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Microbiology of the food chain — Horizontal method for the detection and enumeration of *Enterobacteriaceae* —

Part 2: Colony-count technique

Microbiologie de la chaîne alimentaire — Méthode horizontale pour la recherche et le dénombrement des Enterobacteriaceae —

Partie 2: Technique par comptage des colonies

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Foreword

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

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 meaning of ISO specific terms and expressions related to conformity assessment, as well as information about ISO's adherence to the 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 second edition cancels and replaces the first edition (ISO 21528-2:2004), which has been technically revised with the following main changes:

— the confirmation step has been changed by replacing glucose agar by glucose OF medium;

— precision data based on the results of an interlaboratory study using the method according to this revised edition has been included in an informative annex.

A list of all the parts in the ISO 21528 series can be found on the ISO website.

This corrected version of ISO 21528-2:2017 incorporates the following corrections:

— in 6.4, a water bath capable of being maintained between 44 °C to 47 °C has been added to the list of equipment;

— in 9.3.2, the temperature has been reduced from “47 °C to 50 °C” to “44 °C to 47 °C”.

Introduction

This document is intended to provide general guidance for the examination of products not dealt with by existing International Standards and to be taken into account by organizations preparing microbiological test methods for application to foods or animal feeding stuffs. Because of the large variety of products within this field of application, these guidelines 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 the guidelines provided as far as possible and that deviations from them will only be made if absolutely necessary for technical reasons.

The main changes, listed in the foreword, introduced in this document compared to ISO 21528-2:2004, are considered as minor changes (see ISO 17468).

The harmonization of test methods cannot be immediate, and for certain groups of products, International Standards and/or national standards may already exist that do not comply with this horizontal method. It is hoped that when such standards are reviewed, they will be changed to comply with this 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 and enumeration of *Enterobacteriaceae* —

Part 2: Colony-count technique

WARNING — In order to safeguard the health of laboratory personnel, it is essential that tests for detecting *Enterobacteriaceae* 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.

1 Scope

This document specifies a method for the enumeration of *Enterobacteriaceae*. It is applicable to

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

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

This technique is intended to be used when the number of colonies sought is expected to be more than 100 per millilitre or per gram of the test sample.

The most probable number (MPN) technique, as included in ISO 21528-1, is generally used when the number sought is expected to be below 100 per millilitre or per gram of test sample.

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

ISO 18593, *Microbiology of food and animal feeding stuffs — Horizontal methods for sampling techniques from surfaces using contact plates and swabs*

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
Enterobacteriaceae
microorganism that forms characteristic colonies on violet red bile glucose agar and that ferment glucose and show a negative oxidase reaction when the tests are carried out in accordance with the methods specified in this document

3.2
count of *Enterobacteriaceae*
number of *Enterobacteriaceae* found per millilitre or per gram of the test sample or per unit area swabbed, when the test is carried out according to the method specified in this document

4 Principle

4.1 Preparation of initial suspension and decimal dilutions

An initial suspension and decimal dilutions are prepared from the test sample.

4.2 Isolation and selection for confirmation

Violet red bile glucose (VRBG) agar is inoculated with a specified quantity of the test sample if the product is liquid, or of the initial suspension in the case of other products. An overlay of the same medium is added. Other plates are prepared under the same conditions, using decimal dilutions of the test sample or of the initial suspension.

The dishes are incubated at 37 °C (or 30 °C) for 24 h.

NOTE The incubation temperature of 37 °C for enrichment and isolation/enumeration on the plating medium is generally used when the detection and enumeration of *Enterobacteriaceae* is for a hygiene indicator. Alternatively, a temperature of 30 °C can be chosen when the detection or enumeration of *Enterobacteriaceae* is conducted for technological purposes and includes psychrotrophic *Enterobacteriaceae*. In this document, 37 °C will be used throughout the text.

4.3 Confirmation

Colonies of presumptive *Enterobacteriaceae* are subcultured onto non-selective medium, and confirmed by means of tests for the fermentation of glucose and the presence of oxidase.

