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Meat and meat products — Detection of *salmonellæ* (Reference method)

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FOREWORD

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Draft International Standards adopted by the Technical Committees are circulated to the Member Bodies for approval before their acceptance as International Standards by the ISO Council.

International Standard ISO 3565 was drawn up by Technical Committee ISO/TC 34, *Agricultural food products*, and circulated to the Member Bodies in May 1974.

It has been approved by the Member Bodies of the following countries :

Australia	Germany	Romania
Austria	Hungary	South Africa, Rep. of
Belgium	India	Spain
Bulgaria	Iran	Thailand
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The Member Bodies of the following countries expressed disapproval of the document on technical grounds :

New Zealand
United Kingdom

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Meat and meat products — Detection of *salmonellæ* (Reference method)

1 SCOPE

This International Standard specifies a reference method for the detection of *salmonellæ* in meat and meat products.

2 FIELD OF APPLICATION

The method can be applied to all kinds of meat and meat products.

3 REFERENCE

ISO 3100, *Meat and meat products — Sampling.*

4 DEFINITIONS

4.1 *salmonellæ* : Micro-organisms which form typical colonies on solid selective media and which display the biochemical and serological characteristics described when the test is carried out according to this method.

4.2 detection of *salmonellæ* : Determination of the presence or absence of these micro-organisms, in a particular mass, when the test is carried out according to the method described.

5 PRINCIPLE

The detection of *salmonellæ* necessitates four successive stages, because they are usually present in low numbers, sometimes in an injured state, and often in the presence of considerably larger numbers of other members of *Enterobacteriaceæ.*

5.1 Pre-enrichment : incubation of the samples in a non-selective liquid medium at 37 °C.

5.2 Enrichment : inoculation of two liquid selective media with the incubated pre-enrichment medium followed by incubation at 37 °C or 42 to 43 °C respectively.

5.3 Plating out : inoculation of the two enrichment media onto solid, selective diagnostic media which, after incubation at 37 °C, are examined for the presence of colonies which from their characteristics are considered presumptive *salmonellæ.*

5.4 Confirmation : subculturing of colonies of presumptive *salmonellæ* and determination of their biochemical and serological characteristics.

6 CULTURE MEDIA AND REAGENTS

6.1 Basic materials

For uniformity of results, it is recommended that either dehydrated culture medium components of uniform quality and analytical grade chemicals, or a dehydrated complete medium, be used. The water used shall be distilled water or water of at least equivalent purity.

NOTE — With regard to brilliant green, note the specifications given in the annex. If dehydrated complete media are used, they should be prepared and used as recommended by the media suppliers.

6.2 Culture media

6.2.1 Buffered peptone water

Composition

peptone	10,0 g
sodium chloride	5,0 g
disodium hydrogen phosphate ($Na_2HPO_4 \cdot 12H_2O$)	9,0 g
potassium dihydrogen phosphate (KH_2PO_4)	1,5 g
water	1 000 ml

Preparation

Dissolve the components in the water by boiling.

Adjust the pH so that after sterilization it is 7,0 ± 0,1 at 20 °C.

Transfer the medium in quantities of 225 ml into flasks of 500 ml capacity.

Sterilize the medium for 20 min at 121 ± 1 °C.

6.2.2 Tetrathionate medium (Muller Kauffmann)

6.2.2.1 BASE

Composition

meat extract	5,0 g
peptone	10,0 g
sodium chloride	3,0 g
calcium carbonate	45 g
water	1 000 ml

Preparation

Add the dehydrated base components or the dehydrated complete base to the water and boil until complete dissolution of the soluble components.

Adjust the pH so that after sterilization it is 7,0 ± 0,1 at 20 °C.

Sterilize the base for 20 min at 121 ± 1 °C.

6.2.2.2 SODIUM THIOSULPHATE SOLUTION

Composition

sodium thiosulphate ($Na_2S_2O_3 \cdot 5H_2O$)	50,0 g
water to a final volume of	100 ml

Preparation

Dissolve the sodium thiosulphate in a part of the water.

Dilute to the final volume.

Sterilize the solution for 20 min at 121 ± 1 °C.

6.2.2.3 IODINE SOLUTION

Composition

iodine	20,0 g
potassium iodide	25,0 g
water to a final volume of	100 ml

Preparation

Dissolve the potassium iodide in a minimal volume of water and add the iodine.

Shake until solution is complete.

Dilute to the final volume.

