TECHNICAL SPECIFICATION

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Microbiology of the food chain — Polymerase chain reaction (PCR) for the detection of food-borne pathogens — Detection of pathogenic *Yersinia enterocolitica* and *Yersinia pseudotuberculosis*

Microbiologie de la chaîne alimentaire — Réaction de polymérisation en chaîne (PCR) pour la détection de micro-organismes pathogènes dans les aliments — Détection des Yersinia enterocolitica *et* Yersinia pseudotuberculosis *pathogènes*

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Foreword

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The committee responsible for this document is the European Committee for Standardization (CEN) Technical Committee CEN/TC 275, *Food analysis — Horizontal methods*, in collaboration with 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).

Introduction

Yersinia enterocolitica and *Yersinia pseudotuberculosis* are zoonotic bacterial pathogens causing food-borne infection (yersiniosis) in humans worldwide. The main reservoir for pathogenic *Y. enterocolitica* is domestic pigs[3] and for *Y. pseudotuberculosis* a wide range of domestic and wild animals such as rodents, deer, birds, and various farm animals serve as potential reservoirs.[4] Some of the biotypes of *Y. enterocolitica* are associated with human infection. In contrast, all *Y. pseudotuberculosis* are considered potentially pathogenic to humans.[9]

The chromosomally located gene *ail* (attachment invasion locus) is present in all bio(sero)types of *Y. enterocolitica* associated with disease and a variant of it is also present in *Y. pseudotuberculosis*.[8] The *ail* gene is the target gene used for detection in this Technical Specification, and the developed primer/probe sets target different sites of the *ail* gene for the two pathogens.[7][8][13][14]

Microbiology of the food chain — Polymerase chain reaction (PCR) for the detection of food-borne pathogens — Detection of pathogenic *Yersinia enterocolitica* and *Yersinia pseudotuberculosis*

1 Scope

This Technical Specification specifies two horizontal methods for detection of the pathogenic bioserotypes of *Y. enterocolitica* and one for detection of *Y. pseudotuberculosis* by using real-time PCR-based methods. The described methods allow for the detection of the two pathogens in enrichments and allow the isolation of colonies. *Y. pestis*, the causative agent of bubonic and pneumonic plague harbours a variant of the *ail* gene as well and will be detected by the same primer/probe set as *Y. pseudotuberculosis*. However, *Y. pestis* is normally not associated with food. This Technical Specification is applicable to products for human consumption, animal feeding stuffs, and environmental samples.

2 Normative references

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

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

ISO 10273, *Microbiology of food and animal feeding stuffs — Horizontal method for the detection of presumptive pathogenic* Yersinia enterocolitica

ISO 20837, *Microbiology of food and animal feeding stuffs — Polymerase chain reaction (PCR) for the detection of food-borne pathogens — Requirements for sample preparation for qualitative detection*

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

ISO 22119, *Microbiology of food and animal feeding stuffs — Real-time polymerase chain reaction (PCR) for the detection of food-borne pathogens — General requirements and definitions*

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

3 Terms and definitions

For the purpose of this document, the following terms and definitions given in ISO 22174 and ISO 22119 apply.

4 Principles

4.1 General

The method comprises the following consecutive steps:

a) Microbial enrichment (4.2);

b) Nucleic acid extraction (4.3);

c) Amplification and detection (4.4);

d) Isolation (4.5).

4.2 Microbial enrichment

The number of pathogenic *Y. enterocolitica* and *Y. pseudotuberculosis* bacterial cells is increased by growth in a non-selective or semi-selective liquid nutrient medium.

4.3 Nucleic acid extraction

Bacteria cells are separated from the nutrient broth, lysed, and the nucleic acid extracted for use in the PCR reaction.

4.4 Amplification and detection

The extracted nucleic acid is amplified using a probe-based real-time PCR. Detection of the target sequence is achieved by monitoring a clear increase in the fluorescence signal above the cycle threshold, *Ct*.

NOTE Probe-based real-time PCR combines amplification, detection, and confirmation of the target DNA.

4.5 Isolation

After a PCR-positive result is obtained, the target organism can be isolated by using culture methods as described in this Technical Specification.

5 Reagents

5.1 General

For the stages in 4.1 b)-c), molecular grade reagents and consumables suitable for molecular biology shall be used as given in ISO 20837 and ISO 20838.

Requirements are specified in ISO 20838.

The following media and reagents should be used.

5.2 Culture media

5.2.1 General

See ISO 7218 and ISO 11133 for the preparation, production, and performance testing of culture media.

5.2.2 Diluent

See ISO 6887-1 and the relevant part of ISO 6887 dealing with the product to be examined.

5.2.3 Enrichment media

5.2.3.1 Tryptone-soya broth supplemented with yeast, TSBY

5.2.3.1.1 Composition

Pancreatic digest of casein	17,0 g

Papaic digest of soyabean meal	3,0 g
Sodium chloride, (NaCl)	5,0 g
Dibasic potassium phosphate, (K_2 HPO_4)	2,0 g
Glucose	2,5 g
Yeast extract	6,0 g
Water	1 000 ml

5.2.3.1.2 Preparation

Dissolve the above ingredients in 1 000 ml distilled water. Adjust the pH, if necessary, so that after sterilization it is pH 7,3 ± 0,2. Dispense the medium into tubes or flasks of suitable capacity to obtain portions appropriate for the test samples. Sterilize for 15 min at 121 °C ± 1 °C.

Store the medium in the dark at room temperature and not longer than 4 weeks.

Alternatively, use dehydrated Tryptone soya broth (TSB) 30 g/l supplemented with 0,6 % yeast extract, pH 7,3 ± 0,2.

