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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).

Attention is drawn to the possibility that some of the elements of this document may be the subject of patent rights. ISO shall not be held responsible for identifying any or all such patent rights. Details of any patent rights identified during the development of the document will be in the Introduction and/or on the ISO list of patent declarations received (see www.iso.org/patents).

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.

A suitable positive control is any pathogenic *Y. enterocolitica* strain that has been verified to possess the virulence plasmid, before use.

EXAMPLE Suitable positive control strain is WDCM 00216 (*Y. enterocolitica*, bioserotype 4/O:3) and negative control strain is WDCM 00160 (*Y. enterocolitica*, bioserotype 1B/O:8, plasmid-free) or WDCM 00217 (*Y. intermedia)*.

Since the virulence plasmid (pYV) can be lost during subculturing in the laboratory, continue confirmation also with strains giving a negative reaction in the test.

Additionally, preserve positive strains as frozen stock cultures at an early stage of confirmation [preferably after positive reactions in urease (10.6.3.1) and CR-MOX tests (10.6.3.3)].

An example of preserving strains is given below (other suitable means of preserving cultures can also be used):

— immediately subculture each pure culture into TSB (B.8);

— incubate at 30 °C (7.2) for 24 h ± 2 h;

— add an equal volume of 40 % sterile glycerol (B.9) to get a final glycerol concentration of 20 %;

— mix well and freeze, preferably at −70 °C.

10.6.3.4 Detection of pyrazinamidase

Inoculate a large area of the slant surface (B.13) of the medium with generous loopful (7.6) of bacteria.

Incubate at 30 °C (7.2) for 48 h ± 4 h.

Add 1 ml of freshly prepared (on the day of use) 1 % ammonium iron(II) sulfate solution (B.14).

If the reaction is positive, a pinkish-brown colour develops within 15 min indicating presence of pyrazinoic acid formed by pyrazinamidase enzyme.

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 The possibility of obtaining false positive reactions increases if old 1 % ammonium iron(II) sulfate solution is used.

10.6.3.5 Interpretation of pathogenicity tests

The strain is considered to be a pathogenic *Yersinia* if it is urease positive, and esculin and pyrazinamidase negative. Additionally, the presence of virulence plasmid pYV (10.6.3.3) is a strong indication of pathogenicity.[10,14] For confirmation of pathogenic *Y. enterocolitica*, proceed to 10.6.4.

10.6.4 Confirmation of pathogenic *Y. enterocolitica*

10.6.4.1 General

After determination of pathogenic *Yersinia* species proceed to confirmation of *Y. enterocolitica*. This is necessary only if tests in 10.6.3 have indicated the presence of pathogenic *Yersinia* species.

10.6.4.2 Lysine decarboxylase and arginine dihydrolase

Using a loop (7.6), inoculate each liquid medium just below the surface (B.15). If the tubes are not full of medium and airtight, cover the surface with molten (heated then just cooled so that it remains still liquid) Vaseline®[2)] oil or sterile liquid paraffin.

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

A violet colour after incubation indicates a positive reaction.

A yellow colour indicates a negative reaction.

10.6.4.3 Phenylalanine (Tryptophane) deaminase

Inoculate a large area of the slant surface (B.16) of the medium.

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

Add 2 to 3 drops of 10 % ferric chloride solution (B.17) onto grown bacteria on agar slant surface.

Development of a green colour indicates a positive reaction.

10.6.4.4 Fermentation of sucrose, sorbitol, rhamnose and melibiose

Inoculate each medium (B.18) just below the surface of the liquid.

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

A yellow colour after incubation indicates a positive reaction.

A red colour indicates a negative reaction.

10.6.4.5 Utilization of citrate (Simmon's citrate) (optional)

By means of a loop (7.6), take a well isolated colony of a strain to be tested and mix it well with a drop of saline solution (B.4).

NOTE 1 The washing step with saline removes the excess nutrients from the inoculum and thus helps to avoid false-positive results and interpretation problems. Over incubation can also cause false positive reactions.

NOTE 2 This test can give false positive reactions in some commercially available biochemical identification kits.

Streak the surface of the agar (B.19) with the bacterial suspension and incubate at 30 °C (7.2) for 24 h ± 2 h.

The reaction is positive if there is visible growth on the medium and it turns blue. Faint blue reaction is also considered positive.

EXAMPLE Suitable control strains are *Y. intermedia* WDCM 00217 (positive control) and *Y. enterocolitica* WDCM 00216 (bioserotype 4/O:3, negative control) or WDCM 000160 (*Y. enterocolitica*, bioserotype 1B/O:8, negative control).

NOTE 3 This test is useful in discriminating between *Y. enterocolitica* (negative reaction) and many related species like *Y. intermedia*, *Y. frederiksenii*, *Y. rohdei* and *Y. aldovae* (positive and variable reactions).

10.6.4.6 Alternative confirmation tests

In confirmation of pathogenic *Y. enterocolitica*, it is possible to replace the biochemical tests of 10.6.3 and 10.6.4 by real-time PCR test targeting *ail*-gene in accordance with ISO/TS 18867 (see Figure A.2 and

2) Vaseline® is an example(s) 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.

Reference [6]). However, in case of positive reaction in real-time PCR test, it is mandatory to proceed to detection of pyrazinamidase (10.6.3.4) to obtain a test result according to this document.

If shown to be reliable, miniaturized galleries for the biochemical identification of *Y. enterocolitica* may be used (see ISO 7218). The galleries shall include, as a minimum, the biochemical tests as specified in this document.

NOTE 1 Alternative procedures can be used to confirm the isolate as *Y. enterocolitica*, providing the suitability of the alternative procedure is verified (see ISO 7218).

NOTE 2 Some alternative identification procedures can incorrectly identify the most recent members of *Yersinia* species as *Y. enterocolitica*.

10.6.5 Interpretation of confirmation tests for *Y. enterocolitica*

Y. enterocolitica gives results in accordance with Table 1.

Table 1 — Interpretation of confirmation tests for *Yersinia enterocolitica*

Test	Reaction
Urea (10.6.3.1)	+
Lysine decarboxylase (10.6.4.2)	–
Arginine dihydrolase (10.6.4.2)	–
Phenylalanine/Tryptophane deaminase (10.6.4.3)	–
Sucrose (10.6.4.4)	+ [a]
Sorbitol (10.6.4.4)	+ [a]
Rhamnose (10.6.4.4)	–
Melibiose (10.6.4.4)	–
Citrate (10.6.4.5)	–
[a] Negative strains belonging to pathogenic biotypes may occur.	

10.6.6 Interpretation of confirmation tests for pathogenic *Y. enterocolitica*

Pathogenic *Y. enterocolitica* is detected if at least one colony is urease positive, and esculin and pyrazinamidase negative (10.6.3.5) and presents the characteristics of *Y. enterocolitica* in Table 1 (10.6.5).

For interpretation of real-time PCR result (optional alternative confirmation) see Annex A, Figure A.2 and ISO/TS 18867.

10.7 Biotyping of *Y. enterocolitica* (optional)

10.7.1 General

In addition to tests for esculin (10.6.3.2) and pyrazinamidase (10.6.3.4), complete biotyping scheme includes tests for xylose, tween-esterase/lipase, salicin (optional), trehalose and indole.