Store the solution in a tightly closed opaque container.

6.2.2.4 BRILLIANT GREEN SOLUTION

Composition

brilliant green	0,5 g
water	100 ml

Preparation

Add the brilliant green to the water.

Store the solution at least for one day in the dark to allow auto-sterilization to occur.

6.2.2.5 OX BILE SOLUTION

Composition

ox bile, desiccated	10,0 g
water	100 ml

Preparation

Dissolve the desiccated ox bile in the water by boiling.

Sterilize the solution for 20 min at 121 ± 1 °C.

6.2.2.6 COMPLETE MEDIUM

Composition

base (6.2.2.1)	900 ml
sodium thiosulphate solution (6.2.2.2)	100 ml
iodine solution (6.2.2.3)	20 ml
brilliant green solution (6.2.2.4)	2 ml
ox bile solution (6.2.2.5)	50 ml

Preparation

Add to the base, under aseptic conditions, the other ingredients in the above-mentioned order.

Mix the liquids well after each addition.

Transfer the complete medium in quantities of 100 ml aseptically into sterile flasks of 500 ml capacity.

Store it at 4 °C in the dark until needed but use it within one week after preparation.

6.2.3 Selenite brilliant green medium (Stokes and Osborne)

6.2.3.1 BASE

Composition

peptone	5,0 g
yeast extract	5,0 g
mannitol	5,0 g
sodium taurocholate	1,0 g
sodium hydrogen selenite	4,0 g
water	900 ml

Preparation

Dissolve the first four ingredients (i.e. the dehydrated base components or the dehydrated complete base) in the water by boiling for 5 min.

After cooling, add the sodium hydrogen selenite.

Adjust the pH to 7,0 ± 0,1 at 20 °C.

Store it at 4 °C in the dark until needed but use it within one week after preparation.

6.2.3.2 Buffer solution

Composition

Solution A

potassium dihydrogen orthophosphate (KH_2PO_4)	34,0 g
water	1 000 ml

Dissolve the potassium dihydrogen orthophosphate in the water.

Solution B

dipotassium hydrogen orthophosphate (K_2HPO_4)	43,6 g
water	1 000 ml

Dissolve the dipotassium hydrogen orthophosphate in the water.

Preparation

Mix 2 volumes of solution A and 3 volumes of solution B to obtain a solution with a pH of 7,0 ± 0,1 at 20 °C.

6.2.3.3 Brilliant green solution

For composition and preparation of this solution, see 6.2.2.4.

6.2.3.4 Complete medium

Composition

base (6.2.3.1)	900 ml
buffer solution (6.2.3.2)	100 ml
brilliant green solution (6.2.3.3)	1 ml

Preparation

Add the buffer solution to the base.

Heat to 80 °C.

Cool and add the brilliant green solution.

Transfer the complete medium in quantities of 100 ml to sterile flasks of 500 ml capacity.

Use the medium on the day of preparation.

6.2.4 Brilliant green/phenol red agar (Edel and Kampelmacher)

6.2.4.1 Base

Composition

meat extract	4,0 g
peptone	10,0 g
sodium chloride	3,0 g
disodium hydrogen orthophosphate (Na_2HPO_4)	0,8 g
sodium dihydrogen orthophosphate (NaH_2PO_4)	0,6 g
agar, readily soluble[1]	12,0 g
water	900 ml

Preparation

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

Adjust the pH so that after sterilization it is 7,0 ± 0,1 at 40 °C.

Transfer the base to tubes or flasks of not more than 500 ml capacity.

Sterilize the base for 15 min at 121 ± 1 °C.

6.2.4.2 Sugar/phenol red solution

Composition

lactose	10,0 g
sucrose	10,0 g
phenol red	0,09 g
water to a final volume of	100 ml

Preparation

Dissolve the ingredients in the water.

Heat in a water bath for 20 min at 70 °C.

Cool to 55 °C and use immediately.

6.2.4.3 Brilliant green solution

For composition and preparation of this solution, see 6.2.2.4.

6.2.4.4 Complete medium

Composition

base (6.2.4.1)	900 ml
sugar/phenol red solution (6.2.4.2)	100 ml
brilliant green solution (6.2.4.3)	1 ml

Preparation

Under aseptic conditions, add the brilliant green solution to the sugar/phenol red solution cooled to approximately 55 °C.

Add to the base at 50 to 55 °C and mix.

