INTERNATIONAL STANDARD 5552

INTERNATIONAL ORGANIZATION FOR STANDARDIZATION•МЕЖДУНАРОДНАЯ ОРГАНИЗАЦИЯ ПО СТАНДАРТИЗАЦИИ•ORGANISATION INTERNATIONALE DE NORMALISATION

Meat and meat products — Detection and enumeration of Enterobacteriaceae (Reference methods)

Viandes et produits à base de viande — Recherche et dénombrement des Enterobacteriaceae (Méthodes de référence)

First edition — 1979-04-01

UDC 637.5 : 576.8.08 : 576.851

Ref. No. ISO 5552-1979 (E)

Descriptors : meat, meat products, microbiological analysis, detection, counting, bacteria, Enterobacteriaceae.

Price based on 6 pages

FOREWORD

ISO (the International Organization for Standardization) is a worldwide federation of national standards institutes (ISO member bodies). The work of developing International Standards is carried out through ISO technical committees. Every member body interested in a subject for which a technical committee has been set up 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.

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 5552 was developed by Technical Committee ISO/TC 34, *Agricultural food products*, and was circulated to the member bodies in August 1977.

It has been approved by the member bodies of the following countries :

Canada	Israel	Portugal
Czechoslovakia	Kenya	Romania
Egypt, Arab Rep. of	Malaysia	South Africa, Rep. of
Ethiopia	Mexico	Spain
France	Netherlands	Thailand
Germany, F. R.	New Zealand	Turkey
Hungary	Peru	United Kingdom
India	Philippines	USSR
Iran	Poland	

The member bodies of the following countries expressed disapproval of the document on technical grounds :

Austria
Bulgaria

Printed in Switzerland

Published 1979-06-01

INTERNATIONAL ORGANIZATION FOR STANDARDIZATION • МЕЖДУНАРОДНАЯ ОРГАНИЗАЦИЯ ПО СТАНДАРТИЗАЦИИ • ORGANISATION INTERNATIONALE DE NORMALISATIO

Meat and meat products — Detection and enumeration of Enterobacteriaceae (Reference methods)

ERRATUM

MODIFICATION TO FOREWORD *(Inside front cover)*

The ISO member body for the United Kingdom has now disapproved this International Standard. The United Kingdom should therefore be included in the list of countries whose member bodies have disapproved the document.

Meat and meat products — Detection and enumeration of Enterobacteriaceae (Reference methods)

1 SCOPE

This International Standard specifies reference methods for the detection and enumeration of viable Enterobacteriaceae in meat and meat products.

2 FIELD OF APPLICATION

The methods can be applied to all kinds of meat and meat products but are not intended for surface count of carcasses.

3 REFERENCE

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

4 DEFINITIONS

4.1 Enterobacteriaceae : Micro-organisms which ferment glucose and show a negative oxidase reaction when the test is carried out according to the method specified.

4.2 detection of Enterobacteriaceae : Determination of the presence or absence of Enterobacteriaceae in a particular mass when the test is carried out according to the method specified.

4.3 count of Enterobacteriaceae : The number of Enterobacteriaceae found per gram of meat or meat product when the test is carried out according to the method specified.

5 PRINCIPLE

5.1 Maceration and dilution

Mincing of a test sample and then maceration of a test portion with a sterile diluent, in a mechanical blender. Preparation, from the macerate, of decimal dilutions.

5.2 Detection of the presence or absence of Enterobacteriaceae in a particular mass (0,1 g or 0,01 g or 0,001 g) of meat or meat product

Introduction of 1 ml of the macerate (or the dilution 10^{-2} or 10^{-3}, in triplicate, into tubes containing a selective enrichment broth.

Incubation of the tubes at 37 °C for 24 h, followed by streaking of the cultures onto violet red bile glucose agar. After incubation of the streaked agar plates at 37 °C for 24 h, subjection of suspected colonies to biochemical confirmation tests.

5.3 Enumeration of Enterobacteriaceae

5.3.1 *Most probable number (MPN) technique* — where the number is expected to be in the range of 1 to 1 000 per gram of meat or meat product

Introduction of 1 ml of the macerate and of the dilutions 10^{-2} and 10^{-3}, in triplicate, into tubes containing the same selective enrichment broth as mentioned in 5.2. Incubation of the tubes at 37 °C for 24 h. From the number of confirmed positive tubes (see 5.2), determination of the most probable number of Enterobacteriaceae per gram of sample by using the MPN table (see annex).

