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Microbiology of the food chain — Horizontal method for the detection, enumeration and serotyping of *Salmonella* —

Part 1: Detection of *Salmonella* spp.

Microbiologie de la chaîne alimentaire — Méthode horizontale pour la recherche, le dénombrement et le sérotypage des Salmonella *—*

Partie 1: Recherche des Salmonella *spp.*

Reference number
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Contents

Page

Foreword

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The procedures used to develop this document and those intended for its further maintenance are described in the ISO/IEC Directives, Part 1. In particular the different approval criteria needed for the different types of ISO documents should be noted. This document was drafted in accordance with the editorial rules of the ISO/IEC Directives, Part 2 (see www.iso.org/directives).

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. Details of any patent rights identified during the development of the document will be in the Introduction and/or on the ISO list of patent declarations received (see www.iso.org/patents).

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For an explanation on the meaning of ISO specific terms and expressions related to conformity assessment, as well as information about ISO's adherence to the World Trade Organization (WTO) principles in the Technical Barriers to Trade (TBT) see the following URL: www.iso.org/iso/foreword.html

This document was prepared by the European Committee for Standardization (CEN), Technical Committee CEN/TC 275, *Food analysis — Horizontal methods*, in collaboration with ISO Technical Committee TC 34, *Food products*, Subcommittee SC 9, *Microbiology*, in accordance with the agreement on technical cooperation between ISO and CEN (Vienna Agreement).

This first edition of ISO 6579-1 cancels and replaces ISO 6579:2002 and ISO 6785:2001, which have been technically revised. It also incorporates ISO 6579:2002/Amd 1:2007 and ISO 6579:2002/Cor 1:2004.

The main changes, compared to ISO 6579:2002, are the following.

— ISO 6785 has been incorporated in this document.

— Samples from the primary production stage have been added to the scope.

— Detection of *Salmonella* Typhi and *Salmonella* Paratyphi is described in Annex D.

— Descriptions of preparations of initial suspensions have been removed and references made to relevant parts of ISO 6887, whenever possible.

— The temperature range for incubation of non-selective media has been extended from 37 °C ± 1 °C to 34 °C to 38 °C without further tolerance.

— For selective enrichment, there is a choice between using the broth or the semi-solid agar of Rappaport Vassiliadis medium (RVS or MSRV) for food, animal feed samples, and for environmental samples from the food production area.

— The inoculation of the isolation medium has become less prescriptive; the objective is to obtain well-isolated colonies after incubation.

— For confirmation, it is acceptable to perform the tests on only one suspect colony (instead of one suspect colony of each medium combination). If this isolate tests negative for *Salmonella,* four more suspect isolates from different media combinations shall be tested.

— It is permitted to perform the biochemical confirmation directly on a suspect, well-isolated colony from the selective plating medium. The purity check on the non-selective agar medium can then be performed in parallel.

— Two confirmation tests have become optional (*ß*-galactosidase test and indole reaction) and one confirmation test has been deleted (Voges-Proskauer reaction).

— In this document, serological confirmation (to serogroup level) is described. For guidance on serotyping (to serovar level), reference is made to ISO/TR 6579-3.

— Table 1 has been improved.

— Performance testing for the quality assurance of the culture media has been added to Annex B.

— Performance characteristics of MSRV have been added to Annex C.

A list of all parts in the ISO 6579 series can be found on the ISO website.

Introduction

This document describes a horizontal method for the detection of *Salmonella* spp. in food (including milk and milk products, originally described in ISO 6785), in animal feed, in animal faeces, and in environmental samples from the primary production stage (the latter two were originally described in ISO 6579:2002/Amd 1:2007).

The main changes, listed in the foreword, introduced in this document compared to ISO 6579:2002, are considered as minor (see ISO 17468[37]).

A procedure for the enumeration of *Salmonella* spp. is described in ISO/TS 6579-2.[3]

Guidance for serotyping of *Salmonella* spp. is described in ISO/TR 6579-3.[24]

Microbiology of the food chain — Horizontal method for the detection, enumeration and serotyping of *Salmonella* —

Part 1:
Detection of *Salmonella* spp.

WARNING — In order to safeguard the health of laboratory personnel, it is essential that tests for detecting *Salmonella* 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. Persons using this document should be familiar with normal laboratory practice. This document does not purport to address all of the safety aspects, if any, associated with its use. It is the responsibility of the user to establish appropriate safety and health practices and to ensure compliance with any national regulatory conditions.

1 Scope

This document specifies a horizontal method for the detection of *Salmonella*. It is applicable to the following:

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

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

— samples from the primary production stage such as animal faeces, dust, and swabs.