6.2.4.5 Preparation of agar plates

Add to sterile large-size Petri dishes (7.2.5.1) quantities of about 40 ml of the freshly prepared complete medium (6.2.4.4), having a temperature of approximately 45 °C.

1) The material known by the brand name of Oxoid No. 1 Agar is suitable.

(When large Petri dishes are not available, transfer quantities of about 15 ml of the melted medium (6.2.4.4) to sterile small Petri dishes (7.2.5.2).) Allow to solidify.

Immediately before use, dry the plates carefully, preferably with the lids off and the agar surface downwards, in an oven or incubator at 50 ± 5 °C for 30 min.

If prepared in advance, the undried plates shall not be kept longer than 4 h at room temperature or one day in a refrigerator.

6.2.5 Crystal violet/neutral red/bile/lactose agar

Composition

yeast extract	3,0 g
peptone	7,0 g
bile salts	1,5 g
lactose	10,0 g
sodium chloride	5,0 g
neutral red	0,03 g
crystal violet	0,002 g
agar	15,0 g
water	1 000 ml

Preparation

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

Adjust the pH so that after boiling it is 7,4 ± 0,1 at 40 °C.

Transfer the culture medium to sterile tubes or flasks of not more than 500 ml capacity.

Sterilization of the medium is not desirable.

If prepared in advance, the medium shall not be kept longer than one week in a refrigerator.

Preparation of agar plates

Transfer quantities of about 15 ml of the melted medium (6.2.5) to sterile small Petri dishes (7.2.5.2) and proceed as in 6.2.4.5.

6.2.6 Triple sugar/iron agar (TSI agar)

Composition

meat extract	3,0 g
yeast extract	3,0 g
peptone	20,0 g
sodium chloride	5,0 g
lactose	10,0 g
sucrose	10,0 g
glucose	1,0 g
iron(III) citrate	0,3 g
sodium thiosulphate	0,3 g
phenol red	0,024 g
agar	12,0 g
water	1 000 ml

Preparation

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

Adjust the pH so that after sterilization it is 7,4 ± 0,1 at 40 °C.

Transfer the medium in quantities of 10 ml to tubes of diameter 17 to 18 mm.

Sterilize the medium for 10 min at 121 ± 1 °C.

Allow to set in a sloping position to give a butt of depth 2,5 cm.

6.2.7 Urea agar (Christensen)

6.2.7.1 BASE

Composition

peptone	1,0 g
glucose	1,0 g
sodium chloride	5,0 g
potassium dihydrogen orthophosphate (KH_2PO_4)	2,0 g
phenol red	0,012 g
agar	15,0 g
water	1 000 ml

Preparation

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

Sterilize the base for 20 min at 121 ± 1 °C.

6.2.7.2 UREA SOLUTION

Composition

urea	400 g
water to a final volume of	1 000 ml

Preparation

Dissolve the urea in the water.

Sterilize by filtration and check the sterility.

(For details of the technique of sterilization by filtration, reference should be made to any appropriate textbook on microbiology.)

6.2.7.3 COMPLETE MEDIUM

Composition

base (6.2.7.1)	950 ml
urea solution (6.2.7.2)	50 ml

Preparation

Under aseptic conditions, add the urea solution to the base.

Adjust the pH so that it is 6,8 ± 0,1 at 40 °C.

Transfer the complete medium in quantities of 10 ml to sterile tubes.

Allow to set in a sloping position.

6.2.8 Semi-solid nutrient agar

Composition

meat extract	3,0 g
peptone	5,0 g
agar	8,0 g
water	1 000 ml

Preparation

Dissolve the dehydrated base components in the water by boiling.

Adjust the pH so that after sterilization it is 7,0 ± 0,1 at 40 °C.

Transfer the medium to flasks of not more than 500 ml capacity.

Sterilize the medium for 20 min at 121 ± 1 °C.

Preparation of agar plates

Add to sterile small Petri dishes (7.2.5.1) quantities of about 15 ml of the freshly prepared complete medium. The plates shall not be dried.

6.2.9 Saline solution

Composition

sodium chloride	8,5 g
water	1 000 ml

Preparation

Dissolve the sodium chloride in the water by boiling.

Adjust the pH so that after sterilization it is 7,0 ± 0,1 at 20 °C.

Transfer such quantities of the solution to flasks or tubes that they will contain 90 to 100 ml after sterilization.

Sterilize the solution for 20 min at 121 ± 1 °C.

