EAST AFRICAN COMMUNITY

EDICT OF GOVERNMENT

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EAS 217-6 (2008) (English): Microbiology of food and animal feeding stuffs — Part 6: Horizontal method for the detection of Salmonella spp.

ISO INSIDE

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EAST AFRICAN STANDARD

Microbiology of food and animal feeding stuffs — Part 6: Horizontal method for the detection of *Salmonella* spp.

EAST AFRICAN COMMUNITY

First Edition 2008

Foreword

Development of the East African Standards has been necessitated by the need for harmonizing requirements governing quality of products and services in East Africa. It is envisaged that through harmonized standardization, trade barriers which are encountered when goods and services are exchanged within the Community will be removed.

In order to meet the above objectives, the EAC Partner States have enacted an East African Standardization, Quality Assurance, Metrology and Test Act, 2006 (EAC SQMT Act, 2006) to make provisions for ensuring standardization, quality assurance, metrology and testing of products produced or originating in a third country and traded in the Community in order to facilitate industrial development and trade as well as helping to protect the health and safety of society and the environment in the Community.

East African Standards are formulated in accordance with the procedures established by the East African Standards Committee. The East African Standards Committee is established under the provisions of Article 4 of the EAC SQMT Act, 2006. The Committee is composed of representatives of the National Standards Bodies in Partner States, together with the representatives from the private sectors and consumer organizations. Draft East African Standards are circulated to stakeholders through the National Standards Bodies in the Partner States. The comments received are discussed and incorporated before finalization of standards, in accordance with the procedures of the Community.

Article 15(1) of the EAC SQMT Act, 2006 provides that “Within six months of the declaration of an East African Standard, the Partner States shall adopt, without deviation from the approved text of the standard, the East African Standard as a national standard and withdraw any existing national standard with similar scope and purpose”.

East African Standards are subject to review, to keep pace with technological advances. Users of the East African Standards are therefore expected to ensure that they always have the latest versions of the standards they are implementing.

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Introduction

This East African Standard has been revised and aligned to ISO 6579:2002, *Microbiology of food and animal feeding stuffs — Horizontal method for the detection of Salmonella spp.*

This East African Standard includes the following ISO 6579 updates:

ISO 6579:2002/Cor 1:2004

ISO 6579:2002/Amd 1:2007, *Annex D: Detection of Salmonella spp. in animal faeces and in environmental samples from the primary production stage*

INTERNATIONAL STANDARD ISO 6579:2002
TECHNICAL CORRIGENDUM 1

Published 2004-04-01

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

Microbiology of food and animal feeding stuffs — Horizontal method for the detection of *Salmonella* spp.

TECHNICAL CORRIGENDUM 1

Microbiologie des aliments — Méthode horizontale pour la recherche des Salmonella *spp.*

RECTIFICATIF TECHNIQUE 1

Technical Corrigendum 1 to ISO 6579:2002 was prepared by Technical Committee ISO/TC 34, *Food products*, Subcommittee SC 9, *Microbiology.*

Page 16

In **B.3.1.2, Preparation**, second sentence, replace

"8,2 ± 0,2 at 25 °C."

by

"8,0 ± 0,2 at 25 °C."

ICS 07.100.30 **Ref. No. ISO 6579:2002/Cor.1:2004(E)**

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INTERNATIONAL STANDARD

ISO 6579

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Microbiology of food and animal feeding stuffs — Horizontal method for the detection of *Salmonella* spp.

Microbiologie des aliments — Méthode horizontale pour la recherche des Salmonella *spp.*

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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.

International Standards are drafted in accordance with the rules given in the ISO/IEC Directives, Part 3.

The main task of technical committees is to prepare International Standards. 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.

Attention is drawn to the possibility that some of the elements of this International Standard may be the subject of patent rights. ISO shall not be held responsible for identifying any or all such patent rights.

ISO 6579 was prepared by Technical Committee ISO/TC 34, *Food products*, Subcommittee SC 9, *Microbiology*.

This fourth edition cancels and replaces the third edition (ISO 6579:1993), which has been technically revised.

Annexes A and B form a normative part of this Interntional Standard. Annex C is for information only.

Introduction

Because of the large variety of food and feed products, this horizontal method may not be appropriate in every detail for certain products. In this case, different methods, which are specific to these products, may be used if absolutely necessary for justified technical reasons. Nevertheless, every attempt should be made to apply this horizontal method as far as possible.

When this International Standard is next reviewed, account will be taken of all information then available regarding the extent to which this horizontal method has been followed and the reasons for deviations from this method 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 International Standard 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 method for the detection of *Salmonella* spp.

WARNING — In order to safeguard the health of laboratory personnel, it is essential that tests for detecting *Salmonella*, and especially *Salmonella* Typhi and *Salmonella* Paratyphi, 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.

1 Scope

This International Standard specifies a horizontal method for the detection of *Salmonella*, including *Salmonella* Typhi and *Salmonella* Paratyphi.

Subject to the limitations discussed in the Introduction, this International Standard is applicable to

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

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

WARNING — The method may not recover all *Salmonella* Typhi and Paratyphi.

2 Normative references

The following normative documents contain provisions which, through reference in this text, constitute provisions of this International Standard. For dated references, subsequent amendments to, or revisions of, any of these publications do not apply. However, parties to agreements based on this International Standard are encouraged to investigate the possibility of applying the most recent editions of the normative documents indicated below. For undated references, the latest edition of the normative document referred to applies. Members of ISO and IEC maintain registers of currently valid International Standards.

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 7218:1996, *Microbiology of food and animal feeding stuffs — General rules for microbiological examinations*

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

3 Terms and definitions

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

3.1
Salmonella
microorganisms which form typical or less typical colonies on solid selective media and which display the biochemical and serological characteristics described when tests are carried out in accordance with this International Standard

3.2
detection of *Salmonella*
determination of the presence or absence of *Salmonella* (3.1), in a particular mass or volume of product, when tests are carried out in accordance with this International Standard

4 Principle

4.1 General

The detection of *Salmonella* necessitates four successive stages (see also annex A).

NOTE The *Salmonella* may be present in small numbers and are often accompanied by considerably larger numbers of other *Enterobacteriaceæ* or other families. Furthermore, pre-enrichment is necessary to permit the detection of low numbers of *Salmonella* or injured *Salmonella*.

4.2 Pre-enrichment in non-selective liquid medium

Buffered peptone water is inoculated at ambient temperature with the test portion, then incubated at 37 °C ± 1 °C for 18 h ± 2 h.

For certain foodstuffs the use of other pre-enrichment procedures is necessary. See 9.1.2.

For large quantities, the buffered peptone water should be heated to 37 °C ± 1 °C before inoculation with the test portion.

4.3 Enrichment in selective liquid media

Rappaport-Vassiliadis medium with soya (RVS broth) and Muller-Kauffmann tetrathionate/novobiocin broth (MKTTn broth) are inoculated with the culture obtained in 4.2.

The RVS broth is incubated at 41,5 °C ± 1 °C for 24 h ± 3 h, and the MKTTn broth at 37 °C ± 1 °C for 24 h ± 3 h.

4.4 Plating out and identification

From the cultures obtained in 4.3, two selective solid media are inoculated:

— xylose lysine deoxycholate agar (XLD agar);

— any other solid selective medium complementary to XLD agar and especially appropriate for the isolation of lactose-positive *Salmonella* and *Salmonella* Typhi and *Salmonella* Paratyphi strains; the laboratory may choose which medium to use.

The XLD agar is incubated at 37 °C ± 1 °C and examined after 24 h ± 3 h. The second selective agar is incubated according to the manufacturer's recommendations.