4.4 Calculation

The number of *Enterobacteriaceae* per millilitre or gram of the test sample is calculated from the number of confirmed typical colonies per dish.

5 Diluent, culture media and reagent

For current laboratory practice, see ISO 7218.

Composition of culture media and reagents and their preparation are described in Annex A.

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

6 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.

6.1 Apparatus for dry sterilization (oven) or **wet sterilization** (autoclave), as specified in ISO 7218.

6.2 Incubator, capable of operating at 37 °C ± 1 °C (or 30 °C ± 1 °C).

6.3 Drying cabinet (ventilated by convection) or **incubator**, capable of operating between 25 °C and 50 °C.

6.4 Water baths, one capable of being maintained between 47 °C to 50 °C and another capable of being maintained between 44 °C to 47 °C.

6.5 Test tubes or **flasks,** of appropriate capacity.

6.6 Petri dishes, made of glass or plastic, with a diameter of approximately 90 mm and (optional) large size (diameter approximately 140 mm).

6.7 Loops (of diameter approximately 3 mm) and **wires**, made of platinum/iridium or nickel/chromium, and/or **glass rods**, or equivalent sterile disposable loops or inoculating needles.

6.8 Graduated pipettes or **automatic pipettes**, of nominal capacities 10 ml, 1 ml and 0,1 ml.

6.9 pH-meter, accurate to within ±0,1 pH unit at 25 °C.

6.10 Homogenizer, as specified in ISO 7218.

7 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:

— 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.

8 Preparation of test sample

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

9 Procedure

9.1 General

See ISO 7218.

9.2 Test portion, initial suspension and dilutions

See ISO 6887 (all parts).

Prepare a single decimal dilution series from the test sample if the product is liquid, or from the initial suspension in the case of other products.

9.3 Inoculation and incubation

9.3.1 Take one sterile Petri dish (6.6). Using a sterile pipette (6.8), transfer to the dish 1 ml of the test sample if the product is liquid, or 1 ml of the initial suspension in case of other products.

Repeat the procedure described with the further dilutions, if necessary, using a fresh sterile pipette for each dilution.

If only the initial suspension is used, then inoculate two plates of this dilution (ISO 7218).

9.3.2 Add into each Petri dish approximately 15 ml of the violet red bile glucose (VRBG) agar (A.2) which has been prepared then cooled to 44 °C to 47 °C in the water bath (6.4). The time elapsing between the inoculation of the Petri dishes and the moment when the medium is poured into the dishes shall not exceed 15 min.

Carefully mix the inoculum with the medium by horizontal movements and allow the medium to solidify, with the Petri dishes standing on a cool surface.

9.3.3 After complete solidification of the mixture, add a covering layer of approximately 5 ml to 10 ml of the violet red bile glucose (VRBG) agar (A.2), prepared then cooled as described in 9.3.2, to prevent spreading growth and to achieve semi-anaerobic conditions. Allow to solidify as described above.

9.3.4 Invert the prepared dishes and incubate them at 37 °C (6.2) for 24 h ± 2 h.

9.4 Counting and selection of colonies for confirmation

Characteristic colonies are pink to red or purple (with or without precipitation haloes).

Select the dishes (see 9.3.4) containing less than 150 characteristic colonies; count these colonies. Then choose at random five such colonies from each dish for subculturing (see 9.5) for the biochemical confirmation tests (see 9.6). If there are less than five colonies on the plate, take all presumptive colonies present.

Spreading colonies shall be considered as single colonies. If less than one-quarter of the dish is overgrown by spreading, count the colonies on the unaffected part of the dish and calculate by extrapolation the theoretical number of colonies for the entire plate. If more than one-quarter is overgrown by spreading colonies, discard the count.

Certain *Enterobacteriaceae* may cause decolouration of their colonies or of the medium. Therefore, when no characteristic colonies are present, choose five whitish colonies for confirmation.