Use appropriate control strains for each test in biotyping.

EXAMPLE Suitable positive control strain for all tests is any *Y. enterocolitica* strain representing biotype 1A. *Y. enterocolitica* WDCM 000160 (bioserotype 1B/O:8) is suitable as positive control for all other tests (10.7.1 to 10.7.4) except salicin (10.7.3). *Y. intermedia* WDCM 00217 is suitable as positive control for all other tests (10.7.1 to 10.7.4) except tween-esterase test (10.7.2). An example of a suitable negative control strain for xylose (10.7.1), tween-esterase (10.7.2), salicin (10.7.3) and indole (10.7.4) is WDCM 00216 (*Y. enterocolitica*, bioserotype 4/O:3).

10.7.2 Fermentation of xylose

Inoculate the medium (B.18) just below the surface of the liquid.

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

A yellow colour after incubation indicates a positive reaction.

A red colour indicates a negative reaction.

10.7.3 Tween-esterase test

Streak bacteria onto the surface of the agar (B.20).

Incubate at 25 °C (7.2) for 44 ± 4 h.

The reaction is positive if an opaque zone of precipitate (due to calcium oleate microcrystals) appears.

NOTE Some strains of *Y. enterocolitica* representing pathogenic biotype 1B can give delayed or weak positive reaction in tween-esterase test. Their positive reaction can be better visualized in egg-yolk agar medium (lipase test).[11]

10.7.4 Fermentation of salicin (optional) and trehalose

Inoculate each medium (B.18) just below the surface of the liquid.

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

A yellow colour after incubation indicates a positive reaction.

A red colour indicates a negative reaction.

10.7.5 Indole formation

Inoculate the tube of tryptone/tryptophan medium (B.21). Use the colony mass of at least five colonies for the inoculum.

Incubate at 30 °C (7.2) for 48 h ± 4 h.

Add 1 ml of Kovac's reagent (B.22) and allow to stand at room temperature for 10 min to 15 min.

The formation of a red colour indicates a positive reaction.

A yellow/brown colour indicates a negative reaction.

10.7.6 Interpretation of biotyping tests

Based on the test for esculin (10.6.3.2), pyrazinamidase (10.6.3.4) and tests for xylose, tween-esterase (lipase), trehalose, and indole (10.7.2 to 10.7.5), *Y. enterocolitica* strains can be divided into biotypes (Table 2). According to the test reactions, *Y. enterocolitica* strain is considered pathogenic if it belongs to biotypes 1B, 2, 3, 4 or 5. Additional biochemical tests may be performed.

For epidemiological purposes, determination of the somatic antigens of *Y. enterocolitica* should be investigated. Pathogenic strains, when serotyped by use of appropriate antisera, have been found to usually belong to serotypes O:3, O:8, O:9 or O:5,27.

If tests for esculin, xylose and pyrazinamidase give negative reactions, the result is indicative for biotype 4. This can be confirmed by serotyping since the bioserotype 4/O:3 is most prevalent in many parts of the world.[8]

Table 2 — Biotypes of *Yersinia enterocolitica*

Reaction	Biotype [a]					
	1A [b]	1B	2	3	4	5
Esculin/Salicin	+	–	–	–	–	–
Xylose	+	+	+	+	–	D [d]
Pyrazinamidase	+	–	–	–	–	–
Tween-esterase/Lipase	+	+ [c]	–	–	–	–
Trehalose	+	+	+	+	+	–
Indole	+	+	+	–	–	–

[a] Strains belonging to biotypes 1B, 2, 3, 4, and 5 contain virulence plasmid pYV (10.6.3.3), that may be lost during subculturing in the laboratory.

[b] Generally considered non-pathogenic. However, there are indications that a small proportion of the 1A strains may be pathogenic.[16]

[c] Reaction for tween-esterase may be weak or delayed (see 10.7.3).

[d] D: often weak or delayed.

11 Expression of results

In accordance with the interpretation of the results, indicate pathogenic *Yersinia enterocolitica* detected or not detected in a test portion of x g or x ml of product (see ISO 7218), or on the surface area, or in an object or swab.

12 Performance characteristics of the method

12.1 Interlaboratory study

The performance characteristics of the method were determined in an interlaboratory study (or studies) to determine the specificity, sensitivity and the LOD_{50} of the method. The data are summarized in Annex C. The values derived from the interlaboratory study may not be applicable to food types other than those given in Annex C.

12.2 Sensitivity

The sensitivity is defined as the number of samples found positive divided by the number of true positive samples tested at a given level of contamination. The results are thus dependent on the level of contamination of the sample.

12.3 Specificity

The specificity is defined as the number of samples found negative divided by the number of true negative (or blank) samples tested.

12.4 LOD_{50}

The LOD_{50} is the concentration (cfu/sample) for which the probability of detection is 50 %.

13 Test report

The test report shall specify the following:

— the test method used, with reference to this document, i.e. ISO 10273;

— the sampling method used, if known;

— the size of the test portion and/or the nature of the objects examined;

— all operating conditions not specified in this document, or regarded as optional, together with details of any incidents which may have influenced the test result(s);

— any deviation in the media or the incubation conditions used;

— all information necessary for the complete identification of the sample;

— the test result(s) obtained;

— the date of the test.

The test report shall also state if further tests are to be or have been carried out by a reference laboratory and, if available, what those results were.

14 Quality assurance

The laboratory should have a clearly defined quality control system to ensure that the apparatus, reagents and techniques are suitable for the test. The use of positive controls, negative controls and blanks are part of the test. The performance testing of the culture media is described in B.23 and in ISO 11133.

Annex A
(normative)

