonies.

EXAMPLE Suitable *Y. enterocolitica* control strains are WDCM 00216 (bioserotype 4/O:3), WDCM 00215 (bioserotype 2/O:9), and WDCM 00160 (bioserotype 1B/O:8).

Invert the inoculated plates and place them in the incubator set at 30 °C (7.2) for 24 h ± 2 h.

Examine the incubated plates for characteristic colonies (see 10.5) and purity of culture. This should be done with the help of a stereomicroscope (7.7). Compare the morphology of suspect colonies to colonies of control strains for better distinction between typical and atypical colonies. Discard plates with atypical colonies. If mixed cultures with typical colonies are present, subculture typical colonies onto CIN agar plates (B.6) and incubate as above.

Proceed with one pure culture representing initial typical colonies on the primary plate. Retain the other typical pure cultures (up to five, if available) for confirmation in case the first culture does not confirm. Streak the selected colonies onto the surface of non-selective agar (for example, nutrient agar (B.7), blood agar, or tryptone soya agar) in a manner which will allow well-separated colonies to develop.

Invert the inoculated plates and place them in the incubator set at 30 °C (7.2) for 18 h to 24 h or until growth is satisfactory.

Use pure cultures for the biochemical confirmations and pathogenicity tests.

NOTE 1 It is not necessary to proceed to confirmation from all successive enrichment steps if pathogenic *Y. enterocolitica* from earlier step has been confirmed.

NOTE 2 For epidemiological purposes or during outbreak investigations, confirmation of additional colonies, e.g. five typical or suspect colonies from each selective enrichment/isolation medium combination, can be beneficial.

10.6.3 Determination of pathogenic *Yersinia* species

10.6.3.1 Detection of urease

Streak bacteria onto the slant surface of the agar (B.10). Close the caps of the tubes loosely so that air can enter and aerobic growth conditions prevail.

Incubate at 30 °C (7.2) for 24 h ± 2 h.

Pink-violet or red-pink colours indicate a positive urease reaction.

EXAMPLE Suitable positive control strain is WDCM 00216 (*Y. enterocolitica*, bioserotype 4/O:3) or WDCM 00160 (*Y. enterocolitica*, bioserotype 1B/O:8).

An orange-yellow colour indicates a negative urease reaction.

Retain for further confirmation all urease positive colonies with typical colony morphology.

NOTE 1 Pathogenic *Y. enterocolitica* strains inoculated on some types of commercially available urea agars can need more time (up to 7 days) for positive reaction to develop.

NOTE 2 Pathogenic urease-negative strains of *Y. enterocolitica* do exist, but they are extremely rare (0,01 %).

10.6.3.2 Hydrolysis of esculin

Streak bacteria onto the slant surface (B.12) of the agar.

Incubate at 30 °C (7.2) for 24 h ± 2 h.

A black halo around the colonies indicates a positive reaction.

EXAMPLE Suitable negative control strain is WDCM 00216 (*Y. enterocolitica*, bioserotype 4/O:3) or WDCM 000160 (*Y. enterocolitica*, bioserotype 1B/O:8) and positive control strain is any *Y. enterocolitica* strain representing biotype 1A or *Y. intermedia* WDCM 00217.

NOTE This test for hydrolysis of esculin is equivalent to the test for fermentation of salicin in determining pathogenicity.

10.6.3.3 Detection of virulence plasmid (pYV) by CR-MOX agar test

Congo red binding and formation of pin-point colonies at 37 °C are typical features of pathogenic *Y. enterocolitica.* The virulence plasmid (pYV) determines traits related to the pathogenicity of *Yersinia*, and many of them, including calcium dependent growth are switched on only at 37 °C.

The virulence plasmid (pYV) can be spontaneously lost in the laboratory during storage, lengthy culture and repeated passages. Therefore, the test for virulence plasmid (CR-MOX test) shall be carried out at an early stage of confirmation.

Using a loop (7.6), touch several colonies of the pure culture of the strain selected for further confirmation (urease positive, typical colony morphology). Inoculate the surface of CR-MOX agar (B.11) to obtain well-separated colonies.

Incubate at 37 °C (7.2) for 24 h to 48 h.

If needed, examine the plates for positive, pYV containing colonies after 24 h and continue incubation for further 24 h if positive colonies are not present.

A plate giving a positive reaction contains sharp orange-red (congo red binding) pinpoint colonies (calcium dependent growth at 37 °C) and possibly colourless larger colonies. A plate giving a negative reaction contains only colourless colonies.

NOTE 1 In a pure culture, it is normal that some of the colonies contain cells with plasmid pYV while other colonies in the same culture contain plasmid-free cells. When preparing inoculum for this test, collecting material with a loop from several colonies helps to avoid choosing for plasmid-free bacterial cells.

NOTE 2 For better distinction between positive and negative reactions, it is advantageous to inoculate two parallel CR-MOX plates from the same inoculant of the strain tested and incubate one plate at 37 °C (7.2) and the other at 25 °C (7.2). The plate incubated at 25 °C (7.2) always gives a negative reaction (even if the strain contains pYV). Therefore, the difference between possible positive result at 37 °C (7.2) and negative reaction at 25 °C (7.2) is better visualized.