BRITISH STANDARD

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Microbiology of food and animal feeding stuffs — Horizontal methods for the detection and enumeration of Enterobacteriaceae —

Part 2: Colony-count method

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British Standards

National foreword

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Summary of pages

This document comprises a front cover, an inside front cover, the ISO title page, pages ii to v, a blank page, pages 1 to 10 an inside back cover and a back cover.

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INTERNATIONAL STANDARD

ISO 21528-2

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Microbiology of food and animal feeding stuffs — Horizontal methods for the detection and enumeration of Enterobacteriaceae —

Part 2: Colony-count method

Microbiologie des aliments — Méthodes horizontales pour la recherche et le dénombrement des Enterobacteriaceae —

Partie 2: Méthode par comptage des colonies

Reference number
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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.

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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.

ISO 21528-2 was prepared by Technical Committee ISO/TC 34, *Food products*, Subcommittee SC 9, *Microbiology*.

This first edition of ISO 21528-2, together with ISO 21528-1:2004, cancels and replaces the following standards:

— ISO 5552:1997, *Meat and meat products — Detection and enumeration of Enterobacteriaceae without resuscitation — MPN technique and colony-count technique;*

— ISO 7402:1993, *Microbiology — General guidance for the enumeration of Enterobacteriaceae without resuscitation — MPN technique and colony-count technique;*

— ISO 8523:1991, *Microbiology — General guidance for the detection of Enterobacteriaceae with pre-enrichment.*

ISO 21528 consists of the following parts, under the general title *Microbiology of food and animal feeding stuffs — Horizontal methods for the detection and enumeration of Enterobacteriaceae*:

— *Part 1: Detection and enumeration by MPN technique with pre-enrichment*

— *Part 2: Colony-count method*

Introduction

This part of ISO 21528 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.

When this part of ISO 21528 is next reviewed, account will be taken of all information then available regarding the extent to which the guidelines have been followed and the reasons for deviation from them in the case of particular products.

The harmonization of test methods cannot be immediate, and for certain groups of products International Standards and/or national standards may already exist that do not comply with this horizontal method. It is hoped that when such standards are reviewed they will be changed to comply with this part of ISO 21528 so that eventually the only remaining departures from this horizontal method will be those necessary for well-established technical reasons.

Microbiology of food and animal feeding stuffs — Horizontal methods for the detection and enumeration of Enterobacteriaceae —

Part 2:
Colony-count method

1 Scope

This part of ISO 21528 specifies a method, without pre-enrichment, 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 food production and food handling.

Enumeration is carried out by counting colonies in a solid medium after incubation at 37 °C (or 30 °C)[1)].

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

2 Normative references

The following referenced documents are indispensable for the application 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 6887-1, *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 6887-2, *Microbiology of food and animal feeding stuffs — Preparation of test samples, initial suspension and decimal dilutions for microbiological examination — Part 2: Specific rules for the preparation of meat and meat products*

ISO 6887-3, *Microbiology of food and animal feeding stuffs — Preparation of test samples, initial suspension and decimal dilutions for microbiological examination — Part 3: Specific rules for the preparation of fish and fishery products*

ISO 6887-4, *Microbiology of food and animal feeding stuffs — Preparation of test samples, initial suspension and decimal dilutions for microbiological examination — Part 4: Specific rules for the preparation of products other than milk and milk products, meat and meat products, and fish and fishery products*

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1) The temperature of 37 °C is generally used when the enumeration of Enterobacteriaceae is for a hygienic indicator. Alternatively, a temperature of 30 °C can be chosen when the enumeration of Enterobacteriaceae is conducted for technological purposes and includes psychrotrophic Enterobacteriaceae.

ISO 7218:1996, *Microbiology of food and animal feeding stuffs — General rules for microbiological examinations*, and Amendment 1:2001.

ISO 8261, *Milk and milk products — General guidance for the preparation of test samples, initial suspensions and decimal dilutions for microbiological examination*

ISO/TS 11133-1, *Microbiology of food and animal feeding stuffs — Guidelines on preparation and production of culture media — Part 1: General guidelines on quality assurance for the preparation of culture media in the laboratory*

ISO/TS 11133-2:2003, *Microbiology of food and animal feeding stuffs — Guidelines on preparation and production of culture media — Part 2: Practical guidelines on performance testing of culture media*

3 Terms and definitions

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

3.1
Enterobacteriaceae
microorganisms that form 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 part of ISO 21528

3.2
count of Enterobacteriaceae
number of Enterobacteriaceae found per millilitre or per gram of the test sample when the test is carried out according to the method specified in this part of ISO 21528

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

Violet red bile glucose agar contained in two Petri dishes (poured-plate technique) 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 pairs of 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)[1)] for 24 h ± 2 h.

4.3 Confirmation

Subculture of colonies of presumptive Enterobacteriaceae on non-selective medium, and confirmation by means of tests for fermentation of glucose and 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, ISO/TS 11133-1 and ISO/TS 11133-2.

5.1 Diluent

See ISO 6887-1.

5.2 Culture media

5.2.1 Violet red bile glucose (VRBG) agar

5.2.1.1 Composition

Enzymatic digest of animal tissues	7,0 g
Yeast extract	3,0 g
Bile salts No. 3	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.	

5.2.1.2 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 capacity not more than 500 ml.

Do not sterilize the medium.

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

5.2.1.3 Performance testing for the quality assurance of the culture medium

For the definition of selectivity and productivity refer to ISO/TS 11133-1. For the performance criteria, refer to ISO/TS 11133-2:2003, Table B.1.

5.2.2 Nutrient agar

5.2.2.1 Composition

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.	