6.2.10 Lysine decarboxylation medium

Composition

l-lysine monohydrochloride	5,0 g
yeast extract	3,0 g
glucose	1,0 g
bromocresol purple	0,015 g
water	1 000 ml

Preparation

Dissolve the components in the water by boiling.

Adjust the pH so that after sterilization it is 6,8 ± 0,1 at 20 °C.

Transfer the medium in quantities of 5 ml to narrow culture tubes approximately 8 mm in diameter and 160 mm in length.

Sterilize the medium for 10 min at 121 ± 1 °C.

6.2.11 β-galactosidase reagent

6.2.11.1 Buffer solution

Composition

sodium dihydrogen orthophosphate (NaH_2PO_4)	6,9 g
sodium hydroxide, approximately 0,1 N (4 g/l) solution	3 ml
water to a final volume of	50 ml

Preparation

Dissolve the sodium dihydrogen orthophosphate in approximately 45 ml of water.

Adjust the pH to 7,0 ± 0,1 with approximately 3 ml of the sodium hydroxide solution.

Add water to a final volume of 50 ml.

Store under refrigeration.

6.2.11.2 ONPG Solution

Composition

o-nitrophenyl β-D-galactopyranoside (ONPG)	80 mg
water	15 ml

Preparation

Dissolve the ONPG in the water at 50 °C.

Cool the solution.

6.2.11.3 Complete reagent

Composition

buffer solution (6.2.11.1)	5 ml
ONPG solution (6.2.11.2)	15 ml

Preparation

Add the buffer solution to the ONPG solution.

Store the complete reagent at 4 °C but not for longer than one month.

6.2.12 Voges-Proskauer reaction (rapid method by Barry and Feeney)

6.2.12.1 VP Medium

Composition

peptone	7,0 g
glucose	5,0 g
dipotassium hydrogen orthophosphate (K_2HPO_4)	5,0 g
water	1 000 ml

Preparation

Dissolve the components in the water.

Adjust the pH to 6,9 and filter.

Sterilize the medium for 20 min at 115 °C.

6.2.12.2 CREATINE SOLUTION

Composition

creatine monohydrate	0,5 g
water	100 ml

Preparation

Dissolve the creatine monohydrate in the water.

6.2.12.3 α-NAPHTHOL REAGENT

Composition

α-naphthol	6 g
ethanol, 96 % (*V/V*)	100 ml

Preparation

Dissolve the α-naphthol in the ethanol.

6.2.12.4 KOH REAGENT

Composition

potassium hydroxide	40 g
water	100 ml

Preparation

Dissolve the potassium hydroxide in the water.

6.2.13 Indol reaction

6.2.13.1 TRYPTON-TRYPTOPHAN MEDIUM (by Ljutov)

Composition

trypton	10 g
sodium chloride	5 g
DL-tryptophan	1 g
water	1 000 ml

Preparation

Dissolve the components in the water at 100 °C and filter. Adjust the pH to 7,5.

6.2.13.2 REAGENT (KOVACS)

Composition

p-dimethylaminobenzaldehyde	5 g
hydrochloric acid, ρ 1,18 to 1,19 g/ml	25 ml
*tert*amyl alcohol	75 ml

Preparation

Mix the components.

6.3 Sera

Several anti-*salmonellæ* sera may be obtained commercially, i.e. anti-sera containing one or more "O" groups (so called mono- or polyvalent anti O-sera), anti Vi-sera and anti-sera containing one or more "H" groups (so called mono- or polyvalent anti H-sera). For each serum, follow the instructions for use given by the serum manufacturer.

7 APPARATUS AND GLASSWARE

7.1 Apparatus

7.1.1 Mechanical meat mincer, laboratory size, sterile, fitted with a plate with holes of diameter not exceeding 4 mm.

7.1.2 Mechanical blender, operating at not less than 8 000 rev/min and not more than 45 000 rev/min, with glass or metal blending jars of an appropriate capacity, fitted with lids and resistant to the conditions of sterilization.

7.1.3 Apparatus for sterilization of glassware, blender jars, culture media, etc. and equipment for filter sterilization, for example asbestos pad, membrane filter, or filter candle of a suitable porosity.