Diagrams of the procedures

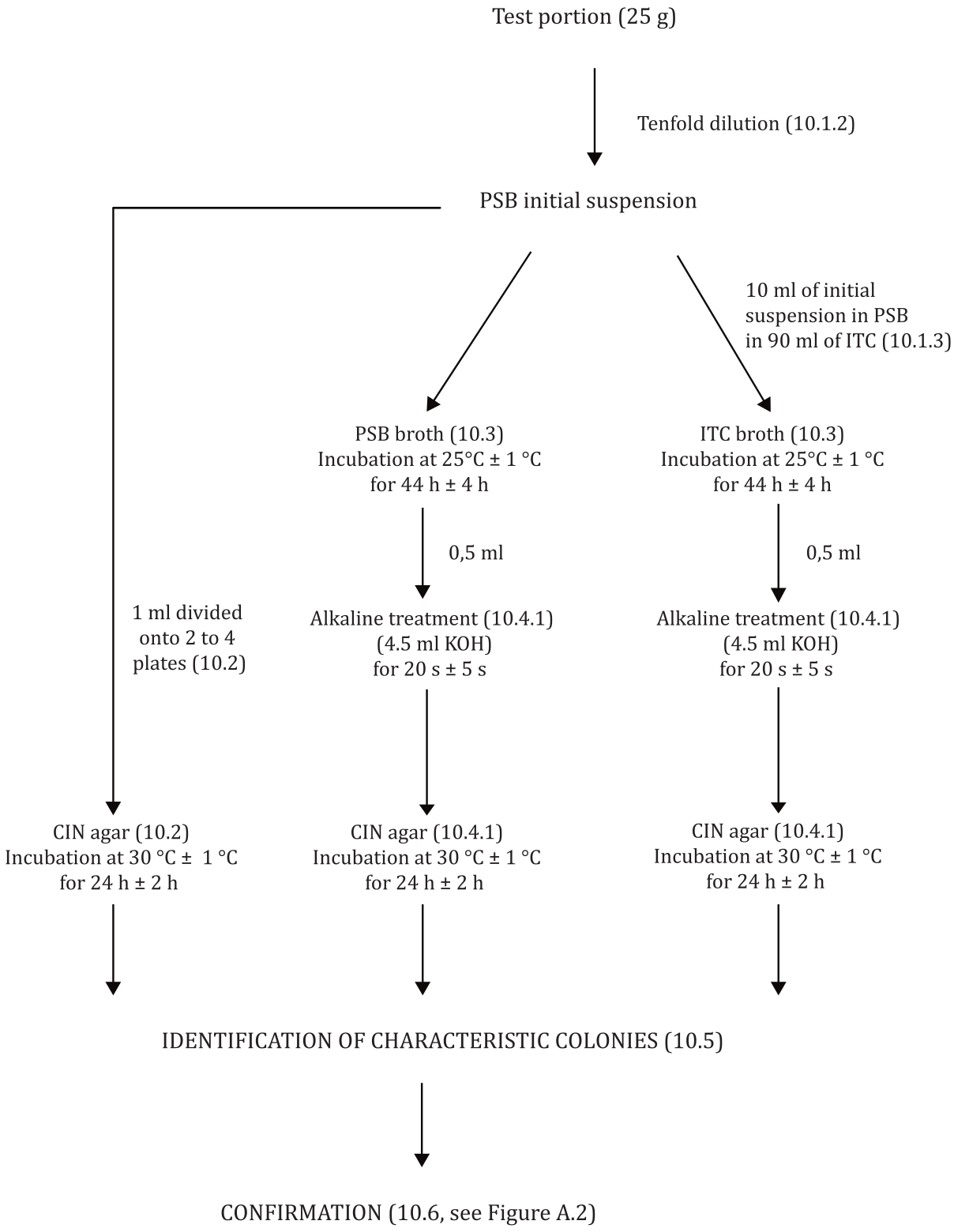
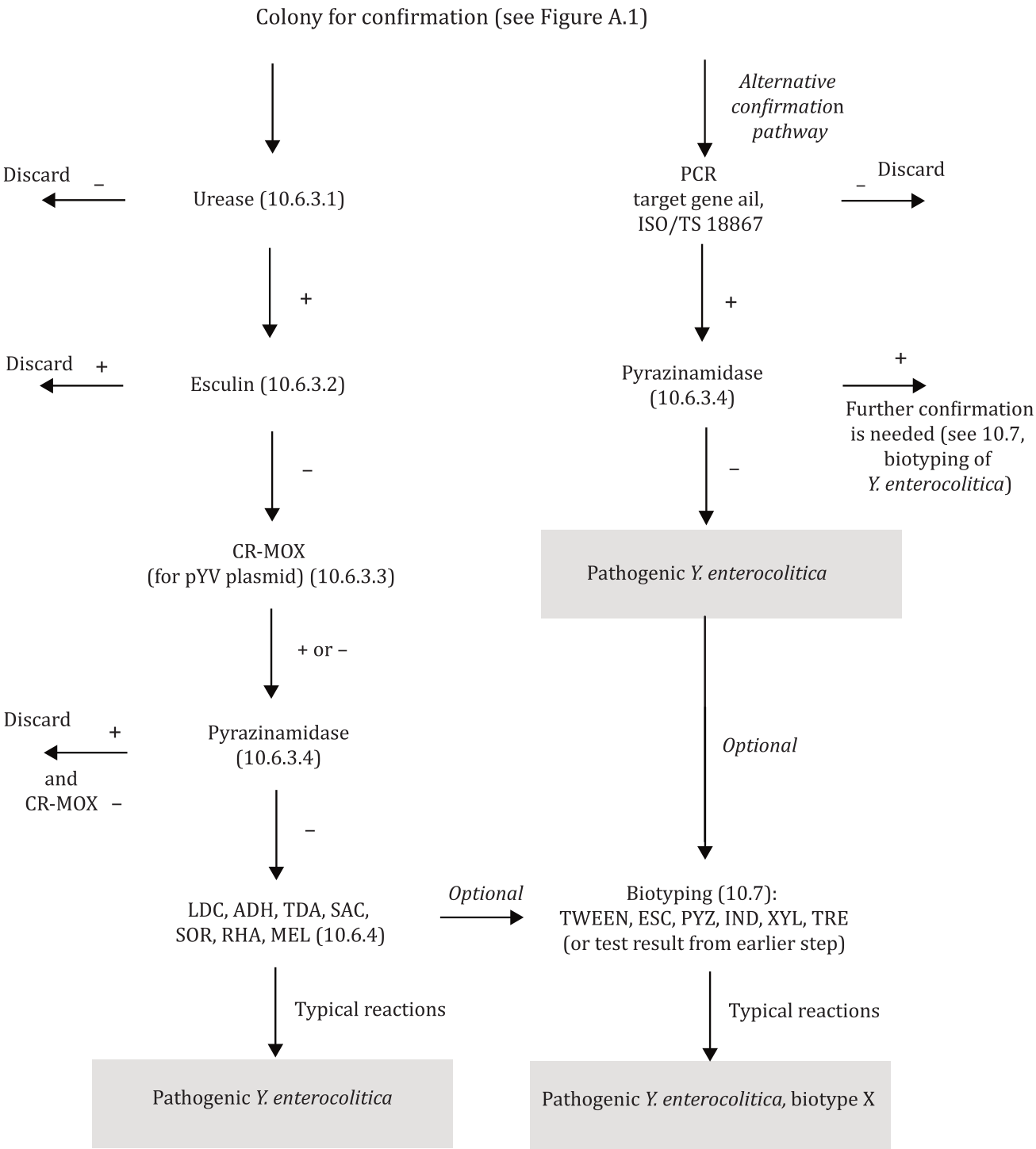


Figure A.1 — Diagram of the procedure of detecting pathogenic *Y. enterocolitica* in food, animal feed and environmental samples



NOTE Real-time PCR targeting *ail*-gene can be used for screening positive samples in PSB broth after incubation of 24 h ± 2 h at 25 °C (7.2). See ISO/TS 18867 for details of the procedure.

Figure A.2 — Diagram of the confirmation of pathogenic *Y. enterocolitica*

Annex B
(normative)

Composition and preparation of culture media and reagents

B.1 General

The general specifications of ISO 11133 are applicable to the preparation and performance testing of the culture media described in this annex. If culture media or reagents are prepared from dehydrated complete media/reagents or if ready-to-use media/reagents are used, follow the manufacturer's instructions regarding preparation, storage conditions, expiry date and use.

The shelf lives of the media indicated in this annex have been shown in some studies. The user should verify these under their own storage conditions (see ISO 11133).

Performance testing for the quality assurance of the culture media is described in B.23.

B.2 Peptone, sorbitol and bile salts (PSB) broth

B.2.1 Composition

Enzymatic digest of casein	5,0 g
Sorbitol	10,0 g
Sodium chloride	5,0 g
Disodium hydrogen phosphate (Na_2HPO_4)	8,23 g
Sodium dihydrogen phosphate monohydrate ($NaH_2PO_4 \cdot H_2O$)	1,2 g
Bile salts	1,5 g
Water	1 000 ml

B.2.2 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 7,6 ± 0,2 at 25 °C.