5.2.2.2 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 7,3 ± 0,2 at 25 °C.

Dispense the culture medium into sterile tubes or flasks (6.5) of capacity not more than 500 ml.

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

5.2.2.3 Preparation of agar plates

Transfer portions of about 15 ml of the culture medium, melted and cooled to approximately 47 °C, to Petri dishes (6.6) and allow to solidify.

Just before use, dry the plates, preferably with the lids off and the agar surface downwards, in a drying cabinet (6.3) until the agar is dry.

If prepared in advance, the undried plates may be stored in conditions that do not change their composition for up to 2 weeks at 5 °C ± 3 °C.

5.2.2.4 Performance testing for the quality assurance of the culture medium

For the definition of selectivity and productivity refer to ISO/TS 11133-1. Fort the performance criteria, refer to ISO/TS 11133-2:2003, Table B.6.

5.2.3 Glucose agar

5.2.3.1 Composition

Enzymatic digest of casein	10,0 g
Yeast extract	1,5 g
Glucose	10,0 g
Sodium chloride	5,0 g
Bromocresol purple	0,015 g
Agar	9 g to 18 g[a]
Water	1 000 ml

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

5.2.3.2 Preparation

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

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

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

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

The medium may be stored for up to 1 week at 5 °C ± 3 °C.

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

5.3 Oxidase reagent

5.3.1 Components

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

5.3.3 Preparation

Dissolve the component in the cold water.

Prepare the reagent just before use.

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

6 Apparatus and glassware

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

6.1 Apparatus for dry sterilization (oven) or **wet sterilization** (autoclave)

See ISO 7218.

6.2 Incubator, capable of operating at 37 °C ± 1 °C.

6.3 Drying cabinet (ventilated by convection) or i**ncubator**, capable of operating between 37 °C and 55 °C.

6.4 Water bath, or similar apparatus, capable of being maintained at between 44 °C and 47 °C.

6.5 Containers (e.g. test tubes), of dimensions 16 mm × 160 mm and 20 mm × 200 mm, or **flasks** or **bottles** of capacity between 150 ml and 500 ml, suitable for the sterilization and storage of culture media.

6.6 Petri dishes, made of glass or plastics, of diameter 90 mm to 100 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 Total-delivery graduated pipettes, of 1 ml nominal capacity, graduated in divisions of 0,1 ml with an outflow opening of 2 mm to 3 mm in diameter.

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

6.10 Homogenizer

See ISO 7218.

7 Sampling

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

Sampling should be carried out in accordance with the specific International Standard appropriate to the product concerned. If there is no specific International Standard, it is recommended that agreement be reached on this subject by the parties concerned.

8 Preparation of test sample

Prepare the test sample in accordance with ISO 6887-1, ISO 6887-2, ISO 6887-3, ISO 6887-4 or ISO 8261 and/or the specific International Standard appropriate to the product concerned. If there is no specific International Standard, it is recommended that agreement be reached on this subject by the parties concerned.

9 Procedure

9.1 General

For guidance in carrying out the procedure, see ISO 7218.

9.2 Test portion, initial suspension and dilutions

See ISO 6887-1, ISO 6887-2, ISO 6887-3, ISO 6887-4 and/or ISO 8261.

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

Take two other sterile Petri dishes. Using a fresh sterile pipette, transfer to each dish 1 ml of the first decimal dilution (10^{-1}) of the test sample if the product is liquid, or 1 ml of the first decimal dilution of the initial suspension (10^{-2}) in case of other products.

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

9.3.2 Pour into each Petri dish approximately 10 ml of the violet red bile glucose medium (5.2.1) which has been prepared then cooled to 44 °C to 47 °C in the water bath (6.4). The time elapsing between 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 15 ml of the violet red bile glucose medium (5.2.1), 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 in the incubator (6.2) set at 37 °C 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 for subculturing (see 9.5) for the biochemical confirmation tests (see 9.6).

Consider the determination to be void if half or more than half of the surface area of a dish is overgrown. If less than half of the surface of a dish is overgrown, count the colonies on the clear part and extrapolate so that the number corresponds to the total surface area of the dish.

Certain Enterobacteriaceae may cause decoloration 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 onto nutrient agar plates (5.2.2) each of the colonies selected for confirmation (see 9.4).

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 loop or wire or a 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 (5.3) or onto a commercially available disc. 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 within 10 s.

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

9.6.2 Fermentation test

Stab, using a wire (6.7), the same colonies selected in 9.5 that gave a negative oxidase test into tubes containing glucose agar (5.2.3).

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

If a yellow colour develops throughout the contents 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/Amd.1.

11 Test report

The test report shall specify:

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

— the sampling method used, if known;

— the test method used, with reference to this part of ISO 21528;

— the incubation temperature used;

— all operating details not specified in this part of ISO 21528, or regarded as optional, together with details of any incidents which may have influenced the test results;

— the test results obtained.

Annex A
(normative)

Confidence limits for the estimation of small numbers of colonies

The confidence limits at the 95 % level for the estimation of small numbers, when the number of colonies retained is less than 15, are given in Table A.1.

Table A.1 — Confidence limits for the estimation of small numbers of colonies

Number of microorganisms	Confidence limits at the 95 % level	
	lower	upper
1	<1	2
2	<1	4
3	<1	5
4	1	6
5	2	9
6	2	10
7	2	12
8	3	13
9	4	14
10	4	16
11	5	18
12	6	19
13	7	20
14	7	21
15	8	23

Bibliography

[1] COWELL N.D. and MORISETTI M.D. *J. Sci. Fd. Agric.*, **20**, 1969, p. 573

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