5.2.3.2 Peptone-sorbitol-bile-salt broth, PSB[15]

5.2.3.2.1 Composition

Peptone	5,0 g
Sorbitol	10,0 g
Sodium chloride, (NaCl)	5,0 g
Disodium hydrogen phosphate (Na_2HPO_4)	8,23 g
Sodium dihydrogen phosphate monohydrate ($NaH_2PO_4.H_2O$)	1,2 g
Bile salts	1,5 g
Water	1 000 ml

5.2.3.2.2 Preparation

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

Adjust the pH, if necessary, so that after sterilization it is pH 7,6 ± 0,2.

Dispense the medium into tubes or flasks of suitable capacity to obtain portions appropriate for the test samples. Sterilize for 15 min at 121 °C ± 1 °C.

5.2.3.3 Cold enrichment broth, PMB [5]

5.2.3.3.1 Composition

Disodium hydrogen phosphate (Na_2HPO_4)	7,6 g
Potassium dihydrogen phosphate (KH_2PO_4)	1,0 g
Sodium chloride (NaCl)	8,5 g

Mannitol	10,0 g
Bile salts N°. 3	1,5 g
Water	1 000 ml

5.2.3.3.2 Preparation

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

Adjust the pH, if necessary, so that after sterilization it is pH 7,6 ± 0,2.

Dispense the medium into tubes or flasks of suitable capacity to obtain portions appropriate for the test samples. Sterilize for 15 min at 121 °C ± 1 °C.

5.2.4 Selective solid medium

5.2.4.1 Cefsulodin Irgasan Novobiocin agar, CIN[10]

5.2.4.1.1 Basic medium, 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, (NaCl)	1,0 g
Magnesium sulfate, ($MgSO_4.7\ H_2O$)	0,01 g
Sodium desoxycholate	0,5 g
Neutral red	0,03 g
Crystal violet	0,001 g
Agar	12,5 g
Water	1 000 ml

5.2.4.1.2 Preparation

Dissolve the components or dehydrated basic medium in the water by boiling. Adjust the pH, if necessary, so that after sterilization it is pH 7,4 ± 0,2 at 25 °C. Dispense the medium into flasks of suitable capacity. Sterilize for 15 min at 121 °C ± 1 °C.

5.2.4.2 Supplements

5.2.4.2.1 **Cefsulodin solution** (15 mg/ml)

Dissolve 1,5 g Cefsulodin in 100 ml water. Sterilize by filtration.

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

Dissolve Irgasan in ethanol, store the solution at about –20 °C for not more than 4 weeks.

5.2.4.2.3 **Novobiocin solution** (2,5 mg/ml)

Dissolve novobiocin in water. Sterilize by filtration.

5.2.4.3 Composition of the complete medium

Basic medium (5.2.4.1.1)	997 ml
Cefsulodin solution (5.2.4.2.1)	1 ml
Irgasan solution (5.2.4.2.2)	1 ml
Novobiocin solution (5.2.4.2.3)	1 ml

5.2.4.4 Preparation

Add each antibiotic solution aseptically to the basic medium, cooled to about 45 °C, and mix. Pour approximately 15 ml of the complete medium into sterile petri dishes.

5.2.5 Potassium hydroxide in saline solution, KOH

5.2.5.1 Composition

Potassium hydroxide (KOH)	0,25 g/0,50 g
Saline solution	100 ml

NOTE It is recommended to use freshly prepared 0,5 % KOH for pathogenic *Y. enterocolitica* and 0,25 % for *Y. pseudotuberculosis.*

5.2.5.2 Preparation

Dissolve the potassium hydroxide in the saline solution. Dispense the solution into flasks of a suitable capacity. Sterilize for 15 min at 121 °C ± 1 °C. Prepare the solution the day before use.

5.3 Nucleic acid extraction

Nucleic acid extraction procedure and reagents appropriate for Gram-negative bacteria shall be used.

NOTE Commercial kits can also be used.

5.4 Reagents for PCR

See ISO 22119 and ISO 20838.

5.5 Primers and probes

The primers and probes for detection of pathogenic *Y. enterocolitica* and *Y. pseudotuberculosis* are listed in Annex B and Annex C.

6 Apparatus and equipment

6.1 General

Microbiology equipment (see ISO 7218, ISO 20837, and ISO 22119), in particular, the following

6.2 Equipment for sample preparation prior to enrichment

Peristaltic blender and sterile bags with filter.

NOTE Filter of small pore size suitable for PCR is recommended.

6.3 Equipment for microbial enrichment

Incubators, capable of operating at 25 °C ± 1 °C and 30 °C ± 1 °C.

6.4 Equipment for nucleic acid extraction

6.4.1 Micro-centrifuge tubes, with capacities of 1,5 ml and 2,0 ml.

6.4.2 Centrifuge, for reaction tubes with a capacity of 1,5 ml and 2,0 ml and capable of achieving an acceleration up to approximately 14 000 × *g*.

6.4.3 Thermoblock, with heating capacity of up to 100 °C.

6.4.4 Graduated pipettes and **pipette filter tips**, for volumes between 1 µl to 1 000 µl.

6.4.5 Mixer.

6.5 Equipment for real-time PCR

6.5.1 Real-time PCR thermal cycler.

6.5.2 96-well plates and/or 8-well strips.

7 Sampling

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

8 Procedure

See diagram in Annex A.

8.1 Sample preparation prior to enrichment

8.1.1 General

It is recommended to analyse at least 25 g or 25 ml of sample. However, if sample amount is limited other sample sizes can be used.

8.1.2 Preparation of the sample

Prepare and homogenize the sample according to ISO 6887-1 and the relevant part of ISO 6887 dealing with the specific product type intended for analysis (see ISO 6887-1 to ISO 6887-5).

A test portion of the sample is added to the enrichment medium to obtain a ratio of the test portion to medium of 1/10.