7.1.4 Drying cabinet, oven or **incubator** for drying the surface of agar plates preferably at 50 ± 5 °C.

7.1.5 Incubator for maintaining the inoculated liquid media, plates and tubes at 37 ± 1 °C.

7.1.6 Incubator or **water bath** for maintaining inoculated liquid media at 42 to 43 °C.

7.1.7 Water baths for heating and cooling solutions and culture media to the appropriate temperatures.

7.2 Glassware

7.2.1 The glassware shall be resistant to repeated sterilization.

7.2.2 Culture tubes and flasks for sterilization and storage of culture media, and **culture tubes** 8 mm in diameter and 160 mm in length for lysine decarboxylation medium (6.2.10).

7.2.3 Measuring cylinder of 100 ml capacity, subdivided in 10 ml, for preparation of the complete media.

7.2.4 Graduated pipettes with a nominal capacity of 10 ml and 1 ml, subdivided respectively in 1,0 and 0,1 ml.

7.2.5 Petri dishes

7.2.5.1 LARGE-SIZE DISH

Dish

external diameter	140 ± 2	mm
external height	30 ± 2	mm
glass thickness	1,5 ± 0,5	mm

The rim shall be ground in a plane parallel to the base.

The bottom of the dish shall be flat and parallel to the base.

Lid

external diameter	150 ± 2	mm
external height	15 ± 2	mm
glass thickness	1,5 ± 0,5	mm

7.2.5.2 SMALL-SIZE DISH

Dish

internal diameter	90 ± 2 mm
external height, minimum	18 mm

The rim shall be ground in a plane parallel to the base.

The bottom of the dish shall be flat and parallel to the base.

Lid

external diameter, maximum	102 mm

7.2.5.3 Alternatively, plastics Petri dishes may be used, even if of slightly different dimensions from the glass dishes described in 7.2.5.1 and 7.2.5.2.

7.3 Sterilization of glassware, etc.

Sterilize the glassware, etc. by one of the following methods :

— wet sterilization at not less than 121 °C for not less than 20 min;

— dry sterilization at not less than 170 °C for not less than 1 h.

8 SAMPLING

Proceed from a representative sample of at least 200 g. See ISO 3100.

The representative sample may be stored in the laboratory at a temperature of 0 to 5 °C, but not for longer than 24 h.

9 PROCEDURE

9.1 Pre-treatment of the sample

Grind and mix the sample twice in the meat mincer (7.1.1). Start the examination of the pre-treated sample as soon as possible; it may be stored, if necessary, at a temperature between 0 and 5 °C, but not for longer than 1 h.

9.2 Test portion

Weigh 25 g of the minced meat or meat product (9.1) into a sterile blender jar (7.1.2).

9.3 Maceration

Add 225 ml of the buffered peptone water (6.2.1) to the jar.

Operate the blender according to its speed, for sufficient time to give a total number of 15 000 to 20 000 revolutions. Thus, even with the slowest blender, this time will not exceed 2,5 min.

NOTE — The presence or absence of *salmonellæ* in smaller quantities of meat (for example 1,0 g, 0,1 g) can be determined by transferring the appropriate quantity (for example 10 ml or 1 ml) of the macerate to 100 ml of buffered peptone water (6.2.1) and proceeding as described, reporting the results (see clause 10) in terms of the actual amount of meat examined.

9.4 Pre-enrichment

9.4.1 Transfer the contents of the blender jar aseptically to a sterile 500 ml flask.

9.4.2 Incubate the flask at 37 ± 1 °C for not less than 16 h and not more than 20 h.

9.5 Enrichment

9.5.1 After the incubation period, transfer 10 ml from the flask to 100 ml of tetrathionate medium (6.2.2), and 10 ml to 100 ml of selenite medium (6.2.3).

9.5.2 Incubate the inoculated tetrathionate medium for up to 2 days at 42 to 43 °C and the inoculated selenite medium for up to 2 days at 37 ± 1 °C.

9.6 Plating out

9.6.1 After an incubation period of 18 to 24 h, streak from each flask (9.5.2), using a loop with a diameter of 2,5 to 3 mm, onto the surface of brilliant green/phenol red agar plates (6.2.4) and, if so desired, streak likewise onto the surface of one other solid medium preferred by the laboratory as a selective diagnostic medium for *salmonellæ*, such as bismuth sulphite agar, S.S. agar, desoxycholate-citrate agar, etc., so that well-isolated colonies are obtained. (When large Petri dishes are not available, two small Petri dishes may be streaked one after the other, using the same loop.)

9.6.2 Incubate the plates with the bottom of the Petri dishes uppermost in an incubator at 37 ± 1 °C.