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Meat and meat products — Enumeration of *Pseudomonas* spp.

Viande et produits à base de viande — Dénombrement des Pseudomonas *spp.*

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.

Draft International Standards adopted by the technical committees are circulated to the member bodies for voting. Publication as an International Standard requires approval by at least 75 % of the member bodies casting a vote.

International Standard ISO 13720 was prepared by Technical Committee ISO/TC 34, *Agricultural food products*, Subcommittee SC 6, *Meat and meat products*.

Annex A of this International Standard is for information only.

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Meat and meat products — Enumeration of *Pseudomonas* spp.

1 Scope

This International Standard describes a method for the enumeration of *Pseudomonas* spp. present in meat and meat products, including poultry.

2 Normative references

The following standards contain provisions which, through reference in this text, constitute provisions of this International Standard. At the time of publication, the editions indicated were valid. All standards are subject to revision, and parties to agreements based on this International Standard are encouraged to investigate the possibility of applying the most recent editions of the standards indicated below. Members of IEC and ISO maintain registers of currently valid International Standards.

ISO 3100-2:1988, *Meat and meat products — Sampling and preparation of test samples — Part 2: Preparation of test samples for microbiological examination.*

ISO 6887:1983, *Microbiology — General guidance for the preparation of dilutions for microbiological examination.*

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

3 Definition

For the purposes of this International Standard, the following definition applies.

3.1 ***Pseudomonas***: Bacteria of the genus of *Pseudomonas* which at 25 °C form colonies in cetrimide, fucidin and cephaloridine (CFC) agar when the test is carried out in accordance with this International Standard.

4 Principle

4.1 Inoculation of the surface of a solid selective culture medium, using duplicate plates, with a specified quantity of the test sample if the product is liquid, or with a specified quantity of the initial suspension in the case of other products.

Inoculation, under the same conditions, using decimal dilutions of the test sample or of the initial suspension, with two plates per dilution.

4.2 Aerobic incubation of the plates at 25 °C for 48 h.

4.3 Calculation of the number of *Pseudomonas* per millilitre, or per gram, of sample from the number of typical and/or atypical colonies obtained on plates at dilution levels chosen so as to give a significant result, and confirmed by the oxidase test and growth on Kligler's agar.

5 Dilution fluid, culture media and reagent

5.1 General

For current laboratory practice, see ISO 7218.

5.2 Dilution fluid

See ISO 6887.

1) To be published. (Revision of ISO 7218:1985)

5.3 Cetrimide, fucidin and cephaloridine (CFC) agar [1]

5.3.1 Basic medium

5.3.1.1 Composition

Gelatin peptone	16,0 g
Casein hydrolysate	10,0 g
Potassium sulfate (K_2SO_4)	10,0 g
Magnesium chloride ($MgCl_2$)	1,4 g
Agar	12,0 g to 18,0 g [1)]
Water	1 000 ml

1) Depending on the gel strength of the agar.

5.3.1.2 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 7,2 ± 0,2 at 25 °C.

Dispense the medium in quantities of 100 ml to flasks or bottles of appropriate capacity.

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

5.3.2 Inhibitor solutions

Do not keep solutions for more than 7 days in the refrigerator.

5.3.2.1 Cetrimide solution: Solution A

5.3.2.1.1 Composition

Cetrimide[1)]	0,1 g
Water	100 ml

1) Mixture consisting chiefly of tetradecyltrimethylammonium bromide together with smaller amounts of dodecyltrimethylammonium bromide and cetrimonium bromide

5.3.2.1.2 Preparation

Dissolve the cetrimide in the water.

Sterilize by filtration.

5.3.2.2 Fucidin solution: Solution B

5.3.2.2.1 Composition

Fucidin ($C_{31}H_{47}NaO_6$)	0,1 g
Water	100 ml

5.3.2.2.2 Preparation

Dissolve the fucidin in the water.

Sterilize by filtration.

5.3.2.3 Cephaloridine solution: Solution C

5.3.2.3.1 Composition

Cephaloridine ($C_{19}H_{17}N_3O_4S_2$)	0,1 g
Water	100 ml

5.3.2.3.2 Preparation

Dissolve the cephaloridine in the water.

Sterilize by filtration.

5.3.3 Complete medium

5.3.3.1 Composition

Basic medium	100 ml
Solution A	1 ml
Solution B	1 ml
Solution C	5 ml

5.3.3.2 Preparation

Under aseptic conditions, add the inhibitor solutions to the basic medium, melted and maintained at 47 °C, and mix carefully.

5.3.4 Preparation of CFC agar plates

Pour approximately 15 ml of the complete medium into sterile Petri dishes (6.9). Leave to set.