Dispense the medium into tubes or flasks (7.3) of suitable capacity to obtain portions appropriate for the test samples (see 10.1.2).

Sterilize for 15 min in an autoclave (7.1) set at 121 °C.

B.3 Irgasan™, ticarcillin and potassium chlorate (ITC) broth; Triclosan, ticarcillin chlorate broth (TTC) broth

B.3.1 Basic medium

B.3.1.1 Composition

Enzymatic digest of casein	10,0 g
Yeast extract	1,0 g
Magnesium chloride hexahydrate ($MgCl_2 \cdot 6H_2O$)	60,0 g
Sodium chloride	5,0 g
Malachite green, 0,2 % aqueous solution	5,0 ml
Water	1 000 ml

B.3.1.2 Preparation

Dissolve the components or the dehydrated basic medium in the water, by heating if necessary.

Adjust the pH, if necessary, so that after sterilization it is 6,9 ± 0,2 at 25 °C.

Dispense the basic medium into flasks (7.3) of suitable capacity to obtain the portions necessary (e.g. 988 ml for 1 l of complete medium).

Sterilize for 15 min in an autoclave (7.1) set at 121 °C.

B.3.2 Ticarcillin solution (1 mg/ml)

B.3.2.1 Composition

Ticarcillin	10,0 mg
Water	10 ml

B.3.2.2 Preparation

Dissolve the ticarcillin in the water. Sterilize by filtration.

B.3.3 Triclosan (Irgasan™) [5-chloro-2-(2,4-dichlorophenoxy)phenol], ethanolic solution (1 mg/ml)

B.3.3.1 Composition

Triclosan	10,0 mg
Ethanol, 95 % (by volume)	10,0 ml

B.3.3.2 Preparation

Dissolve the Triclosan in the ethanol as and when required, or alternatively store the solution at about −20 °C for not more than 4 weeks.

B.3.4 Potassium chlorate solution (100 mg/ml)

B.3.4.1 Composition

Potassium chlorate ($KClO_3$)	10,0 g
Water	100 ml

B.3.4.2 Preparation

Dissolve the potassium chlorate in the water. Sterilize by filtration.

B.3.5 Complete medium

B.3.5.1 Composition

Basic medium (B.3.1)	988 ml
Ticarcillin solution (B.3.2)	1 ml
Triclosan solution (B.3.3)	1 ml
Potassium chlorate solution (B.3.4)	10 ml

B.3.5.2 Preparation

Add the ticarcillin, triclosan and potassium chlorate solutions aseptically to the basic medium cooled to about 47 °C and mix.

Dispense the medium aseptically in 90 ml amounts into flasks of suitable capacity (see 10.1.3), so as to obtain the minimum area/volume ratio (relative anaerobiosis).

B.4 Saline solution

B.4.1 Composition

Sodium chloride (NaCl)	9,0 g
Water	1000 ml

B.4.2 Preparation

Dissolve the sodium chloride in the water.

Dispense the solution into flasks (7.3) of suitable capacity.

Sterilize for 15 min in an autoclave (7.1) set at 121 °C.

B.5 Potassium hydroxide (KOH) in saline solution

B.5.1 Composition

Potassium hydroxide (KOH)	0,5 g
Sterile saline solution (0,9 % NaCl; see B.2)	100 ml

B.5.2 Preparation

Dissolve the KOH in the sterile saline solution.

Dispense the solution into flasks (7.3) of suitable capacity.

Prepare the KOH solution the day before use.

B.6 Cefsulodin, Irgasan™(Triclosan) and novobiocin (CIN) agar

B.6.1 Basic medium

B.6.1.1 Composition

Enzymatic digest of gelatin	17,0 g
Enzymatic digest of casein and animal tissues	3,0 g
Yeast extract	2,0 g
Mannitol	20,0 g
Sodium pyruvate	2,0 g
Sodium chloride	1,0 g
Magnesium sulfate heptahydrate ($MgSO_4 \cdot 7H_2O$)	0,01 g
Sodium desoxycholate	0,5 g
Neutral red	0,03 g
Crystal violet	0,001 g
Agar	12,0 g
Water	1 000 ml

B.6.1.2 Preparation

Dissolve the components or the dehydrated basic medium in the water by boiling.

Adjust the pH, if necessary, so that after sterilization it is 7,4 ± 0,2 at 25 °C.

Dispense the medium into flasks (7.3) of suitable capacity to obtain the portions necessary (e.g. 997 ml for 1 l of complete medium).

Sterilize for 15 min in an autoclave (7.1) set at 121 °C.

B.6.2 Cefsulodin solution (15 mg/ml)

B.6.2.1 Composition

Cefsulodin	1,5 g
Water	100 ml

B.6.2.2 Preparation

Dissolve the cefsulodin in the water. Sterilize by filtration.

B.6.3 Triclosan (Irgasan™) [5-chloro-2-(2,4-dichlorophenoxy)phenol], ethanolic solution (4 mg/ml)

B.6.3.1 Composition

Triclosan	0,4 g
Ethanol, 95 % (by volume)	100 ml

B.6.3.2 Preparation

Dissolve the triclosan in the ethanol as and when required, or alternatively store the solution at about −20 °C for not more than 4 weeks.

B.6.4 Novobiocin solution (2,5 mg/ml)

B.6.4.1 Composition

Novobiocin	0,25 g
Water	100 ml

B.6.4.2 Preparation

Dissolve the novobiocin in the water. Sterilize by filtration.

B.6.5 Complete medium

B.6.5.1 Composition

Basic medium (B.6.1)	997 ml
Cefsulodin solution (B.6.2)	1 ml
Triclosan solution (B.6.3)	1 ml
Novobiocin solution (B.6.4)	1 ml

B.6.5.2 Preparation

Add each antibiotic solution aseptically to the basic medium cooled to about 45 °C and mix.

B.6.5.3 Preparation of CIN agar plates

Pour approximately 15 ml of the complete medium into sterile Petri dishes (7.4). Leave to set.

B.7 Nutrient agar (example of non-selective medium)

B.7.1 Composition

Meat extract	3,0 g
Peptone	5,0 g
Sodium chloride (NaCl) (optional)	5,0 g
Agar	9 g to 18 g[a]
Water	1 000 ml

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

B.7.2 Preparation

Dissolve the components or the dehydrated complete medium in the water by boiling.

Adjust the pH, if necessary, so that after sterilization it is 7,0 ± 0,2 at 25 °C.

Dispense the medium into flasks (7.3) of suitable capacity.

Sterilize for 15 min in an autoclave (7.1) set at 121 °C.

B.7.3 Preparation of nutrient agar plates

Pour approximately 15 ml of the medium, cooled to about 45 °C, into sterile Petri dishes (7.3). Leave to set.

B.8 Tryptic soy broth (TSB) (tryptone soya broth, casein soya bean digest medium)

B.8.1 Composition

Enzymatic digest of casein	17,0 g
Enzymatic digest of soy	3,0 g
Sodium chloride	5,0 g
Dipotassium hydrogen phosphate	2,5 g
Glucose (=Dextrose)	2,5 g
Water	1 000 ml

B.8.2 Preparation

Dissolve the components or the complete dehydrated medium in the water, by heating if necessary.