9.5 Subculturing selected colonies

Streak the selected colonies (9.4) onto the surface of pre-dried non-selective agar medium (A.3), in a manner which will allow well-isolated colonies to develop.

Incubate these plates at 37 °C for 24 h ± 2 h.

Select a well-isolated colony from each of the incubated plates for the biochemical confirmation tests (see 9.6).

9.6 Biochemical confirmation tests

9.6.1 Oxidase reaction

Using a platinum/iridium loop, wire or glass rod (6.7), take a portion of each well-isolated colony (see 9.5) and streak onto a filter paper moistened with the oxidase reagent (A.5) or onto a commercially available disc or stick. A nickel/chromium loop or wire shall not be used.

Consider the test to be negative when the colour of the filter paper does not turn dark blue purple within 10 s.

Consult the manufacturer's instructions for ready-to-use discs or sticks.

9.6.2 Fermentation test

Using a wire (6.7), stab the same colonies selected in 9.5 that gave a negative oxidase test into tubes containing Glucose OF medium (A.4). Overlay the surface of the medium with minimal 1 cm of sterile mineral oil (A.6).

Incubate these tubes at 37 °C for 24 h ± 2 h.

If a yellow colour develops throughout the content of the tube, regard the reaction as being positive.

9.6.3 Interpretation of biochemical tests

Colonies that are oxidase-negative and glucose-positive are confirmed as *Enterobacteriaceae*.

10 Expression of results

See ISO 7218. Calculate and report the results as the number of *Enterobacteriaceae* in cfu per gram, millilitre, per square centimetre or per sampling device.

11 Performance characteristics of the method

11.1 Interlaboratory study

Results of the interlaboratory study to determine the precision of the method are summarized in Annex B. Repeatability and reproducibility limits were determined using five types of food contaminated at various levels. The values derived from the interlaboratory study may not be applicable to concentration ranges and food types other than those given in Annex B.

NOTE In this document, the word "type" is combined with "food" to improve the readability of this document. However, the word "food" is interchangeable with "feed" and the other areas of the food chain as mentioned in the scope of this document.

11.2 Repeatability limit

The absolute difference between two independent single ($\log_{10}$-transformed) test results (number of cfu per gram or per millilitre) or the ratio of the higher to the lower of the two test results on the normal scale, obtained using the same method on identical test material in the same laboratory by the same operator using the same apparatus within the shortest feasible time interval, will, in not more than 5 % of cases, exceed the repeatability limit *r*.

As a general indication of repeatability limit (*r*), the following values (derived from the mean of the variance estimates for all levels per matrix tested in the interlaboratory study (see Annex B) may be used when testing samples:

r = 0,37 (expressed as a difference between $\log_{10}$-transformed test results); or

r = 2,33 (expressed as a ratio between test results).

EXAMPLE A test result of 10 000 or 1,0 × 10^4 or $\log_{10}$ 4,0 cfu per gram of sample material was observed in a given laboratory. Under repeatability conditions, the difference between $\log_{10}$-transformed results should not be greater than ±0,37 $\log_{10}$ units. So the result from a second test of the same sample should be between 3,63 (4,0 - 0,37) and 4,37 (4,0 + 0,37) $\log_{10}$ units.

For non-log-transformed results, the ratio between the first test result and the second test result from the same sample should not be greater than 2,33. So the second test result should be between 4 290 (= 10 000/2,33) and 23 300 (10 000 × 2,33) cfu per gram.

11.3 Reproducibility limit

The absolute difference between two single ($\log_{10}$-transformed) test results (number of cfu per gram or per millilitre) or the ratio of the higher to the lower of the two test results on the normal scale, obtained using the same method on identical test material in different laboratories with different operators using different equipment, will, in not more than 5 % of cases, exceed the reproducibility limit R.

As a general indication of reproducibility limit (R), the following values (derived from the mean of the variance estimates for all levels per matrix tested in the interlaboratory study (see Annex B) are used when testing food samples in general:

R = 0,87 (expressed as a difference between $\log_{10}$-transformed test results); or

R = 7,38 (expressed as a ratio between test results).