5.3.2 *Colony count* — where the number is expected to be $> 1\,000$ per gram

Introduction of 1 ml of the macerate and of the dilutions 10^{-2}, 10^{-3}, 10^{-4} and 10^{-5} into empty Petri dishes, followed by addition of violet red bile glucose agar and covering of this agar by an overlay of violet red bile glucose agar. Incubation of the dishes at 37 °C for 24 h. From the number of confirmed (see 5.2) typical colonies per Petri dish, calculation of the number of Enterobacteriaceae per gram of sample.

6 CULTURE MEDIA, DILUTION FLUID AND REAGENTS

6.1 Basic materials

6.1.1 For uniformity of results, it is recommended that uniform dehydrated culture media components or dehydrated complete culture media be used.

6.1.2 The basic materials — peptone, tryptone, yeast extract, ox bile, bile-salts, and water — shall meet the requirements for preparations of bacteriological culture media. Chemicals shall be of analytical reagent grade.

6.2 Culture media

6.2.1 Buffered brilliant green bile glucose broth

Composition

peptone	10,0 g
glucose	5,0 g
disodium hydrogen phosphate (Na_2HPO_4)	6,45 g
potassium hydrogen phosphate (KH_2PO_4)	2,0 g
ox bile, desiccated	20,0 g
brilliant green	0,015 g
water	1 000 ml

Preparation

Dissolve the components or the complete medium in the water by boiling. The medium shall not be heated longer than 30 min. Cool the medium rapidly.

Adjust the pH so that after boiling it is 7,2 ± 0,1 at 20 °C.

Transfer portions of 10 ml to sterile culture tubes.

Sterilization of the medium is not desirable.

The medium may be stored for up to 1 week at 0 to 5 °C.

6.2.2 Violet red bile glucose agar

Composition

peptone	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	15,0 g
water	1 000 ml

Preparation

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

Adjust the pH so that after boiling it is 7,4 ± 0,1 at 45 °C.

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

Sterilization of the medium is not desirable.

This medium shall be freshly prepared.

Preparation of agar plates

Transfer portions of about 10 ml of the culture medium, melted and cooled to approximately 45 °C, to Petri dishes (7.2.3) and allow to solidify.

Immediately before use, dry the plates, preferably with the lids off and the agar surface downwards, in an oven or incubator (7.1.4) at 50 °C for 30 min.

If prepared in advance, the undried plates shall not be kept longer than 4 h at room temperature or 1 day at 0 to 5 °C.

6.2.3 Glucose agar

Composition

tryptone	10,0 g
yeast extract	1,5 g
glucose	10,0 g
sodium chloride	5,0 g
bromo-cresol purple	0,015 g
agar	15,0 g
water	1 000 ml

Preparation

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

Adjust the pH so that after sterilization it is 7,0 ± 0,1 at 45 °C.

Transfer the culture medium in quantities of 15 ml to culture tubes.

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

Allow the medium to set in the tubes in a vertical position.

These tubes may be stored for up to 1 week at 0 to 5 °C.

6.2.4 Nutrient agar

Composition

beef extract	3,0 g
peptone	5,0 g
agar	15,0 g
water	1 000 ml

Preparation

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

Adjust the pH so that after sterilization it is 7,0 ± 0,2 at 45 °C.

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

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

Preparation of agar plates

Transfer portions of about 15 ml of the culture medium, melted and cooled to approximately 45 °C, to Petri dishes (7.2.3) and allow to solidify.

Immediately before use, dry the plates, preferably with the lids off and the agar surface downwards, in an oven or incubator (7.1.4) at 50 °C for 30 min.

If prepared in advance, the undried plates shall not be kept longer than 4 h at room temperature or 1 day at 0 to 5 °C.

6.3 Dilution fluid

Composition

peptone	1,0 g
sodium chloride	8,5 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 part of the dilution fluid in quantities of 100 ml to 300 ml flasks for the maceration and the remainder to tubes or small flasks in such quantities that these contain 9,0 ml after sterilization.

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

6.4 Oxidase reagent

Composition

N,N,N′,N′-tetramethyl-*p*-phenylene diamine dihydrochloride	1,0 g
water	100 ml

Preparation

Dissolve the reagent in cold water.

The reagent shall be prepared immediately before use.

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 a rotational frequency of not less than 8 000 min^{-1} and not more than 45 000 min^{-1}, 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.

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

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

7.1.6 Water bath for cooling the melted culture medium to 45 °C.

7.2 Glassware

The glassware shall be resistant to repeated sterilization.

7.2.1 Culture tubes and flasks for the sterilization and storage of culture media and dilution fluid.

7.2.2 Graduated pipettes, calibrated for bacteriological use only, with a nominal capacity of 1 ml, subdivided in 0,1 ml and with an outflow opening of diameter 2 to 3 mm.