Immediately before use, dry the agar plates, preferably with the lids removed and with the agar surfaces facing downwards, in the oven (6.2) set at a temperature between 35 °C and 55 °C, until the droplets have disappeared from the surface of the medium. Do not dry them any further. The agar plates can also be dried in a laminar-flow safety cabinet for 30 min with half-open lids, or overnight with the lids in place.

If prepared in advance, the undried agar plates shall not be kept for longer than 1 month at 0 °C to 5 °C.

5.4 Nutrient agar

5.4.1 Composition

Meat extract	3,0 g
Peptone	5,0 g
Sodium chloride (NaCl)	5,0 g
Agar	12,0 g to 18,0 g [1)]
Water	1 000 ml

1) Depending on the gel strength of the agar.

5.4.2 Preparation

Dissolve the dehydrated 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 culture medium into tubes or bottles of capacity not more than 500 ml.

Sterilize for 20 min in the autoclave (6.1) set at 121 °C.

5.4.3 Preparation of nutrient agar plates

Transfer portions of about 15 ml of the recently prepared culture medium to Petri dishes of diameter 90 mm (6.9) and allow to solidify.

Immediately before use, dry the agar plates, as described in 5.3.4.

If prepared in advance, the undried agar plates shall not be kept for longer than 1 month at 0 °C to 5 °C.

5.5 Kligler's agar

5.5.1 Composition

Beef extract	3,0 g
Yeast extract	3,0 g
Pancreatic casein peptone	20,0 g
Sodium chloride (NaCl)	5,0 g
Lactose	10,0 g
Glucose	1,0 g
Ammonium iron(II) sulfate hexahydrate [$(NH_4)_2SO_4 \cdot FeSO_4 \cdot 6H_2O$]	0,5 g
Sodium thiosulfate pentahydrate ($Na_2S_2O_3 \cdot 5H_2O$)	0,5 g
Phenol red	0,025 g
Agar	12,0 g to 18,0 g [1)]
Water	1 000 ml

1) Depending on the gel strength of the agar.

5.5.2 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 7,4 ± 0,2 at 25 °C.

Dispense the medium in 10 ml amounts into test tubes (6.7).

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

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

CAUTION — Do not prepare this medium more than 7 or 8 days before use. Otherwise it shall be melted and reactivated in a boiling water bath, then allowed to resolidify in the proper position.

5.6 Reagent for the detection of oxidase

5.6.1 Composition

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

5.6.2 Preparation

Dissolve the reagent in the water immediately before use.

6 Apparatus and glassware

See ISO 7218.

All glassware shall be resistant to repeated sterilization. See also ISO 6887.

Usual microbiological laboratory equipment and, in particular, the following.

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

See ISO 7218.

6.2 Drying cabinet or **ventilated oven** (for drying the agar plates), capable of being maintained between 35 °C ± 1 °C and 55 °C ± 1 °C, or a **laminar air-flow cabinet**

6.3 Incubator, capable of operating at 25 °C ± 1 °C.

6.4 Water bath, capable of operating at 47 °C ± 2 °C.

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

6.6 Loops, of platinum/iridium, nickel/chromium or sterile plastic, approximately 3 mm in diameter, and **wires** of the same material, or a **glass rod**.

NOTE 1 A nickel/chromium loop is not suitable for use in the oxidase test (see 9.4.2.1).

6.7 Test tubes and **bottles**, of appropriate capacity.

6.8 Total-delivery graduated pipettes, calibrated for bacteriological use only, of nominal capacity 1 ml, graduated in 0,1 ml divisions, and with an outflow opening of diameter 2 mm to 3 mm.

6.9 Petri dishes, made of glass or plastic, of diameter 90 mm to 100 mm.

6.10 Spreaders, made of glass or plastic, for example, hockey sticks made from a glass rod of approximately 3,5 mm diameter and 20 cm length, bent at right angles about 3 cm from one end and with the cut ends made smooth by heating.

7 Sampling

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

Sampling is not part of the method specified in this International Standard. A recommended method of sampling is given in ISO 3100-1[2].

Store the sample, if necessary, in such a way that deterioration and change in composition are prevented.

8 Preparation of test sample

See ISO 3100-2.

9 Procedure

9.1 Test portion, initial suspension and dilutions

Prepare the initial suspension and dilutions in accordance with ISO 6887.

9.2 Inoculation and incubation

9.2.1 Take two CFC agar plates (5.3.4). Using a sterile pipette (6.8), transfer to each plate 0,1 ml of the initial suspension.

Take two other CFC agar plates. Using a fresh sterile pipette, transfer to each plate 0,1 ml of the first decimal solution of the initial suspension (10^{-2}).