EXAMPLE 1 A test result of 10 000 or 1,0 × 10^4 or $\log_{10}$ 4,0 cfu per gram of sample material was observed in a first laboratory. Under reproducibility conditions, the difference between $\log_{10}$-transformed results should not be greater than ±0,87 $\log_{10}$ units. So the result from a second laboratory should be between 3,13 (4,0 – 0,87) and 4,87 (4,0 + 0,87) $\log_{10}$ units.

For non-log-transformed results, the ratio between the test result from this first laboratory and a second laboratory should not be greater than 7,38. So the result from the second laboratory should be between 1 360 (= 10 000/7,38) and 73 800 (10 000 × 7,38) cfu per gram.

EXAMPLE 2 A laboratory wants to know the maximum value it may find for a sample, which is still in compliance with a pre-set limit (e.g. a limit of 1 000 or $\log_{10}$ 3). For this, the R value (on the log scale) has to be multiplied by a factor of 0,59.

The factor 0,59 reflects the fact that a test with a one-sided 95 % interval is used to test whether the limit is exceeded; it is obtained from the following formula: $0{,}59 = \frac{1{,}64}{1{,}96 \times \sqrt{2}}$

The maximum value is 0,51 (0,87 × 0,59) as a difference between $\log_{10}$-transformed test results or 3,26 ($10^{0{,}51}$) as a ratio between test results. So results up to $\log_{10}$ 3,51 ($\log_{10}$ 3 + $\log_{10}$ 0,51) or 3 260 (1 000 × 3,26) do not indicate non-compliance with the limit.

12 Test report

The test report shall specify:

— the test method used, with a reference to this document, i.e. ISO 21528-2;

— the sampling method used, if known;

— 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 deviations (e.g. in the media or the incubation conditions used);

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

— the test result(s) obtained.

13 Quality assurance

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

Annex A
(normative)

Culture media and reagents

A.1 Diluent

As specified in ISO 6887-1 and ISO 18593.

A.2 Violet red bile glucose (VRBG) agar

A.2.1 Composition

Enzymatic digest of animal tissues	7,0 g
Yeast extract	3,0 g
Bile salts	1,5 g
Glucose	10,0 g
Sodium chloride	5,0 g
Neutral red	0,03 g
Crystal violet	0,002 g
Agar	9 g to 18 g [a]
Water	1 000 ml

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

A.2.2 Preparation

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

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

Dispense the culture medium into sterile tubes or flasks (6.5) of appropriate capacity.

Do not sterilize the medium.

Prepare the medium just before use. Use the molten medium within 4 h of its preparation.

A.2.3 Performance testing for the quality assurance of the culture medium

For the definition of selectivity and productivity, refer to ISO 11133. Table A.1 shows the performance criteria of violet red bile glucose (VRBG) agar.

Table A.1 — Performance criteria of violet red bile glucose (VRBG) agar

Medium	Microorganisms	Function	Incubation	Control strains	WDCM[a] number	Reference media	Method of control	Criteria[g]	Characteristic reactions
VRBG (Solid medium)	*Enterobacteriaceae*	Productivity	(24 ± 2) h / (37 ± 1) °C	*Escherichia coli* *Salmonella* Typhimurium[c,d] *Salmonella* Enteritidis[c,d]	00012[b] 00013 00031 00030	TSA[e]	Quantitative	P_R [f] ≥ 0,5	Pink to red colonies with or without precipitation halo
		Selectivity		*Enterococcus faecalis*[d]	00009 or 00087		Qualitative	Total inhibition (0)	

[a] Refer to the reference strain catalogue 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 by the user laboratory (minimum).

[c] Some national restrictions and directions may require the use of a different serovar. Make reference to national requirements relating to the choice of Salmonella serovars.

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

[e] TSA = tryptone soya agar.

[f] PR = productivity ratio.