NOTE For information, Brilliant green agar (BGA), bismuth sulfite agar, etc., could be used as the second plating-out medium.

4.5 Confirmation of identity

Colonies of presumptive *Salmonella* are subcultured, then plated out as described in 4.4, and their identity is confirmed by means of appropriate biochemical and serological tests.

5 Culture media, reagents and sera

5.1 General

For current laboratory practice, see ISO 7218.

5.2 Culture media and reagents

NOTE Because of the large number of culture media and reagents, it is considered preferable, for clarity, to give their compositions and preparations in annex B.

5.2.1 Non-selective pre-enrichment medium: Buffered peptone water

See B.1.

5.2.2 First selective enrichment medium: Rappaport-Vassiliadis medium with soya (RVS broth)

See B.2.

5.2.3 Second selective enrichment medium: Muller-Kauffmann tetrathionate novobiocin broth (MKTTn broth)

See B.3.

5.2.4 Solid selective plating-out media

5.2.4.1 First medium: Xylose lysine deoxycholate agar (XLD agar)

See B.4.

5.2.4.2 Second medium

The choice of the second appropriate medium is left to the discretion of the testing laboratory. The manufacturer's instructions should be precisely followed regarding its preparation for use.

5.2.5 Nutrient agar

See B.5.

5.2.6 Triple sugar/iron agar (TSI agar)

See B.6.

5.2.7 Urea agar (Christensen)

See B.7.

5.2.8 L-Lysine decarboxylation medium

See B.8.

5.2.9 Reagent for detection of β-galactosidase (or prepared paper discs used in accordance with the manufacturer's instructions)

See B.9.

5.2.10 Reagents for Voges-Proskauer (VP) reaction

See B.10.

5.2.11 Reagents for indole reaction

See B.11.

5.2.12 Semi-solid nutrient agar

See B.12.

5.2.13 Physiological saline solution

See B.13.

5.3 Sera

Several types of agglutinating sera containing antibodies for one or several O-antigens are available commercially; i.e. anti-sera containing one or more “O” groups (called monovalent or polyvalent anti-O sera), anti-Vi sera, and anti-sera containing antibodies for one or several H-factors (called monovalent or polyvalent anti-H sera).

Every attempt should be made to ensure that the anti-sera used are adequate to provide for the detection of all *Salmonella* serotypes. Assistance towards this objective may be obtained by using only anti-sera prepared by a supplier recognized as competent (for example, by an appropriate government agency).

6 Apparatus and glassware

Disposable apparatus 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)**

See ISO 7218.

6.2 Drying cabinet or **oven**, ventilated by convection, capable of operating between 37 °C and 55 °C.

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

6.4 Water bath, capable of operating at 41,5 °C ± 1 °C, or **incubator**, capable of operating at 41,5 °C ± 1 °C.

6.5 Water baths, capable of operating at 44 °C to 47 °C.

6.6 Water bath, capable of operating at 37 °C ± 1 °C.

It is recommended to use a water bath (6.4, 6.5 and 6.6) containing an antibacterial agent because of the low infective dose of *Salmonella*.

6.7 Sterile loops, of diameter approximately 3 mm or 10 µl, or **sterile pipettes**.

6.8 pH-meter, having an accuracy of calibration of ± 0,1 pH unit at 20 °C to 25 °C.

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

Bottles or flasks with non-toxic metallic or plastic screw-caps may be used.

6.10 Graduated pipettes or **automatic pipettes**, of nominal capacities 10 ml and 1 ml, graduated respectively in 0,5 ml and 0,1 ml divisions.

6.11 Petri dishes, of small size (diameter 90 mm to 100 mm) and/or large size (diameter 140 mm).

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. See the specific International Standard dealing with the product concerned. If there is no specific International Standard, it is recommended that the parties concerned come to an agreement on this subject.

8 Preparation of test sample

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

9 Procedure (see diagram in annex A)

9.1 Test portion and initial suspension

9.1.1 General

See ISO 6887-1 and the specific International Standard dealing with the product concerned. See ISO 8261 for milk and milk products.

For preparation of the initial suspension, in the general case use as diluent the pre-enrichment medium specified in 5.2.1 and 4.2 (buffered peptone water).

If the specified mass of test portion is other than 25 g, use the necessary quantity of pre-enrichment medium to yield a 1/10 dilution.

To reduce the examination workload when more than one 25 g test portion from a specified lot of food has to be examined, and when evidence is available that compositing (pooling the test portions) does not affect the result for that particular food, the test portions may be composited. For example, if 10 test portions of 25 g are to be examined, combine the 10 units to form a composite test portion of 250 g and add 2,25 l of pre-enrichment broth. Alternatively, the 0,1 ml (in 10 ml of RVS broth) and 1 ml (in 10 ml of MKTTn broth) portions of the pre-enrichment broth from the 10 separate test portions (see 9.3.1) may be composited for enrichment in 100 ml of selective enrichment media.

9.1.2 Specific preparations of the initial suspension for certain foodstuffs

NOTE The following specific preparations concern only the case of *Salmonella*. Specific preparations applicable for the determination of any microorganisms are described in ISO 6887-2, ISO 6887-3, ISO 6887-4 and ISO 8261.

9.1.2.1 Cocoa and cocoa-containing products (e.g. more than 20 %)

Add to the buffered peptone water (5.2.1) preferably 50 g/l of casein (avoid the use of acid casein), or 100 g/l of sterile skim milk powder and add, after about 2 h incubation, 0,018 g/l of Brilliant green if the foodstuff is likely to be highly contaminated with Gram-positive flora.

9.1.2.2 Acidic and acidifying foodstuffs

Ensure that the pH does not fall to below 4,5 during pre-enrichment.

NOTE The pH of acidic and acidifying foodstuffs is more stable if double-strength buffered peptone water is used.

9.2 Non-selective pre-enrichment

Incubate the initial suspension (9.1) at 37 °C ± 1 °C for 18 h ± 2 h.

9.3 Selective enrichment

9.3.1 Transfer 0,1 ml of the culture obtained in 9.2 to a tube containing 10 ml of the RVS broth (5.2.2); transfer 1 ml of the culture obtained in 9.2 to a tube containing 10 ml of MKTTn broth (5.2.3).

9.3.2 Incubate the inoculated RVS broth (9.3.1) at 41,5 °C ± 1 °C for 24 h ± 3 h and the inoculated MKTTn broth at 37 °C ± 1 °C for 24 h ± 3 h. Care should be taken that the maximum allowed incubation temperature (42,5 °C) is not exceeded.

9.4 Plating out and identification

9.4.1 After incubation for 24 h ± 3 h, using the culture obtained in the RVS broth (9.3.2), inoculate by means of a loop (6.7) the surface of one large-size Petri dish (6.11) containing the first selective plating-out medium (XLD agar, see 5.2.4.1), so that well-isolated colonies will be obtained.

In the absence of large dishes, use two small dishes one after the other, using the same loop.

Proceed in the same way with the second selective plating-out medium (5.2.4.2) using a sterile loop and Petri dishes as above.

9.4.2 After incubation for 24 h ± 3 h, using the culture obtained in the MKTTn broth (9.3.2), repeat the procedure described in 9.4.1 with the two selective plating-out media.

9.4.3 Invert the dishes (9.4.1 and 9.4.2) so that the bottom is uppermost, and place them in the incubator (6.3) set at 37 °C for the first plating-out medium (5.2.4.1). The manufacturer's instructions shall be followed for the second plating-out medium (5.2.4.2).

9.4.4 After incubation for 24 h ± 3 h, examine the plates (9.4.3) for the presence of typical colonies of *Salmonella* and atypical colonies that may be *Salmonella* (see Note). Mark their position on the bottom of the dish.