Adjust the pH, if necessary, so that after sterilization it is pH 7,3 ± 0,2 at 25 °C.

Dispense in 10 ml amounts into tubes (7.3).

Sterilize for 15 min in an autoclave set at 121 °C.

B.9 Sterile glycerol

Dispense the glycerol in 100 ml into flasks or bottles (7.3) and sterilize for 15 min in an autoclave (7.1) set at 121 °C.

B.10 Urea agar (Christensen)

B.10.1 Basic medium

B.10.1.1 Composition

Peptone	1,0 g
Glucose	1,0 g
Sodium chloride	5,0 g
Potassium dihydrogen phosphate (KH_2PO_4)	2,0 g
Phenol red	0,012 g
Agar	12,0 g
Water	1000 ml

B.10.1.2 Preparation

Dissolve the components or the dehydrated basic medium in the water, by heating if necessary.

Adjust the pH, if necessary, so that after sterilization the pH of the complete medium is 6,8 ± 0,2 at 25 °C.

Sterilize for 15 min in an autoclave (7.1) set at 121 °C.

B.10.2 Urea solution

B.10.2.1 Composition

Urea	400,0 g
Water, to a final volume of	1 000 ml

B.10.2.2 Preparation

Dissolve the urea in the water. Sterilize by filtration.

B.10.3 Complete medium

B.10.3.1 Composition

Basic medium (B.10.1)	950 ml
Urea solution (B.10.2)	50 ml

B.10.3.2 Preparation

Add the urea solution aseptically to the basic medium, cooled to 44 °C to 47 °C.

Dispense the complete medium into sterile tubes (7.3) in quantities of 10 ml.

Allow to set in a sloping position.

B.11 Congo red-magnesium oxalate (CR-MOX) agar

B.11.1 Basic medium

B.11.1.1 Composition

Tryptone soya agar	40,0 g
Magnesium chloride hexahydrate ($MgCl_2 \cdot 6H_2O$)	4,1 g
Sodium oxalate ($C_2Na_2O_4$)	2,7 g
Galactose	2,0 g
Water	1 000 ml

B.11.1.2 Preparation

Dissolve the components or the dehydrated basic medium in the water, by heating if necessary.

Adjust the pH, if necessary, so that after sterilization the pH of the complete medium is 7,3 ± 0,2 at 25 °C.

B.11.2 Congo red solution (25 mg/ml)

B.11.2.1 Composition

Congo red	2,5 g
Water	100 ml

B.11.2.2 Preparation

Dissolve the congo red in the water.

NOTE The congo red solution can be stored in a dark glass bottle for one year.

B.11.3 Complete medium

B.11.3.1 Composition

Basic medium (B.11.1)	1 000 ml
Congo red solution (B.11.2)	2 ml

B.11.3.2 Preparation

Add congo red solution to the basic medium and mix.

Sterilize for 15 min in an autoclave (7.1) set at 121 °C.

B.11.3.3 Preparation of CR-MOX agar plates

Pour approximately 25 ml of the complete medium, cooled to about 47 °C, into sterile Petri dishes (7.4). Leave to set.

B.12 Bile and esculin agar

B.12.1 Composition

Meat extract	3,0 g
Enzymatic digest of animal tissue	5,0 g
Esculin	1,0 g
Bile salts	40,0 g
Iron(III) citrate	0,5 g
Agar	12,0 g
Water	1 000 ml

B.12.2 Preparation

Dissolve the components or the dehydrated complete medium in the water by gentle boiling.

Adjust the pH, if necessary, so that after sterilization it is 6,6 ± 0,2 at 25 °C.

Dispense the medium in 10 ml amounts into tubes (7.3) of suitable capacity.

Sterilize for 15 min in an autoclave (7.1) set at 121 °C.

Leave to stand in a tilted position so as to obtain a butt 2,5 cm deep.

B.13 Casein-soya agar for detection of pyrazinamidase

B.13.1 Composition

Enzymatic digest of casein	15,0 g
Enzymatic digest of soy	5,0 g
Pyrazinecarboxamide ($C_5H_5N_3O$)	1,0 g
Sodium chloride	5,0 g
Agar	12,0 g
Tris-maleate buffer (0,2 mol/l, pH 6)	1 000 ml

B.13.2 Preparation

Dissolve the components or the dehydrated complete medium in the water by boiling.

Adjust the pH, if necessary, so that after sterilization it is 7,3 ± 0,2 at 25 °C.

Dispense the medium in 10 ml amounts into flasks (7.3) of suitable capacity.

Sterilize for 15 min in an autoclave (7.1) set at 121 °C.

After sterilization, leave to stand in a tilted position so as to obtain a long slant.

B.14 Ammonium iron(II) sulfate solution for detection of pyrazinamidase

B.14.1 Composition

Ammonium iron(II) sulfate	1,0 g
Water	100 ml

B.14.2 Preparation

Immediately prior to use, dissolve the ammonium iron(II) sulfate in the water.

B.15 Decarboxylase basal medium (lysine or arginine)

B.15.1 Composition

L-lysine or L-arginine	5,0 g
Peptone	5,0 g
Yeast extract	3,0 g
Glucose	1,0 g
Bromocresol purple	0,02 g
Water	1 000 ml

B.15.2 Preparation

Dissolve the components in the water, by heating if necessary.

Adjust the pH, if necessary, so that after sterilization it is 6,8 ± 0,2 at 25 °C.

Dispense the medium in 2 ml to 5 ml amounts into tubes (7.3) of appropriate capacity.

Sterilize for 15 min in an autoclave (7.1) set at 121 °C.

B.16 Phenylalanine (tryptophane) deaminase agar

B.16.1 Composition

Yeast extract	3,0 g
L-phenylalanine or	1,0 g
DL-phenylalanine	2,0 g
Disodium hydrogen phosphate (Na_2HPO_4)	1,0 g
Sodium chloride	5,0 g
Agar	12,0 g
Water	1 000 ml

B.16.2 Preparation

Dissolve the components in the water, by heating if necessary.

Adjust the pH, if necessary, so that after sterilization it is 7,3 ± 0,2 at 25 °C.

Dispense the medium in 5 ml amounts into tubes (7.3) of appropriate capacity.

Sterilize for 15 min in an autoclave (7.1) set at 121 °C.

Allow to set in a sloping position.

B.17 Ferric chloride, 10 %

B.17.1 Composition

Ferric chloride ($FeCl_3$)	10,0 g
Water	90 ml

B.17.2 Preparation

Dissolve the ferric chloride in the water.

B.18 Media for fermentation of carbohydrates (peptone water with phenol red and carbohydrates)

B.18.1 Basic medium

B.18.1.1 Composition

Peptone	10,0 g
Sodium chloride	5,0 g
Phenol red	0,02 g
Water	1 000 ml

B.18.1.2 Preparation

Dissolve the components or the dehydrated basic medium in the water, by heating if necessary.

Adjust the pH, if necessary, so that after sterilization it is 6,8 ± 0,2 at 25 °C.

Dispense the basic medium into flasks (7.3) of suitable capacity.

Sterilize for 10 min in an autoclave (7.1) set at 121 °C.