[g] Growth/turbidity is categorized as 0: no growth/no turbidity; 1: weak growth/slight turbidity; 2: growth/good turbidity.

A.3 Non-selective agar medium

A.3.1 General

The choice of the non-selective agar medium for purity check is left to the discretion of the testing laboratory. The manufacturer's instructions should be followed regarding its preparation for use. An example of a non-selective agar medium is nutrient agar (NA).

A.3.2 Composition nutrient agar

Meat extract	3,0 g
Enzymatic digest of animal tissues	5,0 g
Sodium chloride	5,0 g
Agar	9 g to 18 g[a]
Water	1 000 ml

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

A.3.3 Preparation

Dissolve the components or the dehydrated complete medium in the water by heating, with frequent agitation.

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

Transfer the culture medium into sterile tubes or flasks (6.5) of appropriate capacity.

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

A.3.4 Preparation of agar plates

Cool the medium to 47 °C to 50 °C in a water bath (6.4), mix and pour into sterile Petri dishes (6.6). Allow to solidify.

Immediately before use, dry the agar plates carefully (preferably with the lids off and the agar surface downwards) in the oven (6.3) and set between 25 °C and 50 °C until the surface of the agar is dry.

Store the poured plates, protected from drying, at 5 °C ± 3 °C for up to 4 weeks.

A.3.5 Performance testing for the quality assurance of the culture medium

For the definition of selectivity and productivity, refer to ISO 11133. Table A.2 shows the performance criteria of nutrient agar.

Table A.2 — Performance criteria of nutrient agar

Medium	Microorganisms	Function	Incubation	Control strains	WDCM[a] number	Method of control	Criteria[c]
Nutrient agar (Solid medium)	*Enterobacteriaceae*	Productivity	(24 ± 2) h / (37 ± 1) °C	*Escherichia coli*	00012[b] 00013	Qualitative	Good growth (2)

[a] Refer to the reference strain catalogue 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 by the user laboratory (minimum).

[c] Growth/turbidity is categorized as: 0: no growth/no turbidity; 1: weak growth/slight turbidity; 2: growth/good turbidity.

A.4 Glucose OF medium

A.4.1 Composition

Enzymatic digest of casein	2,0 g
Dipotassium hydrogen phosphate (K2HPO4)	0,3 g
Glucose	10,0 g
Sodium chloride	5,0 g
Bromthymol blue	0,08 g
Agar	3 g to 4 g[a]
Water	1 000 ml

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

A.4.2 Preparation

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

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

Dispense the culture medium in tubes (6.5) of appropriate capacity (e.g. 10 ml of culture medium for tubes of 16 mm × 160 mm).

Sterilize for 15 min in an autoclave (6.1) set at 121 °C. Leave the tubes in vertical position.

The medium may be stored for up to 4 weeks at 5 °C ± 3 °C.

Just before use, heat the medium in boiling water or flowing steam for 15 min to remove oxygen, then cool rapidly to the incubation temperature.

A.4.3 Performance testing for the quality assurance

Performance of the Glucose OF medium shall be verified before use (ISO 11133).

Examples of suitable control strains are *Escherichia coli* WDCM 00012 (positive control) and *Pseudomonas aeruginosa* WDCM 00025 (negative control; yellow colour only at the top of the tube).

A.5 Oxidase reagent

A.5.1 Composition

N,N,N′,N′-Tetramethyl-*p*-phenylenediamine dihydrochloride	1,0 g
Water	100 ml

A.5.2 Preparation

Dissolve the component in the cold water.

Prepare the reagent just before use.

Commercially available discs or sticks may be used. In this case, follow the manufacturer's recommendations.

A.5.3 Performance testing for the quality assurance

Performance of the oxidase reagents shall be verified before use (ISO 11133).

Examples of suitable control strains are *Pseudomonas aerigunosa* WDCM 00025 (positive control), *Escherichia coli* WDCM 00012 (negative control).

A.6 Sterile mineral oil

Mineral oil can be sterilized for 1 h to 2 h at 160 °C in a hot air oven.