Typical colonies of *Salmonella* grown on XLD agar have a black centre and a lightly transparent zone of reddish colour due to the colour change of the indicator.

NOTE *Salmonella* H_2S negative variants (e.g. *S*. Paratyphi A) grown on XLD agar are pink with a darker pink centre. Lactose-positive *Salmonella* grown on XLD agar are yellow with or without blackening.

Incubate the second selective solid medium at the appropriate temperature and examine after the appropriate time to check for the presence of colonies which, from their characteristics, are considered to be presumptive *Salmonella*.

9.5 Confirmation

9.5.1 General

If shown to be reliable, commercially available identification kits for the biochemical examination of *Salmonella* may be used. The use of identification kits concerns the biochemical confirmation of colonies. These kits should be used following the manufacturer's instructions.

NOTE The recognition of colonies of *Salmonella* is to a large extent a matter of experience, and their appearance may vary somewhat, not only from serovar to serovar, but also from batch to batch of the selective culture medium used.

9.5.2 Selection of colonies for confirmation

For confirmation, take from each dish (two small-sized dishes or one large-sized dish) of each selective medium (see 9.4) at least one colony considered to be typical or suspect and a further four colonies if the first is negative.

It is recommended that at least five colonies be identified in the case of epidemiological studies. If on one dish there are fewer than five typical or suspect colonies, take for confirmation all the typical or suspect colonies.

Streak the selected colonies onto the surface of pre-dried nutrient agar plates (5.2.5), in a manner which will allow well-isolated colonies to develop. Incubate the inoculated plates (9.4.3) at 37 °C ± 1 °C for 24 h ± 3 h.

Use pure cultures for biochemical and serological confirmation.

9.5.3 Biochemical confirmation

9.5.3.1 General

By means of an inoculating wire, inoculate the media specified in 9.5.3.2 to 9.5.3.7 with each of the cultures obtained from the colonies selected in 9.5.2.

9.5.3.2 **TSI agar** (5.2.6)

Streak the agar slant surface and stab the butt. Incubate at 37 °C ± 1 °C for 24 h ± 3 h.

Interpret the changes in the medium as follows.

a) Butt

- yellow — glucose positive (glucose used)
- red or unchanged — glucose negative (glucose not used)
- black — formation of hydrogen sulfide
- bubbles or cracks — gas formation from glucose

b) Slant surface

- yellow — lactose and/or sucrose positive (lactose and/or sucrose used)
- red or unchanged — lactose and sucrose negative (neither lactose nor sucrose used)

Typical *Salmonella* cultures show alkaline (red) slants and acid (yellow) butts with gas formation (bubbles) and (in about 90 % of the cases) formation of hydrogen sulfide (blackening of the agar) (9.5.3.8).

When a lactose-positive *Salmonella* is isolated (see 4.4), the TSI slant is yellow. Thus, preliminary confirmation of *Salmonella* cultures shall not be based on the results of the TSI agar test only (see 9.5.3).

9.5.3.3 **Urea agar** (5.2.7)

Streak the agar slant surface. Incubate at 37 °C ± 1 °C for 24 h ± 3 h and examine at intervals.

If the reaction is positive, splitting of urea liberates ammonia, which changes the colour of phenol red to rose-pink and later to deep cerise. The reaction is often apparent after 2 h to 4 h.

9.5.3.4 L-Lysine decarboxylation medium (5.2.8)

Inoculate just below the surface of the liquid medium. Incubate at 37 °C ± 1 °C for 24 h ± 3 h.

Turbidity and a purple colour after incubation indicates a positive reaction. A yellow colour indicates a negative reaction.

9.5.3.5 Detection of β-galactosidase (5.2.9)

Suspend a loopful of the suspected colony in a tube containing 0,25 ml of the saline solution (5.2.13).

Add 1 drop of toluene and shake the tube. Put the tube in a water bath (6.6) set at 37 °C and leave for several minutes (approximately 5 min). Add 0,25 ml of the reagent for detection of β-galactosidase and mix.

Replace the tube in the water bath set at 37 °C and leave for 24 h ± 3 h, examining the tube at intervals.

A yellow colour indicates a positive reaction. The reaction is often apparent after 20 min.

If prepared paper discs (5.2.9) are used, follow the manufacturer's instructions.

9.5.3.6 Medium for Voges-Proskauer (VP) reaction (5.2.10)

Suspend a loopful of the suspected colony in a sterile tube containing 3 ml of the VP medium.

Incubate at 37 °C ± 1 °C for 24 h ± 3 h.

After incubation, add two drops of the creatine solution, three drops of the ethanolic solution of 1-naphthol and then two drops of the potassium hydroxide solution; shake after the addition of each reagent.

The formation of a pink to bright red colour within 15 min indicates a positive reaction.

9.5.3.7 Medium for indole reaction (5.2.11)

Inoculate a tube containing 5 ml of the tryptone/tryptophan medium with the suspected colony.

Incubate at 37 °C ± 1 °C for 24 h ± 3 h. After incubation, add 1 ml of the Kovacs reagent.

The formation of a red ring indicates a positive reaction. A yellow-brown ring indicates a negative reaction.

9.5.3.8 Interpretation of the biochemical tests

Salmonella generally show the reactions given in Table 1.

9.5.4 Serological confirmation and serotyping

9.5.4.1 General

The detection of the presence of *Salmonella* O-, Vi- and H-antigens is tested by slide agglutination with the appropriate sera, from pure colonies (9.5.2) and after auto-agglutinable strains have been eliminated. Use the antisera according to the producer's instructions if different from the description below.

9.5.4.2 Elimination of auto-agglutinable strains

Place one drop of the saline solution (5.2.13) onto a carefully cleaned glass slide. Disperse in the drop, by means of a loop (6.7), part of the colony to be tested, in order to obtain a homogeneous and turbid suspension.

NOTE It is also possible to disperse part of the colony to be tested in a drop of water, and then to mix this solution with one drop of saline solution (5.2.13).

Rock the slide gently for 30 s to 60 s. Observe the result against a dark background, preferably with the aid of a magnifying glass.

If the bacteria have clumped into more or less distinct units, the strain is considered auto-agglutinable, and shall not be submitted to the following tests as the detection of the antigens is not feasible.

Table 1 — Interpretation of biochemical tests

Test [a] (9.5.3.2 to 9.5.3.7)	***Salmonella* strain**									
	***S.* Typhi**		***S.* Paratyphi A**		***S.* Paratyphi B**		***S.* Paratyphi C**		**Other strains**	
	Reaction	% [b]	Reaction	% [b]	Reaction	% [c]	Reaction	% [c]	Reaction	% [b]
TSI acid from glucose	+	100	+	100	+		+		+	100
TSI gas from glucose	– [d]	0	+	100	+		+		+	92
TSI acid from lactose	–	2	–	100	–		–		–	1
TSI acid from sucrose	–	0	–	0	–		–		–	1
TSI hydrogen sulfide produced	+	97	–	10	+		+		+	92
Urea hydrolysis	–	0	–	0	–		–		–	1
Lysine decarboxylation	+	98	–	0	+		+		+	95
β-Galactosidase reaction	–	0	–	0	–		–		–	2 [e]
Voges-Proskauer reaction	–	0	–	0	–		–		–	0
Production of indole	–	0	–	0	–		–		–	1

[a] From reference [5].

[b] These percentages indicate that not all isolates of *Salmonella* serotype show the reactions marked + or –. These percentages may vary between and within serotypes of food poisoning serotypes from different locations.

[c] The percentages are not known from available literature.

[d] *Salmonella* Typhi is anaerogenic.