B.18.2 Carbohydrate solutions (melibiose, rhamnose, salicin, sorbitol, sucrose, trehalose or xylose, 100 mg/ml)

B.18.2.1 Composition

Carbohydrate (melibiose, rhamnose, salicin, sorbitol, sucrose, trehalose or xylose)	10,0 g
Water	100 ml

B.18.2.2 Preparation

Prepare separate solutions of each carbohydrate by adding it to the distilled water.

Sterilize by filtration.

B.18.3 Complete medium

B.18.3.1 Composition

Basic medium (B.18.1)	900 ml
Carbohydrate solution (B.18.2)	100 ml

B.18.3.2 Preparation

For each carbohydrate, add the carbohydrate solution aseptically to the basic medium cooled to about 45 °C and mix.

Dispense the complete medium aseptically in 10 ml amounts into tubes or bottles (7.3) of suitable capacity.

B.19 Simmons' citrate medium

B.19.1 Composition

Sodium citrate	2,0 g
Sodium chloride	5,0 g
Dipotassium hydrogen phosphate (K_2HP0_4)	1,0 g
Bromothymol blue	0,08 g
Ammonium dihydrogen phosphate ($NH_4H_2PO_4$)	1,0 g
Magnesium sulfate	0,2 g
Agar	12,0 g
Water	1 000 ml

B.19.2 Preparation

Dissolve the components or the dehydrated complete medium in the water by boiling.

Adjust the pH, if necessary, so that after sterilization it is 6,8 ± 0,2 at 25 °C.

Sterilize for 15 min in an autoclave (7.1) set at 121 °C.

B.19.3 Preparation of Simmons' citrate plates

Pour approximately 15 ml of the medium, cooled to about 45 °C, into sterile Petri dishes (7.4). Leave to set.

B.20 Medium for tween-esterase test

B.20.1 Basic medium

B.20.1.1 Composition

Peptic digest of meat	10,0 g
Sodium chloride (NaCl)	5,0 g
Calcium chloride ($CaCl_2$)	0,1 g
Agar	12 g
Water	1 000 ml

B.20.1.2 Preparation

Dissolve the components in the water, by heating if necessary.

Adjust the pH, if necessary, so that after sterilization it is 7,4 ± 0,2 at 25 °C.

Sterilize for 30 min in an autoclave set at 121 °C.

B.20.2 Complete medium

B.20.2.1 Composition

Basic medium (D.22.1)	990 ml
Sorbitol mono-oleate[a]	10 ml

[a] For example, Tween80™ 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.

B.20.2.2 Preparation

Add the Sorbitol mono-oleate to the liquid base, and mix.

Sterilize for 30 min in an autoclave set at 110 °C.

B.20.3 Preparation of tween-esterase plates

Pour approximately 15 ml of the medium, cooled to about 45 °C, into sterile Petri dishes (7.4). Leave to set.

B.21 Tryptone/tryptophan medium

B.21.1 Composition

Enzymatic digest of casein (tryptone)	10,0 g
Sodium chloride	5,0 g
DL-tryptophan	3,0 g
Water	1 000 ml

B.21.2 Preparation

Dissolve the components in the water by boiling if necessary.

Adjust the pH so that after sterilization it is 7,5 ± 0,2 at 25 °C.

Dispense in 5 ml amounts into test tubes or bottles (7.3) of appropriate capacity.

Sterilize for 15 min in an autoclave (7.1) set at 121 °C.

B.22 Kovac's reagent

B.22.1 Composition

4-dimethylaminobenzaldehyde	5,0 g
Hydrochloric acid, P = 1,18 g/ml to 1,19 g/ml	25 ml
2-methylbutan-2-ol	75 ml

B.22.2 Preparation

Dissolve 4-dimethylaminobenzaldehyde in 2-methylbutan-2-ol in a water bath set at 60 °C.

Cool to room temperature and place the flask in an ice bath. Then add carefully the hydrochloric acid, mix it slowly.

Store at 3 °C ± 2 °C in an amber-coloured flask. Avoid the use of rubber bottle caps as they spoil the reagent.

B.23 Performance testing for the quality assurance of the culture media

See Table B.1. For the definition of selectivity and productivity refer to ISO 11133. The inoculum volume should be the same as that used in the method for that medium and should contain the number of target or non-target organisms specified in ISO 11133:2014, 5.4.

Table B.1 — Performance testing for the quality assurance of the culture media

Medium	Function	Incubation	Control strains	WDCM numbers[a]	Criteria[d]
ITC	Productivity	44 h ± 4 h/ 25 °C ± 1 °C	*Yersinia enterocolitica* + *Escherichia coli*[c] + *Pseudomonas aeruginosa*	00216[b] + 00012 or 00013 + 00025	> 10 characteristic colonies on CIN (see 10.5)
			Yersinia enterocolitica + *Escherichia coli*[c] + *Pseudomonas aeruginosa*	00160 + 00012 or 00013 + 00025	
	Selectivity		*Proteus mirabilis*	00023	Total (0) or partial inhibition (<10 colonies) on TSA
PSB	Productivity	44 h ± 4 h/ 25 °C ± 1 °C	*Yersinia enterocolitica* + *Pseudomonas aeruginosa*	00216[b] + 00025	> 10 characteristic colonies on CIN (see 10.5)
			Yersinia enterocolitica + *Pseudomonas aeruginosa*	00160 + 00025	
CIN	Productivity	24 h ± 2 h/ 30 °C ± 1 °C	*Yersinia enterocolitica*	00216[b] 00160	Good growth (2) of characteristic colonies (see 10.5).
	Selectivity		*Escherichia coli*[c]	00012 or 00013	Total or partial inhibition (0 - 1), no characteristic colonies
			Staphylococcus aureus	00034	Total inhibition (0)
Nutrient agar	Productivity	24 h ± 2 h/ 30 °C ± 1 °C	*Yersinia enterocolitica*	00216[b] 00160	Good growth (2)

[a] Refer to the reference strain catalogue available at www.wfcc.info for information on culture collection strain numbers and contact details; WDCM: World Data Centre for Microorganisms.

[b] Strain to be used as a minimum.

[c] Strain free of choice; one of the strains has to be used as a minimum.

[d] Growth is categorized as 0: no growth; 1: weak growth (partial inhibition); 2: good growth (see ISO 11133).

NOTE The colony morphology of strain WDCM 00160 (bioserotype 1B/O:8) is not representative of major pathogenic bioserotypes of *Y. enterocolitica* (bioserotypes 4/O:3 and 2/O:9) on CIN agar. See 10.6.2 for examples of control strains for these bioserotypes (to be used as reference for colony morphology during sample analyses).

Annex C
(informative)

Method validation studies and performance characteristics

An interlaboratory study involving 13 to 14 laboratories in 5 countries was carried out. The following food types were included in the studies: raw milk, minced meat and lettuce. The food samples were each tested at two different levels of contamination, plus a negative control. The study was organized in 2013 to 2014 by the Finnish Food Safety Authority Evira as part of the CEN Mandate M381 funded by the European Commission.

The samples were contaminated in different study rounds by strain of *Y. enterocolitica* bioserotype 4/O:3 (raw milk samples) and two different strains of *Y. enterocolitica* bioserotype 2/O:9 (minced meat and lettuce samples).