Repeat these operations with subsequent dilutions, using a new sterile pipette for each decimal dilution.

9.2.2 Spread the liquid over the surface of the agar plate with a sterile spreader (6.10) until the surface is completely dry.

9.2.3 Invert the dishes, prepared in this way and cooled to 25 °C, and incubate them in the incubator (6.3) set at 25 °C for 48 h.

9.3 Counting and selection of colonies

After the specified incubation period, count the colonies on each plate and retain plates containing 15 to 300 colonies.

Select at random five colonies from each retained plate.

9.4 Confirmation

9.4.1 Subculturing

Streak on nutrient agar plates (5.4.3) each of the colonies selected for confirmation.

Incubate these plates at 25 °C for 24 h. Select a well-isolated colony from each of the incubated plates for biochemical confirmation (see 9.4.2).

9.4.2 Biochemical confirmation

9.4.2.1 Oxidase reaction

9.4.2.1.1 Composition

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

9.4.2.1.2 Preparation

Dissolve the reagent in the water. The reagent shall be prepared immediately prior to use.

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

9.4.2.1.3 Procedure

Moisten a piece of filter paper with the reagent. Take a sample of the bacterial culture obtained from an agar medium using a platinum wire or a glass or plastic rod (a nickel/chrome wire gives false positives) and deposit it on the moistened filter paper.

9.4.2.1.4 Interpretation

In the case of the presence of oxidase, a violet to purple colour appears within a period of between 5 s and 10 s. If the colour has not changed after 10 s, the test is considered as being negative.

9.4.2.2 Use of sugars in Kligler's agar

Streak the slant surface (5.5.2) of the agar with the same colonies as in 9.4.2 and stab the butt to the bottom of the agar. Incubate at 25 °C for 24 h.

9.4.2.3 Interpretation

Colonies which show a positive oxidase reaction and which, when inoculated into Kligler's agar, show development only on the surface (aerobic) shall be considered as *Pseudomonas* colonies.

10 Expression of results

10.1 Method of calculation

After identification, calculate for each of the dishes the number of microorganisms identified, a, using the formula:

$$a = \frac{b}{A} \times C$$

where

A is the number of colonies selected for confirmation (5 in the present case);

b is the number of colonies complying with the identification criteria;

C is the total number of colonies counted.

Calculate the number, N, of identified microorganisms present in the test sample as the weighted mean from two successive dilutions using the formula:

$$N = \frac{\sum a}{V(n_1 + 0{,}1n_2)d}$$

where

Σa is the sum of the colonies counted after identification on all the dishes retained;

V is the volume of inoculum applied to each dish, in millilitres;

n_1 is the number of dishes retained at the first dilution;

n_2 is the number of dishes retained at the second dilution;

d is the dilution factor corresponding to the first dilution retained.

Round off the results calculated to two significant figures. For this, if the last figure is below 5, the preceding figure is not modified; if the last figure is 5 or more, the preceding figure is increased by one unit. Proceed stepwise until two significant figures are obtained.

Take as the result the number of microorganisms per millilitre (liquid product) or per gram (other product), expressed as a number between 1,0 and 9,9 multiplied by the appropriate power of 10.

10.2 Estimated counts

10.2.1 If the two dishes, at the level of the test sample (liquid product) or of the initial suspension (other product), contain less than 15 colonies, calculate the arithmetical mean y of the colonies counted on two dishes.

Express the results as follows:

— for liquid products: estimated number of microorganisms per millilitre $N_E = y$

— for the other products: estimated number of microorganisms per gram $N_E = y/d$

where d is the dilution factor of the initial suspension.

10.2.2 If the two dishes at the level of the test sample (liquid product) or of the initial suspension (other product) do not contain any colonies, express the result as follows:

— less than 1 microorganism per millilitre (liquid product)

— less than $1/d$ microorganisms per gram (other product)

where d is the dilution factor of the initial suspension.

10.3 Confidence limits

See ISO 7218.

11 Test report

The test report shall specify

— the method in accordance with which sampling was carried out;

— the method used;

— the duration of incubation;

— the test result(s) obtained; and

— if the repeatability has been checked, the final quoted result obtained.

It shall also mention all operating details not specified in this International Standard, or regarded as optional, together with details of any incidents which may have influenced the test result(s).

The test report shall include all information necessary for the complete identification of the sample.

Annex A
(informative)

Bibliography

[1] MEAD, G.C. and ADAMS, B.W. A selective medium for the rapid isolation of *Pseudomonas* associated with poultry meat spoilage. *Br. Poult. Sci.*, **18**, 1977, pp. 661-670.

[2] ISO 3100-1:1991, *Meat and meat products — Sampling and preparation of test samples — Part 1: Sampling.*