[e] The *Salmonella enterica* subspecies *arizonæ* gives a positive or negative lactose reaction but is always β-galactosidase positive. For the study of these strains it may be useful to carry out complementary tests.

9.5.4.3 Examination for O-antigens

Using one non-autoagglutinating pure colony, proceed according to 9.5.4.2, using one drop of the anti-O serum (5.3) instead of the saline solution (5.2.13).

If agglutination occurs, the reaction is considered positive.

Use the poly- and monovalent sera one after the other.

9.5.4.4 Examination for Vi-antigens

Proceed according to 9.5.4.2, but using one drop of the anti-Vi serum (5.3) instead of the saline solution.

If agglutination occurs, the reaction is considered positive.

9.5.4.5 Examination for H-antigens

Inoculate the semi-solid nutrient agar (5.2.12) with a pure non-auto-agglutinable colony. Incubate the medium at 37 °C ± 1 °C for 24 h ± 3 h.

Use this culture for examination for the H-antigens, proceeding according to 9.5.4.2, but using one drop of the anti-H serum (5.3) instead of the saline solution.

If agglutination occurs, the reaction is considered positive.

9.5.5 Interpretation of biochemical and serological reactions

Table 2 gives the interpretation of the confirmatory tests (9.5.3 and 9.5.4) carried out on the colonies used (9.5.2).

Table 2 — Interpretation of confirmatory tests

Biochemical reactions	Auto-agglutination	Serological reactions	Interpretation
Typical	No	O-, Vi- or H-antigen positive	Strains considered to be *Salmonella*
Typical	No	All reactions negative	May be *Salmonella*
Typical	Yes	Not tested (see 9.5.4.2)	
No typical reactions	No/Yes	O-, Vi- or H-antigen positive	
No typical reactions	No/Yes	All reactions negative	Not considered to be *Salmonella*

9.5.6 Definitive confirmation

Strains which are considered to be *Salmonella*, or which may be *Salmonella* (see Table 2), shall be sent to a recognized *Salmonella* reference centre for definitive typing.

This dispatch shall be accompanied by all possible information concerning the strain(s) and whether it is an outbreak or in food.

10 Expression of results

In accordance with the results of the interpretation, indicate the presence or absence of *Salmonella* in a test portion of x g or x ml of product (see ISO 7218).

See annex C for the precision data obtained from the interlaboratory trial.

11 Test report

The test report shall specify:

— the sampling method used, if known;

— any deviation in the enrichment media or the incubation conditions used;

— all operating conditions not specified in this International Standard, or regarded as optional, together with details of any incidents which may have influenced the results;

— the results obtained.

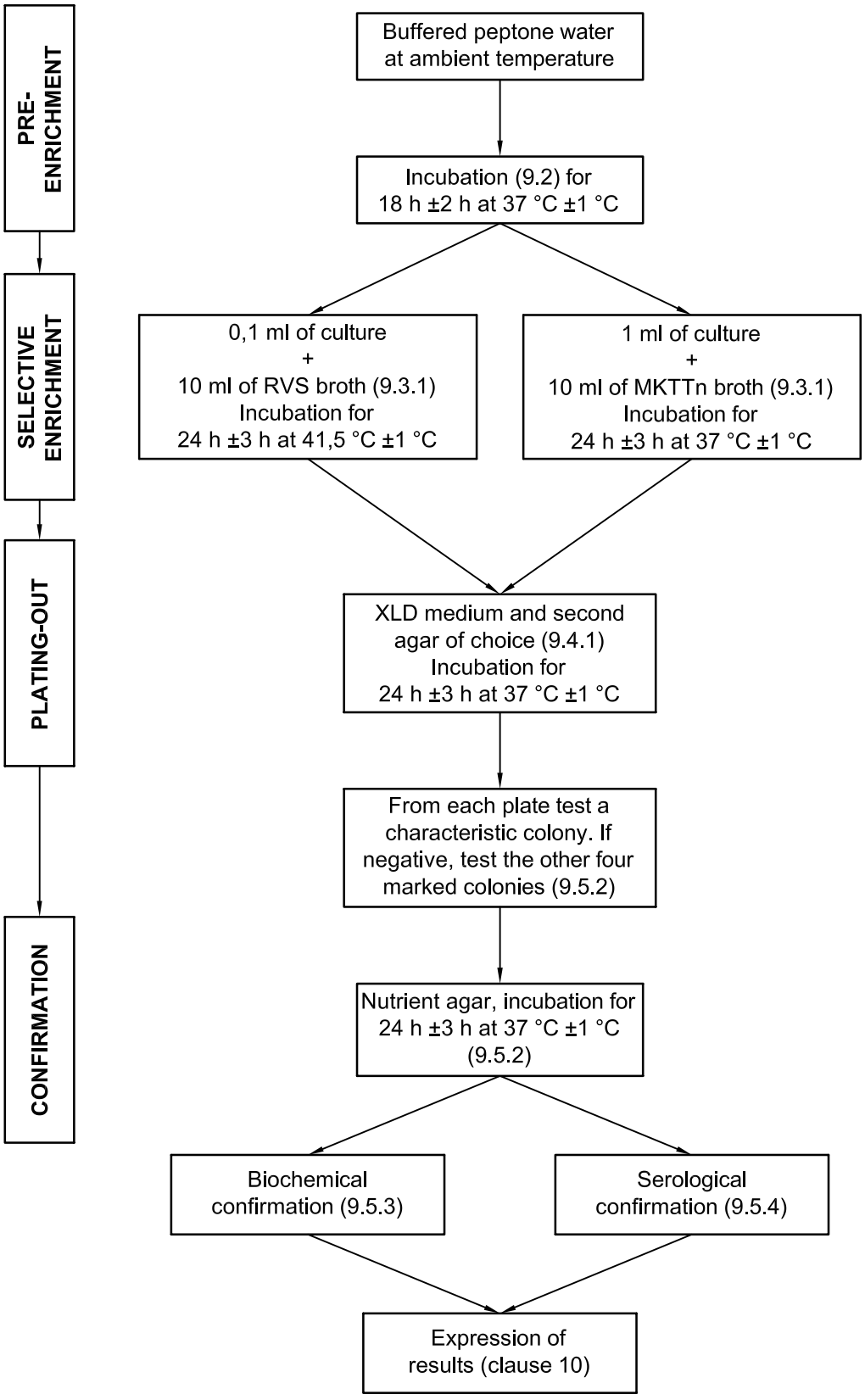
The test report shall also state whether a positive result was obtained only when using a plating-out medium (5.2.4) not specified in this International Standard.

12 Quality assurance

To check the ability of the laboratory to detect *Salmonella* with the methods and media described in this International Standard, introduce reference samples into control flasks of the pre-enrichment medium (see 5.2.1). Proceed with the control flasks as for the test cultures.

Annex A
(normative)

Diagram of procedure



Annex B
(normative)

Composition and preparation of culture media and reagents

B.1 Buffered peptone water

B.1.1 Composition

Enzymatic digest of casein	10,0 g
Sodium chloride	5,0 g
Disodium hydrogen phosphate dodecahydrate ($Na_2HPO_4 \cdot 12H_2O$)	9,0 g
Potassium dihydrogen phosphate (KH_2PO_4)	1,5 g
Water	1 000 ml

B.1.2 Preparation

Dissolve the components 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 medium into flasks (6.9) of suitable capacity to obtain the portions necessary for the test.

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

B.2 Rappaport-Vassiliadis medium with soya (RVS broth)

B.2.1 Solution A

B.2.1.1 Composition

Enzymatic digest of soya	5,0 g
Sodium chloride	8,0 g
Potassium dihydrogen phosphate (KH_2PO_4)	1,4 g
Dipotassium hydrogen phosphate (K_2HPO_4)	0,2 g
Water	1 000 ml

B.2.1.2 Preparation

Dissolve the components in the water by heating to about 70 °C if necessary.