7.2.3 Petri dishes

Dish

internal diameter	90 ± 2 mm
external height not less than	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 not more than	102 mm

Plastics Petri dishes may also be used, even if of slightly different dimensions from those mentioned.

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 a least 200 g. See ISO 3100.

The sample may be stored at a temperature of 0 to 5 °C, but not for longer than 1 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, to the nearest 0,1 g, about 10 g of the pre-treated sample (9.1) into a sterile blender jar (7.1.2).

9.3 Maceration and dilution

9.3.1 Add to the test portion nine times the quantity, by mass, of the dilution fluid (6.3). Operate the blender, according to its rotational frequency, 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.

9.3.2 Take, directly after maceration, duplicate portions of 1 ml of the macerate (9.3.1) with a sterile 1 ml pipette and add each portion to a tube containing 9 ml of the sterile dilution fluid (6.3), avoiding contact between pipette and dilution fluid.

9.3.3 Mix the liquids carefully with a fresh sterile pipette, filling and emptying the pipette ten times, and transfer, with the same pipette, 1 ml of each of the dilutions 10^{-2} to other tubes containing 9 ml of the sterile dilution fluid, avoiding contact between pipette and dilution fluid.

9.3.4 Mix the liquids carefully with a fresh sterile pipette as in 9.3.3 and repeat the operations until the required number of dilutions up to 10^{-5} has been made.

9.4 Inoculation and incubation

9.4.1 *Presence or absence test*

Transfer, with a fresh sterile pipette, six 1 ml portions of the macerate (9.3.1) (dividing them into two triplicate sets), or triplicate 1 ml portions of one of the two dilutions 10^{-2} or 10^{-3} (9.3.2 or 9.3.3) of both dilutions series, to tubes containing buffered brilliant green bile glucose broth (6.2.1). Keep the six tubes for 24 h in an incubator at 37 ± 1 °C.

9.4.2 *Most probable number*

Transfer, with a fresh sterile pipette, six 1 ml portions of the macerate (9.3.1) (dividing them into two triplicate sets), and triplicate 1 ml portions of each of the two dilutions 10^{-2} and 10^{-3} (9.3.2 and 9.3.3) of both dilution series, to tubes containing buffered brilliant green bile glucose broth (6.2.1). Start with the highest dilution (10^{-3}) and proceed to the lowest (the macerate), filling and emptying the pipette three times before transferring the 1 ml portions to the tubes containing the culture medium. Keep the eighteen tubes for 24 h in an incubator at 37 ± 1 °C.

9.4.3 *Colony count*

9.4.3.1 Transfer, with a fresh sterile pipette, four 1 ml portions of the macerate (9.3.1) (dividing them into two duplicate sets) and duplicate 1 ml portions of each of the four dilutions 10^{-2}, 10^{-3}, 10^{-4} and 10^{-5} (9.3.2, 9.3.3 and 9.3.4) of both dilution series, to empty sterile Petri dishes. Start with the highest dilution (10^{-5}) and proceed to the lowest (the macerate), filling and emptying the pipette three times before transferring the 1 ml portions to the dishes.

9.4.3.2 Within 5 min, pour into the dishes 10 ml of the violet red bile glucose agar (6.2.2) which has been melted and cooled carefully to approximately 45 °C.

9.4.3.3 Mix the contents of the Petri dishes thoroughly immediately after the addition of the culture medium. Make sure that the dishes are in a horizontal position while the mixture is solidifying.

9.4.3.4 After solidification of the mixture, add a cover layer of 15 to 20 ml of the violet red bile glucose agar (6.2.2), melted and cooled as in 9.4.3.2, to prevent spreading growth and to obtain semi-anaerobic conditions.

9.4.3.5 Keep the twenty plates, prepared according to 9.4.3.1 to 9.4.3.4, bottom uppermost for 24 h in an incubator at 37 ± 1 °C.

9.5 Confirmation

9.5.1 *Plating out of the broth cultures*

9.5.1.1 PRESENCE OR ABSENCE TEST

Streak a loopful of each of the six incubated cultures of 9.4.1 on agar plates (6.2.2) and incubate the six plates for 24 h at 37 ± 1 °C.

9.5.1.2 MOST PROBABLE NUMBER

Streak a loopful of each of the eighteen incubated cultures of 9.4.2 on agar plates (6.2.2) and incubate the eighteen plates for 24 h at 37 ± 1 °C.