The protocol followed the procedure (see Annex A) and all the operational details of the current version of this standard. The values of the performance characteristics, for each sample type, derived from this interlaboratory study are shown in Tables C.1 to C.3. Data obtained by some collaborators have been excluded from the calculations only on the basis of clearly identified technical reasons (deviations to the protocol).

In the interlaboratory study, confirmation by using both alternative pathways (see Figure A.2) was validated by comparison. For this, collaborators used ISO/TS 18867:2015, Annex B, Methods 1 or 2 and pyrazinamidase testing (10.6.3.4) parallel to the biochemical confirmation pathway (10.6). Results are summarized in Table C.4. Concordant results were obtained from 410 parallel confirmation results.

Table C.1 — Results of data analysis obtained with raw milk

Performance characteristic	Blank 0 cfu/25 g	Low level contamination 9 cfu/25 g	High level contamination 59 cfu/25 g
Number of participating collaborators	14	14	14
Number of collaborators retained after evaluation of the data	12[a]	12[a]	12[a]
Number of samples	112	112	112
Number of samples retained after evaluation of the data	96	96	96
Sensitivity, %	–	68	96
Specificity, %	100	–	–
LOD_{50}, (95 % confidence interval) in cfu/sample	–	9,4 (7,4 to 12,0)	

[a] Two laboratories were excluded; one due to deviation in the protocol and other due to the inconsistency in the result reporting.

Table C.2 — Results of data analysis obtained with minced meat

Performance characteristic	Blank 0 cfu/25 g	Low level contamination 16 cfu/25 g	High level contamination 85 cfu/25 g
Number of participating collaborators	13	13	13
Number of collaborators retained after evaluation of the data	13	13	13
Number of samples	104	104	104
Number of samples retained after evaluation of the data	104	104	104
Sensitivity, %	–	77	97
Specificity, %	96[a]	–	–
LOD_{50}, (95 % confidence interval) in cfu/sample		9,9 (7,8 to 12,5)	

[a] False positives were obtained in three different laboratories (one laboratory 2 samples out of 8; two laboratories 1 sample out of 8). The isolated strains available for analysis from these samples were indistinguishable from the strain used in contamination of the samples. This suggests cross-contamination of the samples, either at the organizer's level or at the participating laboratories' level.

Table C.3 — Results of data analysis obtained with lettuce

Performance characteristic	Blank 0 cfu/25 g	Low level contamination 110 cfu/25 g	High level contamination 1 100 cfu/25 g
Number of participating collaborators	13	13	13
Number of collaborators retained after evaluation of the data	13	13	13
Number of samples	104	104	104
Number of samples retained after evaluation of the data	103[a]	103[a]	104
Sensitivity, %	–	81	98
Specificity, %	98[b]	–	–
LOD_{50}, (95 % confidence interval) in cfu/sample	–	63 (49 to 81)	

[a] One sample at blank level and one sample at low level for one laboratory were excluded from analysis due to lack of confirmation tests.

[b] False positives were obtained in two different laboratories (1 sample out of 8). The isolated strains available for analysis from the other of these samples were indistinguishable from the strain used in contamination of the samples. This suggests cross-contamination of the samples, either at the organizer's level or at the participating laboratory level.

Table C.4 — Results for confirmation of pathogenic *Y. enterocolitica* using alternative pathways[a]

Performance characteristic	No. of collaborators	No. of samples with parallel confirmation[a]	No. of parallel confirmation reactions	No. of inconsistent results
Confirmation results/raw milk	7	91	119	0
Confirmation results/minced meat	8	122	130	0
Confirmation results/lettuce	8	106	161	0
Confirmation results/all matrices		319	410	0

[a] See Figure A.2 for the explanation of alternative confirmation pathways. In confirmation of the colonies, the collaborators used ISO/TS 18867:2015, Annex B, Methods 1 or 2 and pyrazinamidase testing (10.6.3.4) parallel to the biochemical confirmation (10.6).

Annex D
(informative)

Procedure for cold enrichment

D.1 Introduction

The cold enrichment procedure described in this annex may be used to supplement the general procedure in investigations, such as in foodborne outbreaks. Either CEB (D.3.1) or PSB broth (B.2) can be used.

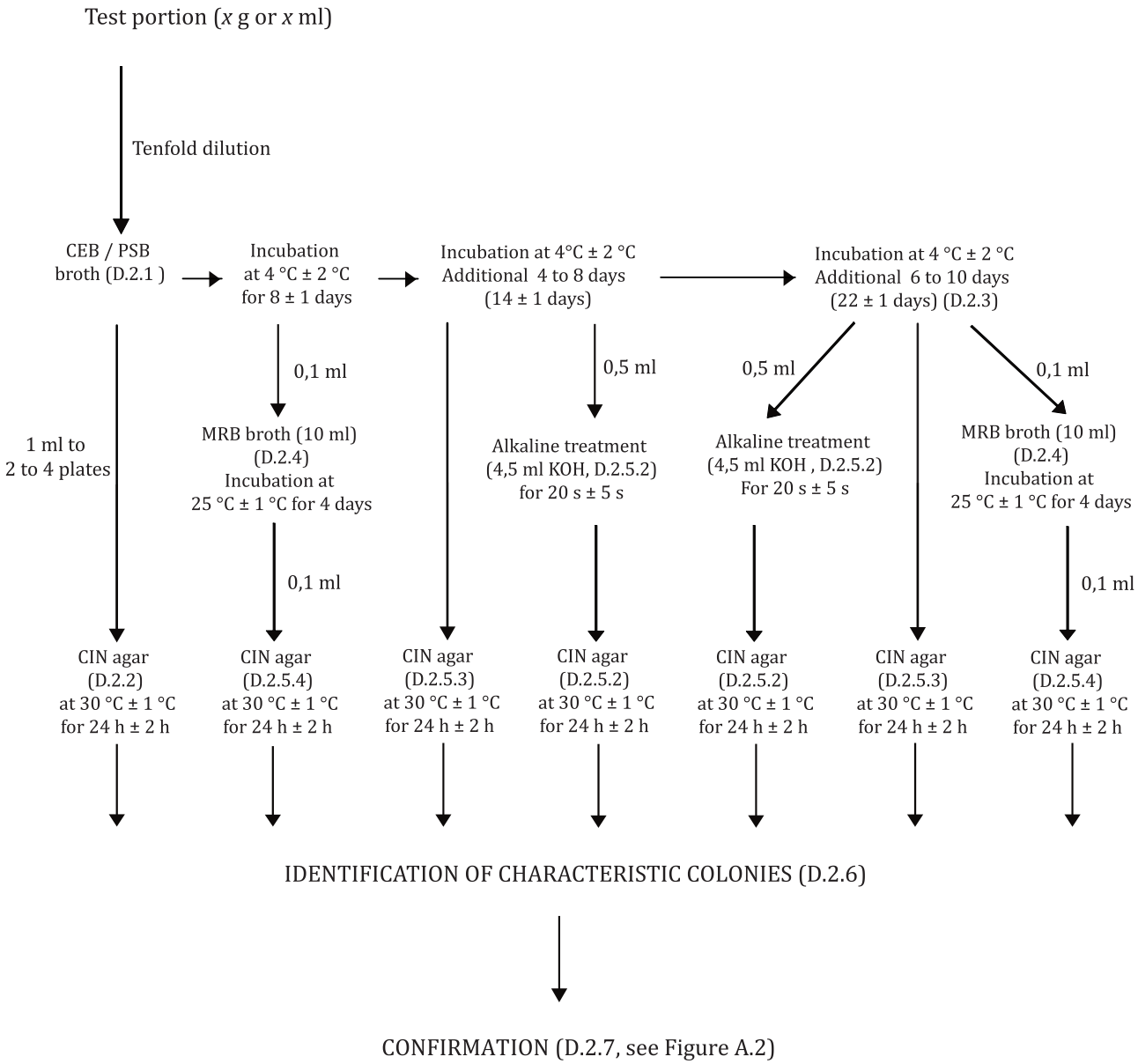


Figure D.1 — Diagram of procedure for cold enrichment

NOTE Real-time PCR targeting *ail*-gene can be used for screening positive samples in PSB broth after incubation of 24 h at 25 °C. See ISO/TS 18867 for details of the procedure.