The solution shall be prepared on the day of preparation of the complete RVS medium.

B.2.2 Solution B

B.2.2.1 Composition

Magnesium chloride hexahydrate ($MgCl_2 \cdot 6H_2O$)	400,0 g
Water	1 000 ml

B.2.2.2 Preparation

Dissolve the magnesium chloride in the water.

As this salt is very hygroscopic, it is advisable to dissolve the entire contents of $MgCl_2 \cdot 6H_2O$ from a newly opened container, according to the formula. For instance, 250 g of $MgCl_2 \cdot 6H_2O$ is added to 625 ml of water, giving a solution of total volume of 788 ml and a mass concentration of about 31,7 g per 100 ml of $MgCl_2 \cdot 6H_2O$.

The solution may be kept in a dark glass bottle with tight stopper at room temperature for at least 2 years.

B.2.3 Solution C

B.2.3.1 Composition

Malachite green oxalate	0,4 g
Water	100 ml

B.2.3.2 Preparation

Dissolve the malachite green oxalate in the water.

The solution may be kept in a brown glass bottle at room temperature for at least 8 months.

B.2.4 Complete medium

B.2.4.1 Composition

Solution A (B.2.1)	1 000 ml
Solution B (B.2.2)	100 ml
Solution C (B.2.3)	10 ml

B.2.4.2 Preparation

Add to 1 000 ml of solution A, 100 ml of solution B and 10 ml of solution C.

Adjust the pH, if necessary, so that after sterilization it is 5,2 ± 0,2.

Before use, dispense into test tubes (6.9) in 10 ml quantities.

Sterilize for 15 min in the autoclave (6.1) set at 115 °C.

Store the prepared medium at 3°C ± 2°C. Use the medium the day of its preparation.

NOTE The final medium composition is: enzymatic digest of soya, 4,5 g/l; sodium chloride, 7,2 g/l; potassium dihydrogen phosphate ($KH_2PO_4 + K_2HPO_4$), 1,44 g/l; anhydrous magnesium chloride ($MgCl_2$), 13,4 g/l or magnesium chloride hexahydrate ($MgCl_2 \cdot 6H_2O$), 28,6 g/l; malachite green oxalate, 0,036 g/l.

B.3 Muller-Kauffmann tetrathionate-novobiocin broth (MKTTn) [7]

B.3.1 Base medium

B.3.1.1 Composition

Meat extract	4,3 g
Enzymatic digest of casein	8,6 g
Sodium chloride (NaCl)	2,6 g
Calcium carbonate ($CaCO_3$)	38,7 g
Sodium thiosulfate pentahydrate ($Na_2S_2O_3 \cdot 5H_2O$)	47,8 g
Ox bile for bacteriological use	4,78 g
Brilliant green	9,6 mg
Water	1 000 ml

B.3.1.2 Preparation

Dissolve the dehydrated basic components or the dehydrated complete medium in the water by boiling for 5 min.

Adjust the pH, if necessary, so that it is 8,2 $\pm$ 0,2 at 25 °C.

Thoroughly mix the medium.

The base medium may be stored for 4 weeks at 3 °C $\pm$ 2 °C.

B.3.2 Iodine-iodide solution

B.3.2.1 Composition

Iodine	20,0 g
Potassium iodide (KI)	25,0 g
Water	100 ml

B.3.2.2 Preparation

Completely dissolve the potassium iodide in 10 ml of water, then add the iodine and dilute to 100 ml with sterile water. Do not heat.

Store the prepared solution in the dark at ambient temperature in a tightly closed container.

B.3.3 Novobiocin solution

B.3.3.1 Composition

Novobiocin sodium salt	0,04 g
Water	5 ml

B.3.3.2 Preparation

Dissolve the novobiocin sodium salt in the water and sterilize by filtration.

Store for up to 4 weeks at 3 °C $\pm$ 2 °C.

B.3.4 Complete medium

B.3.4.1 Composition

Base medium (B.3.1)	1 000 ml
Iodine-iodide solution (B.3.2)	20 ml
Novobiocin solution (B.3.3)	5 ml

B.3.4.2 Preparation

Aseptically add 5 ml of the novobiocin solution (B.3.3) to 1 000 ml of base medium (B.3.1). Mix, then add 20 ml of the iodine-iodide solution (B.3.2). Mix well.

Dispense the medium aseptically into sterile flasks (6.9) of suitable capacity to obtain the portions necessary for the test.

The complete medium shall be used the day of its preparation.

B.4 Xylose lysine deoxycholate agar (XLD agar) [7]

B.4.1 Base medium

B.4.1.1 Composition

Yeast extract powder	3,0 g
Sodium chloride (NaCl)	5,0 g
Xylose	3,75 g
Lactose	7,5 g
Sucrose	7,5 g
L-Lysine hydrochloride	5,0 g
Sodium thiosulfate	6,8 g
Iron(III) ammonium citrate	0,8 g
Phenol red	0,08 g
Sodium deoxycholate	1,0 g
Agar	9 g to 18 g [1)]
Water	1 000 ml

B.4.1.2 Preparation

Dissolve the dehydrated base components or the dehydrated complete base in the water by heating, with frequent agitation, until the medium starts to boil. Avoid overheating.

Adjust the pH, if necessary, so that after sterilization it is 7,4 $\pm$ 0,2 at 25 °C.

Pour the base to tubes or flasks (6.9) of appropriate capacity.

Heat with frequent agitation until the medium boils and the agar dissolves. Do not overheat.

---

1) Depending on the gel strength of the agar.

B.4.2 Preparation of the agar plates

Transfer immediately to a water bath (6.5) at 44 °C to 47 °C, agitate and pour into plates. Allow to solidify.

Immediately before use, dry the agar plates carefully (preferably with the lids off and the agar surface downwards) in the oven (6.2) set between 37 °C and 55 °C until the surface of the agar is dry.

Store the poured plates for up to 5 days at 3 °C ± 2 °C.

B.5 Nutrient agar

B.5.1 Composition

Meat extract	3,0 g
Peptone	5,0 g
Agar	9 g to 18 g [1)]
Water	1 000 ml

B.5.2 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.

Transfer the culture medium into tubes or bottles (6.9) of appropriate capacity.

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

B.5.3 Preparation of nutrient agar plates

Transfer about 15 ml of the melted medium to sterile small Petri dishes (6.11) and proceed as in B.4.2.

B.6 Triple sugar/iron agar (TSI agar)

B.6.1 Composition

Meat extract	3,0 g
Yeast extract	3,0 g
Peptone	20,0 g
Sodium chloride (NaCl)	5,0 g
Lactose	10,0 g
Sucrose	10,0 g
Glucose	1,0 g
Iron(III) citrate	0,3 g
Sodium thiosulfate	0,3 g
Phenol red	0,024 g
Agar	9 g to 18 g [1)]
Water	1 000 ml

B.6.2 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 7,4 ± 0,2 at 25 °C.

Dispense the medium into test tubes or dishes in quantities of 10 ml.

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

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

B.7 Urea agar (Christensen)

B.7.1 Base medium

B.7.1.1 Composition

Peptone	1,0 g
Glucose	1,0 g
Sodium chloride (NaCl)	5,0 g
Potassium dihydrogen phosphate (KH_2PO_4)	2,0 g
Phenol red	0,012 g
Agar	9 g to 18 g [1)]
Water	1 000 ml

B.7.1.2 Preparation

Dissolve the components or the dehydrated complete base 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.