9.5.2 *Selection of colonies for confirmation*

9.5.2.1 PRESENCE OR ABSENCE TEST

From each of the incubated plates (9.5.1.1) on which typical deep red colonies (with deep red precipitate haloes) have developed, select at random five such colonies for the biochemical confirmation tests (9.5.3).

9.5.2.2 MOST PROBABLE NUMBER

Proceed as in 9.5.2.1 with the incubated plates from 9.5.1.2.

9.5.2.3 COLONY COUNT

Select, if possible, a set of four agar plates (9.4.3.5) (i.e. the duplicate plates of both dilution series) at a dilution level such that 30 to 300 typical colonies measuring 0,5 mm or more in diameter have developed on each plate, and count these suspected colonies.

If a choice is possible between two successive dilution levels, select those plates inoculated with the greater amount of the sample under examination.

Consider the determination to be void if half or more than half the surface area of a plate is overgrown. If less than half of the surface area of a plate is overgrown, count the colonies on the clear half and convert the number to correspond to the total surface area of the plate.

If, even on plates corresponding to the highest dilution level (10^{-5}), it is impossible to make a count because more than 300 colonies have developed per plate, the determination must be repeated using dilutions higher than 10^{-5}.

From each of the four plates selected, streak $\sqrt{n}$ typical colonies – n being the number of colonies counted on the plate – on nutrient agar plates (6.2.4). $\sqrt{n}$ shall not be less than 5; i.e. not fewer than 20 agar plates shall be streaked. Incubate these plates for 24 h at 37 ± 1 °C. Select a suspected typical deep red colony from each of the incubated plates for the biochemical confirmation tests (9.5.3).

9.5.3 *Biochemical confirmation tests*

9.5.3.1 OXIDASE REACTION

9.5.3.1.1 Streak a loopful of the selected typical colonies from the incubated plates of 9.5.2 on nutrient agar plates (6.2.4). Incubate these plates for 24 h at 37 ± 1 °C.

9.5.3.1.2 Streak a loopful of the growth from each of the incubated plates of 9.5.3.1.1 on a filter paper moistened with the oxidase reagent (6.4).

Consider the test to be negative when the colour has not turned dark purple within 5 to 10 s.

9.5.3.2 FERMENTATION TEST

Stab the same colonies as mentioned in 9.5.3.1.2 with an inoculation wire into the tubes containing freshly steamed glucose agar (6.2.3) and incubate for 24 h at 37 ± 1 °C.

A yellow colour through the whole tube, mostly accompanied by the formation of gas, is regarded as a positive reaction.

10 EXPRESSION OF RESULTS

10.1 Calculation of the most probable number

10.1.1 If one of the selected typical colonies of a sub-culture (9.5.2.2) is oxidase-negative and glucose-positive, the culture from which the sub-culture is derived shall be regarded as being positive Enterobacteriaceae.

10.1.2 Using the table in the annex, for each dilution series determine from the number of Enterobacteriaceae-positive tubes in the different dilutions, the most probable number of these micro-organisms per gram of sample of meat or meat product.

10.1.3 Calculate the average for the two dilution series.

10.2 Calculation of the colony count

10.2.1 If more than 75 % of the selected typical colonies (9.5.2.3) are oxidase-negative and glucose-positive, and thus confirmed as Enterobacteriaceae, the number of these micro-organisms present shall be taken to be the same as the number of presumptive Enterobacteriaceae given by the count made in 9.5.2.3.

10.2.2 In all other cases, the number shall be calculated from the percentage of oxidase-negative and glucose-positive colonies in relation to the total number of suspected colonies.

10.2.3 Calculate the average count of Enterobacteriaceae from the counts made on the four plates in 9.5.2.3.

10.2.4 Round the result as follows :

a) if the number is less than 100, round it to the nearest multiple of five;

b) if the number is more than 100 and does not end with a 5, round it to the nearest multiple of ten;

c) if the number is more than 100 and ends with a 5, round it to the nearest multiple of twenty.

10.2.5 Calculate the number of Enterobacteriaceae per gram of sample of meat or meat product by multiplying the number obtained according to 10.2.4 by the dilution factor (10^1, 10^2, 10^3, 10^4 or 10^5).

10.3 Reporting of results

10.3.1 *Presence or absence of Enterobacteriaceae in* 0,1 g *or in* 0,01 g *or in* 0,001 g

10.3.1.1 If three tubes, or two tubes, or one tube, of both dilution series (9.4.1) yield colonies confirmed as Entero-bacteriaceae (see 10.1.1), report the result as :

"Enterobacteriaceae were detected in 3 times [or 2 times, or once, respectively] 0,1 g [or 0,01 g, or 0,001 g, depending on the dilution chosen] of the meat or meat product."