D.2 Detection of pathogenic *Y. enterocolitica* by cold enrichment

D.2.1 Test portion and initial suspension

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

For preparation of the initial suspension, in the general case, use as diluent the pre-enrichment medium specified in D.3.1 (CEB) or B.2 (PSB).

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

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

D.2.2 Direct plating on selective agar

See 10.2.

D.2.3 Enrichment

Incubate the initial suspension of CEB or PSB (D.2.1) at 4 °C for 22 d ± 1 d.

D.2.4 Second enrichment

After 8 ± 1 days and 22 ± 1 days incubation of CEB or PSB (D.2.1), transfer 0,1 ml of enrichment to 10 ml of MRB broth (D.3.2) and incubate at 25 °C for 4 days.

D.2.5 Plating out and incubation of the plates

D.2.5.1 General

After incubation of the enrichment media (D.2.3 and D.2.4), proceed as follows.

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

D.2.5.2 Plating from CEB or PSB by KOH treatment on CIN agar

After 14 ± 1 and 22 d ± 1 d of incubation at 4 °C, transfer 0,5 ml of CEB or PSB enrichment (D.2.3) by using a sterile pipette (7.5) 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 CEB or 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.

NOTE 1 Best results are achieved by using KOH solution prepared the day before.

NOTE 2 During alkaline 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 extra CIN plate with 0,1 ml of inoculum.

D.2.5.3 Plating from CEB or PSB without KOH treatment

After 14 d ± 1 d and 22 d ± 1 d of incubation at 4 °C, inoculate CEB or PSB enrichment (D.2.3) with a sterile loop (7.6) on the surface of a CIN agar plate (B.6) to obtain well-separated colonies.

D.2.5.4 Plating from the second enrichment (MRB)

After 4 days of incubation in the second enrichment MRB (D.2.4), using a sterile pipette (7.5) inoculate 0,1 ml of MRB on the surface of a CIN agar plate (B.6) and spread the inoculum by means of a cotton bud or a spreader (7.6) to obtain well-separated colonies.

D.2.5.5 Incubation of the plates

Invert the plates (D.2.5.2 to D.2.5.4) and place them in the incubator (7.2) set at 30 °C (7.2) for 24 h ± 2 h.

D.2.6 Identification of characteristic colonies

See 10.5.

D.2.7 Confirmation of pathogenic *Y. enterocolitica*

See 10.6 and Figure A.2.

D.2.8 Biotyping of *Y. enterocolitica*

See 10.7.

D.3 Culture media

D.3.1 Cold enrichment broth (CEB)

D.3.1.1 Composition

Disodium hydrogen phosphate (Na_2HPO_4)	7,6 g
Potassium dihydrogen phosphate (KH_2PO_4)	1,0 g
Sodium chloride	8,5 g
Sorbitol	20,0 g
Bile salts (bile salt no. 3)	1,5 g
Water	1 000 ml

D.3.1.2 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 7,6 ± 0,2 at 25 °C.

Dispense the medium into containers of suitable capacity to obtain portions appropriate for the test samples (see D.3.1).

Sterilize for 15 min in an autoclave (7.1) set at 121 °C.

D.3.2 Modified rappaport broth (MRB) with magnesium chloride

D.3.2.1 Basic medium

D.3.2.1.1 Composition

Enzymatic digest of casein	10,0 g
Yeast extract	1,0 g
Disodium hydrogen phosphate (Na_2HPO_4)	2,0 g
Malachite green	0,013 g
Water	800 ml

D.3.2.1.2 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 the pH of the complete medium is 5,8 ± 0,1 at 25 °C.

Sterilize for 15 min in an autoclave (7.1) set at 121 °C.

D.3.2.2 Magnesium chloride solution (0,4 g/ml)

D.3.2.2.1 Composition

Magnesium chloride hexahydrate ($MgCl_2 \cdot 6H_2O$)	80,0 g
Water	200 ml

D.3.2.2.2 Preparation

Dissolve the magnesium chloride in the water.

Sterilize for 15 min in an autoclave (7.1) set at 121 °C.

D.3.2.3 Complete medium

D.3.2.3.1 Composition

Basic medium (D.3.2.1)	800 ml
Magnesium chloride solution (D.3.2.2)	200 ml

D.3.2.3.2 Preparation

Add the magnesium chloride solution aseptically to the basic medium and mix.

Dispense the medium aseptically in 10 ml amounts into tubes (7.3).

The solutions shall be cooled to room temperature before mixing to avoid formation of precipitate.

D.3.3 Performance testing for the quality assurance of the cold enrichment media

For the definition of selectivity and productivity refer to ISO 11133. The inoculum volume should be the same as that used in the method for that medium and should contain the number of target or non-target organisms specified in ISO 11133:2014, 5.4.

Table D.1 — Performance testing for the quality assurance of the cold enrichment media

Medium	Function	Incubation	Control strains	WDCM numbers[a]	Criteria[d]
MRB	Productivity	44 h ± 4 h/ 25 °C ± 1 °C	*Yersinia enterocolitica* + *Escherichia coli*[c] + *Pseudomonas aeruginosa*	00160[b] + 00012 or 00013 + 00025	> 10 characteristic colonies on CIN (see 10.5)
	Selectivity		*Pseudomonas aeruginosa* *Proteus mirabilis*	00025 00023	Total inhibition (0) or partial inhibition (< 10 colonies) on TSA
CEB	Productivity	44 h ± 4 h/ 25 °C ± 1 °C	*Yersinia enterocolitica* + *Pseudomonas aeruginosa*	00160[b] + 00025	> 10 characteristic colonies on CIN (see 10.5)

[a] Refer to the reference strain catalogue available at www.wfcc.info for information on culture collection strain numbers and contact details; WDCM: World Data Centre for Microorganisms.

[b] Strain to be used as a minimum.

[c] Strain free of choice; one of the strains has to be used as a minimum.

[d] Growth is categorized as 0: no growth; 1: weak growth (partial inhibition); 2: good growth (see ISO 11133).

NOTE The colony morphology of strain WDCM 00160 (bioserotype 1B/O:8) is not representative of major pathogenic bioserotypes of *Y. enterocolitica* (bioserotypes 4/O:3 and 2/O:9) on CIN agar. See 10.6.2 for examples of control strains for these bioserotypes (to be used as reference for colony morphology during sample analyses).

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