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

B.7.2 Urea solution

B.7.2.1 Composition

Urea	400 g
Water, to a final volume of	1 000 ml

B.7.2.2 Preparation

Dissolve the urea in the water. Sterilize by filtration and check the sterility.

See ISO 7218:1996, 7.3.2.

B.7.3 Complete medium

B.7.3.1 Composition

Base (B.7.1)	950 ml
Urea solution (B.7.2)	50 ml

B.7.3.2 Preparation

Add, under aseptic conditions, the urea solution to the base, previously melted and then cooled to 44 °C to 47 °C.

Dispense the complete medium into sterile tubes (6.9) in quantities of 10 ml.

Allow to set in a sloping position.

B.8 L-Lysine decarboxylation medium

B.8.1 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

B.8.2 Preparation

Dissolve the components 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.

Transfer the medium in quantities of 2 ml to 5 ml to narrow culture tubes (6.9) with screw caps.

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

B.9 β-Galactosidase reagent

B.9.1 Buffer solution

B.9.1.1 Composition

Sodium dihydrogen phosphate (NaH_2PO_4)	6,9 g
Sodium hydroxide, 10 mol/l solution	about 3 ml
Water, to a final volume of	50 ml

B.9.1.2 Preparation

Dissolve the sodium dihydrogen phosphate in approximately 45 ml of water in a volumetric flask.

Adjust the pH to 7,0 ± 0,2 at 25 °C with the sodium hydroxide solution.

Add water to a final volume of 50 ml.

B.9.2 ONPG solution

B.9.2.1 Composition

o-Nitrophenyl β-D-galactopyranoside (ONPG)	0,08 g
Water	15 ml

B.9.2.2 Preparation

Dissolve the ONPG in the water at approximately 50 °C.

Cool the solution.

B.9.3 Complete reagent

B.9.3.1 Composition

Buffer solution (B.9.1)	5 ml
ONPG solution (B.9.2)	15 ml

B.9.3.2 Preparation

Add the buffer solution to the ONPG solution.

B.10 Reagents for Voges-Proskauer (VP) reaction

B.10.1 VP medium

B.10.1.1 Composition

Peptone	7,0 g
Glucose	5,0 g
Dipotassium hydrogen phosphate (K_2HPO_4)	5,0 g
Water	1 000 ml

B.10.1.2 Preparation

Dissolve the components in the water, by heating if necessary.

Adjust the pH, if necessary, so that after sterilization it is 6,9 $\pm$ 0,2 at 25 °C.

Transfer the medium to tubes (6.9) in quantities of 3 ml.

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

B.10.2 Creatine solution (*N*-amidinosarcosine)

B.10.2.1 Composition

Creatine monohydrate	0,5 g
Water	100 ml

B.10.2.2 Preparation

Dissolve the creatine monohydrate in the water.

B.10.3 1-Naphthol, ethanolic solution

B.10.3.1 Composition

1-Naphthol	6 g
Ethanol, 96 % (volume fraction)	100 ml

B.10.3.2 Preparation

Dissolve the 1-naphthol in the ethanol.

B.10.4 Potassium hydroxide solution

B.10.4.1 Composition

Potassium hydroxide	40 g
Water	100 ml

B.10.4.2 Preparation

Dissolve the potassium hydroxide in the water.

B.11 Reagents for indole reaction

B.11.1 Tryptone/tryptophan medium

B.11.1.1 Composition

Tryptone	10 g
Sodium chloride (NaCl)	5 g
DL-Tryptophan	1 g
Water	1 000 ml

B.11.1.2 Preparation

Dissolve the components in the boiling water.

Adjust the pH, if necessary, so that after sterilization it is 7,5 $\pm$ 0,2 at 25 °C.

Dispense 5 ml of the medium into each of several tubes (6.9).

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

B.11.2 Kovacs reagent

B.11.2.1 Composition

4-Dimethylaminobenzaldehyde	5 g
Hydrochloric acid, ρ = 1,18 g/ml to 1,19 g/ml	25 ml
2-Methylbutan-2-ol	75 ml

B.11.2.2 Preparation

Mix the components.

B.12 Semi-solid nutrient agar

B.12.1 Composition

Meat extract	3,0 g
Peptone	5,0 g
Agar	4 g to 9 g [1)]
Water	1 000 ml

B.12.2 Preparation

Dissolve the components 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.

Transfer the medium to flasks (6.9) of appropriate capacity.

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

B.12.3 Preparation of agar plates

Pour into small sterile Petri dishes (6.11), about 15 ml of the freshly prepared medium. Do not allow the agar plates to dry.

B.13 Physiological saline solution

B.13.1 Composition

Sodium chloride (NaCl)	8,5 g
Water	1 000 ml

B.13.2 Preparation

Dissolve the sodium chloride in the water.

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

Dispense quantities of the solution into flasks or tubes (6.9) so that they will contain 90 ml to 100 ml after sterilization.

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

Annex C
(informative)

Results of interlaboratory trial

An international collaborative test was organized in 2000 by AFSSA Ploufragan in Europe, and BioControl Systems in the USA, in the frame of the European project SMT CT 96 2098 [6]. This test involved 11 laboratories in 9 countries in Europe and 10 laboratories in the USA, and was carried out on fresh cheese curd, dried egg powder, raw poultry meat and a reference material. The food samples were each tested at two different levels of contamination, plus a negative control.

The values of the performance characteristics derived from this collaborative test are shown per type of sample in Tables C.1 to C.4. Data obtained by some laboratories have been excluded from the calculations only on the basis of clearly identified technical reasons (deviations to the protocol).

Table C.1 — Results of data analysis obtained with fresh cheese curd samples

	Fresh cheese curd (blank)	**Fresh cheese curd** (low level contamination)	**Fresh cheese curd** (high level contamination)
Number of laboratories having returned results	23	23	23
Number of samples per laboratory	5	5	5
Number of excluded laboratories	2	2	2
Number of laboratories retained after exclusion	21	21	21
Number of accepted samples	105	105	105
Accuracy (specificity), %	100	—	—
Accuracy (sensitivity), %	—	74,3	83,8
Accordance, %	100	83,8	95,2
Concordance, %	100	60,5	71,7

Table C.2 — Results of data analysis obtained with dried egg powder samples

	Dried egg powder (blank)	**Dried egg powder** (low level contamination)	**Dried egg powder** (high level contamination)
Number of laboratories having returned results	26	26	26
Number of samples per laboratory	5	5	5
Number of excluded laboratories	5	5	5
Number of laboratories retained after exclusion	21	21	21
Number of accepted samples	105	105	104
Accuracy (specificity), %	100	—	—
Accuracy (sensitivity), %	—	98,1	99
Accordance, %	100	96,2	98,1
Concordance, %	100	96,2	98,1

Table C.3 — Results of data analysis obtained with raw poultry meat samples

	Raw poultry meat (blank)	**Raw poultry meat** (low level contamination)	**Raw poultry meat** (high level contamination)
Number of laboratories having returned results	25	25	25
Number of samples per laboratory	5	5	5
Number of excluded laboratories	5	5	5
Number of laboratories retained after exclusion	20	20	20
Number of accepted samples	100	99	100
Accuracy (specificity), %	100	—	—
Accuracy (sensitivity), %	—	98	100
Accordance, %	100	96,9	100
Concordance, %	100	96	100

Table C.4 — Results of data analysis obtained with reference materials

	Reference material (capsules containing about 5 cfu of *S*. Typhimurium)
Number of laboratories having returned results	26
Number of samples per laboratory	5
Number of excluded laboratories	1
Number of laboratories retained after exclusion	25
Number of accepted samples	125
Accuracy (specificity), %	—
Accuracy (sensitivity), %	94,4
Accordance, %	88,8
Concordance, %	89,1

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[2] 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*

[3] 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*

[4] ISO/TR 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*

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INTERNATIONAL STANDARD

ISO 6579

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AMENDMENT 1
2007-07-15

Microbiology of food and animal feeding stuffs — Horizontal method for the detection of *Salmonella* spp.

AMENDMENT 1: Annex D: Detection of *Salmonella* spp. in animal faeces and in environmental samples from the primary production stage

Microbiologie des aliments — Méthode horizontale pour la recherche des Salmonella *spp.*

AMENDEMENT 1: Annexe D: Recherche des Salmonella *spp. dans les matières fécales des animaux et dans des échantillons environnementaux au stade de la production primaire*

Reference number
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Foreword

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International Standards are drafted in accordance with the rules given in the ISO/IEC Directives, Part 2.

The main task of technical committees is to prepare International Standards. 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.

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.

Amendment 1 to ISO 6579:2002 was prepared by Technical Committee ISO/TC 34, *Food products*, Subcommittee SC 9, *Microbiology*.

Microbiology of food and animal feeding stuffs — Horizontal method for the detection of *Salmonella* spp.

AMENDMENT 1: Annex D: Detection of *Salmonella* spp. in animal faeces and in environmental samples from the primary production stage

Page 1, Clause 2

Replace the introductory text as follows and add the two references.

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/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*

Page 27, after Annex C

Add the following as Annex D.

Annex D
(normative)

Detection of *Salmonella* spp. in animal faeces and in environmental samples from the primary production stage

D.1 Introduction

The method given in the main text of this International Standard is primarily intended for the isolation of *Salmonella* spp. from food and feeding stuffs and is not always suitable for the detection of *Salmonella* spp. from other matrices.

This annex is applicable to the detection of *Salmonella* spp. in

— animal faeces (such as from poultry, pigs, cattle), and

— environmental samples in the area of the primary production stage (such as dust).

The method in this annex is based upon Clause 9, with a different selective enrichment medium. Therefore, where possible, reference will be made to Clause 9.

The selective enrichment medium as described in this annex (modified semi-solid Rappaport-Vassiliadis: MSRV) is intended for the detection of motile Salmonellae and is not appropriate for the detection of non-motile Salmonellae.

NOTE The non-motile *Salmonella* biovars of *Salmonella* Gallinarum (*Salmonella* Gallinarum biovar gallinarum and *Salmonella* Gallinarum biovar pullorum do not seem to survive long in environmental samples and will therefore rarely be detected in faecal or environmental (such as dust) samples (regardless of the method). The number of other non-motile *Salmonella* serovars in faecal samples seems to be generally low. For example, in Reference [7] in which circa 1 000 faecal samples of poultry layer flocks and circa 900 faecal samples of broiler flocks were analysed, less than 1 % of the total number of samples were positive in a selective broth and at the same time negative on MSRV (and likely to be non-motile). Similar results were found in a Dutch study with circa 3 200 faecal samples of pigs (non-published data). On the other hand, in the case of the study in Reference [7], up to almost 40 % of positive samples would not have been detected (i.e. false negatives) if only a selective broth (in this case Rappaport Vassiliadis) had been used instead of a semi-solid medium.

D.2 Principle

D.2.1 General

The detection of *Salmonella* in animal faeces and in samples of the primary production stage necessitates four stages, as described in Clause 4.

D.2.2 Pre-enrichment in non-selective liquid medium

Buffered peptone water (BPW) is inoculated at ambient temperature with the test portion, then incubated at 37 °C ± 1 °C for 18 h ± 2 h.

D.2.3 Enrichment on selective semi-solid medium

Modified semi-solid Rappaport-Vassiliadis (MSRV) agar plates are inoculated with the culture obtained in D.2.2.

The MSRV is incubated at 41,5 °C ± 1 °C for 24 h ± 3 h. If a plate is negative after 24 h, it is incubated for a further 24 h ± 3 h.

D.2.4 Selective plating and identification

From the culture obtained in D.2.3, two selective solid media are inoculated:

— xylose lysine deoxycholate (XLD) agar;

— any other solid selective medium complementary to XLD agar (see 4.4).

The XLD agar is incubated at 37 °C ± 1 °C and examined after 24 h ± 3 h.

The second selective medium is incubated in accordance with the manufacturer's instructions.

D.2.5 Confirmation of identity

Colonies of presumptive *Salmonella* are subcultured, then plated-out as described in D.2.4, and their identity is confirmed by means of appropriate biochemical and serological tests.

D.3 Culture media, reagents and sera

D.3.1 General

For current laboratory practice, see ISO 7218.

All media and reagents needed for this annex are described in Annex B, except for modified semi-solid Rappaport-Vassiliadis (MSRV) medium, which is described in D.3.2. Alternatively, dehydrated complete media or diluents may be used. Follow, in that respect, the manufacturer's instructions.

NOTE The composition of MSRV, as described in Reference [8], contained 20 mg/l of novobiocin. However, from a scientific point of view, 10 mg/l novobiocin is preferred. In studies performed at the CRL-*Salmonella*, more *Salmonella*-positive results were found in pig faeces samples when tested with MSRV containing 10 mg/l than with MSRV containing 20 mg/l novobiocin (see Reference [9]). Furthermore, when testing different animal faeces (pigs, chicken, cattle) and naturally contaminated dust, the migration zones on MSRV containing 10 mg/l novobiocin were (much) larger than on MSRV containing 20 mg/l novobiocin (Reference [9]). The influence of novobiocin on bacterial motility was earlier described in Reference [10].

For the preparation of the selective plating agar media (see B.4, XLD-agar), standard size Petri dishes may be used (90 mm or 100 mm) instead of large size Petri dishes (140 mm).

D.3.2 Modified semi-solid Rappaport-Vassiliadis medium (MSRV)

D.3.2.1 Base medium

D.3.2.1.1 Composition

Enzymatic digest of animal and plant tissue	4,6	g
Acid hydrolysate of casein	4,6	g
Sodium chloride (NaCl)	7,3	g
Potassium dihydrogenphosphate (KH_2PO_4)	1,5	g
Magnesium chloride anhydrous ($MgCl_2$)	10,9	g
Malachite green oxalate	0,04	g
Agar	2,7	g
Water	1 000	ml

D.3.2.1.2 Preparation

Suspend the ingredients into the water.

Heat to boiling with agitation. **Do not autoclave**.

Do not hold the medium at high temperatures longer than necessary.

Cool the medium to 47-50 °C.

D.3.2.2 Novobiocin solution

D.3.2.2.1 Composition

Novobiocin sodium salt	0,05	g
Water	10	ml

D.3.2.2.2 Preparation

Dissolve the novobiocin sodium salt in the water.

Sterilize by filtration through a filter with a pore size of 0,22 µm.

The solution may be stored for up to 4 weeks at 5 °C ± 3 °C or in small portions (e.g. of 2 ml) at −20 °C for up to one year.

D.3.2.3 Complete medium

D.3.2.3.1 Composition

Base medium (D.3.2.1)	1 000	ml
Novobiocin solution (D.3.2.2)	2	ml

D.3.2.3.2 Preparation

Aseptically add 2 ml of the novobiocin solution (D.3.2.2) to 1 000 ml of base medium (D.3.2.1) at 47 °C to 50 °C. Mix carefully.

The final pH shall be 5,2 (5,1 to 5,4) at 20 °C to 25 °C.

Pour into plates up to a volume of 15 ml to 20 ml in Petri dishes with a diameter of 90 mm.

Allow the medium to solidify before moving and handle with care.

Store the plates, **with surface upwards**, for up to 2 weeks at 5 °C ± 3 °C in the dark.

Do not invert the plates, as the semi-solid agar is too liquid to do so.

Any plates in which the semi-solid agar has liquefied or fragmented shall not be used.

Immediately before use, and only if necessary, dry the surface of the agar plates carefully, for example by placing them with the lids off and the agar surface **upwards** in a laminar air flow cabinet. Take care not to overdry the medium.

D.4 Apparatus and glassware

Use the apparatus listed in Clause 6, and the following.

D.4.1 Sterile loops, of 1 µl.

D.5 Sampling

See Clause 7.

D.6 Preparation of test sample

See Clause 8.

In general, an amount of sample is added to a quantity of BPW to yield a 1/10 dilution (e.g. 25 g of sample added to 225 ml of BPW). However, for some type of samples it may be necessary to use another ratio.

D.7 Procedure

D.7.1 Non-selective pre-enrichment

Pre-warm the BPW to room temperature before use.

Mix samples well by the most suitable means for the sample type.

Weigh the sample and add it to the appropriate quantity of BPW (see D.6). Incubate the jars at 37 °C ± 1 °C for 18 h ± 2 h.

D.7.2 Selective enrichment

Allow the MSRV plates to equilibrate at room temperature if they were stored at a lower temperature.

Inoculate the MSRV plates with 3 drops of incubated BPW culture. The 3 drops should total 0,1 ml and should be placed separately and equally spaced on the surface of the medium.

When taking a subculture from BPW, it is very important not to disturb particulate samples. Therefore, containers should be moved carefully, and not mixed, shaken or swirled. Aim to extract an inoculum from the largest volume of free fluid nearest the interface between container and surface of culture, but it is advisable to go deeper if there are particulates floating on the surface.

Incubate the inoculated MSRV plates at 41,5 °C ± 1 °C for 24 h ± 3 h.

Do not invert the plates.

Positive plates will show a grey-white, turbid zone extending out from the inoculated drop. The turbid zone is characterized by a white halo with a clearly defined edge.

If the plates are negative after 24 h, re-incubate for a further 24 h ± 3 h.

D.7.3 Selective plating

Allow the xylose lysine deoxycholate agar (XLD) plates and the second selective plating medium (see 5.2.4.2) to equilibrate at room temperature if they were stored at a lower temperature. If necessary, dry the surface of the plates before use.

Subculture positive MSRV plates:

Observe the MSRV plate (if necessary on a clear white surface or light box). Determine where the furthest point of spread of opaque growth from the inoculation points is, and dip a 1 µl loop just inside the border of the opaque growth. Withdraw the loop ensuring that no large lumps of MSRV are extracted. Inoculate the surface of an XLD plate so that well-isolated colonies will be obtained. Proceed in the same way with the second selective plating medium using a new sterile loop.

NOTE By plating-out little material from MSRV (using a 1 µl loop), well-isolated colonies can be obtained by using only one standard size Petri dish (90 mm to 100 mm) with selective plating agar. The use of large dishes (140 mm) will therefore not be necessary.

Incubate the XLD plates inverted at 37 °C ± 1 °C for 24 h ± 3 h.

Incubate the second selective plating medium in accordance with the manufacturer's instructions.

Return negative MSRV plates to the 41,5 °C incubator and incubate for a further 24 h ± 3 h. Perform the selective plating procedure if, after 48 h of incubation, these MSRV plates become positive.

Typical colonies of *Salmonella* grown on XLD-agar have a black centre and a lightly transparent zone of reddish colour due to the colour change of the indicator.

Salmonella H_2S negative variants (e.g. *Salmonella* Paratyphi A) grown on XLD agar are pink with a darker pink centre. Lactose-positive *Salmonella* grown on XLD agar are yellow with or without blackening (also see 9.4.4).

Check the second selective plating medium after the appropriate incubation time for the presence of colonies which, from their characteristics, are considered to be presumptive *Salmonella*.

D.7.4 Confirmation

For confirmation of the typical colonies, isolated on the selective plating media, follow the instructions as given in 9.5. In 9.5.2, it is prescribed to streak isolated colonies from the selective plating media onto nutrient agar before performing the biochemical confirmation. However, this extra cultural step is not necessary if well-isolated colonies (of a pure culture) are available on the selective plating media. If this is the case, perform the biochemical confirmation directly on a typical (suspect), well-isolated colony of each selective plating medium.

D.8 Expression of results

See Clause 10.

D.9 Test report

See Clause 11.

D.10 Quality assurance

See Clause 12.

For the performance testing of media, follow the recommendations of ISO/TS 11133-1 and ISO/TS 11133-2. However, in these documents, procedures are given for selective broths as well as for selective agar media for the detection of *Salmonella,* but not for semi-solid media like MSRV. The procedure given below may be used for testing the performance of MSRV and is based upon the procedure and test strains as described for selective (enrichment) media for the detection of *Salmonella* (e.g. RVS and MKTTn, see B.2 and B.3) in ISO/TS 11133-2.

The procedure given below has been extracted from ISO/TS 11133-2:2003, 5.4.2.1, but with an adapted concentration of the test strains. The procedure, test strains and criteria are summarized in Table D.1.

— Inoculation of target microorganisms: Inoculate MSRV for each test organism with *ca.* 10^4 cfu/ 0,1 ml (for preparation of the inoculum, see ISO/TS 11133-2:2003, 5.2.1).

— Inoculation of non-target microorganisms: Inoculate MSRV for each test organism with 10^5 to 10^6 cfu/ 0,1 ml (for preparation of the inoculum, see ISO/TS 11133-2:2003, 5.2.1).

— Inoculation of target and non-target microorganisms as a mixed culture: Inoculate MSRV with a mixed culture containing *ca.* 10^4 cfu/ 0,1 ml of target microorganisms and 10^5 to 10^6 cfu/ 0,1 ml of non-target microorganisms (for preparation of the inoculums, see ISO/TS 11133-2:2003, 5.2.1).

Incubate the MSRV plates at 41,5 °C ± 1 °C and assess the plates after 24 h ± 3 h and after 48 h ± 6 h.

Table D.1 — Performance testing of MSRV

Function	Control strains	Final concentration in the inoculum of 0,1 ml	Incubation of MSRV	Criteria
Specificity	*S.* Typhimurium ATCC 14028 or *S.* Enteritidis ATCC 13076	10^4 cfu	41,5 °C ± 1 °C, 2 × 24 h ± 3 h	Grey-white, turbid zone extending out from the inoculated drop. After 48 h, the turbid zones of the 3 drops will be (almost) fully migrated over the plate.
Selectivity	*E. coli* ATCC 25922 or ATCC 8739 *E. faecalis* ATCC 29212 or ATCC 19433	10^5 to 10^6 cfu	41,5 °C ± 1 °C, 2 × 24 h ± 3 h	Possible growth at the place of the inoculated drop without a turbid zone.
Productivity	*S.* Typhimurium ATCC 14028 or *S.* Enteritidis ATCC 13076 + *E. coli* ATCC 25922 or ATCC 8739 + *P. aeruginosa* ATCC 27853	10^4 cfu $10^5 - 10^6$ cfu $10^5 - 10^6$ cfu	41,5 °C ± 1 °C, 2 × 24 h ± 3 h	Grey-white, turbid zone extending out from the inoculated drop. After 48 h, the turbid zones of the 3 drops will be (almost) fully migrated over the plate. Possible extra: subculture with 1 µl loop just inside the border of the opaque growth and spread onto XLD. Incubate at 37 °C ± 1 °C for 24 h ± 3 h. Criteria: growth of characteristic colonies in majority.

Remark: In general, *S.* Typhimurium will show faster growth and larger migration zones than *S.* Enteritidis.

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Delete Reference [4] and renumber the others.

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