With this horizontal method, most of the *Salmonella* serovars are intended to be detected. For the detection of some specific serovars, additional culture steps may be needed. For *Salmonella* Typhi and *Salmonella* Paratyphi, the procedure is described in Annex D.

The selective enrichment medium modified semi-solid Rappaport-Vassiliadis (MSRV) agar is intended for the detection of motile *Salmonella* and is not appropriate for the detection of non-motile *Salmonella* strains.

2 Normative references

The following documents are referred to in the text in such a way that some or all of their content constitutes requirements of this document. For dated references, only the edition cited applies. For undated references, the latest edition of the referenced document (including any amendments) applies.

ISO 6887 (all parts), *Microbiology of food and animal feed — Preparation of test samples, initial suspension and decimal dilutions for microbiological examination*

ISO 7218, *Microbiology of food and animal feeding stuffs — General requirements and guidance for microbiological examinations*

ISO 11133:2014, *Microbiology of food, animal feed and water — Preparation, production, storage and performance testing of culture media*

3 Terms and definitions

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

ISO and IEC maintain terminological databases for use in standardization at the following addresses:

— IEC Electropedia: available at http://www.electropedia.org/

— ISO Online browsing platform: available at http://www.iso.org/obp

3.1
Salmonella
microorganism which forms typical or less typical colonies on solid selective media and which displays the characteristics described when confirmation tests are carried out in accordance with this document

3.2
detection of *Salmonella*
determination of *Salmonella* (3.1), in a particular mass or volume of product or surface area or object (e.g. boot socks), when tests are carried out in accordance with this document

4 Principle

4.1 General

The detection of *Salmonella* requires four successive stages as specified in Annex A.

NOTE *Salmonella* can be present in small numbers and is often accompanied by considerably larger numbers of other *Enterobacteriaceae* or bacteria of other families. Pre-enrichment is used to permit the detection of low numbers of *Salmonella* or injured *Salmonella*.

4.2 Pre-enrichment in non-selective liquid medium

Buffered peptone water at ambient temperature is inoculated with the test portion, then incubated between 34 °C and 38 °C for 18 h.

For large quantities (e.g. 1 l or more), it is recommended to pre-warm the BPW to 34 °C to 38 °C before mixing it with the test portion.

4.3 Enrichment in/on selective media

Rappaport-Vassiliadis medium with soya (RVS broth) or Modified Semi-solid Rappaport-Vassiliadis (MSRV) agar and Muller-Kauffmann tetrathionate-novobiocin broth (MKTTn broth) are inoculated with the culture obtained in 4.2.

The RVS broth or the MSRV agar is incubated at 41,5 °C for 24 h and the MKTTn broth at 37 °C for 24 h.

For some products, it may be necessary to incubate the selective enrichment medium/media for an additional 24 h.

NOTE MSRV agar is intended for the detection of motile *Salmonella* strains and is not appropriate for the detection of non-motile *Salmonella* strains.

4.4 Plating out on selective solid media

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

— Xylose Lysine Deoxycholate agar (XLD agar);

— any other solid selective medium complementary to XLD agar (for examples, see Annex E).

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

4.5 Confirmation

Colonies of presumptive *Salmonella* are subcultured and their identity is confirmed by means of appropriate biochemical and serological tests.

5 Culture media, reagents, and antisera

For current laboratory practice, see ISO 7218 and ISO 11133.

Composition of culture media and reagents and their preparation are described in Annex B.

6 Equipment and consumables

Disposable equipment 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)**.

As specified in ISO 7218.

6.2 Drying cabinet or **oven**, capable of operating between 25 °C and 50 °C.

6.3 Incubator(s), capable of operating in the range 34 °C to 38 °C and at 37 °C ± 1 °C.

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

6.5 Water bath, capable of operating at 47 °C to 50 °C.

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

6.7 Water bath, capable of operating at 45 °C ± 1 °C.

It is recommended to use a water bath (6.4 to 6.7) containing an antibacterial agent because of the low infective dose of *Salmonella*.

6.8 Refrigerator, capable of operating at 5 °C ± 3 °C.

6.9 Freezer, capable of operating at -20 °C ± 5 °C.

6.10 Sterile loops, of approximate diameter, 3 mm (10 µl volume), and of 1 µl volume and **inoculation needle** or **wire**.

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

6.12 Sterile tubes, **bottles**, or **flasks** with caps of appropriate capacity.

6.13 Sterile graduated pipettes or **automatic pipettes**, of nominal capacities of 25 ml, 10 ml, 1 ml, and 0,1 ml.

6.14 Sterile Petri dishes, with a diameter of approximately 90 mm and (optional) large size (diameter approximately 140 mm).

7 Sampling

Sampling is not part of the method specified in this document (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.

A recommended sampling method is given in ISO/TS 17728[26] for food and animal feed, in ISO 707[27] for milk and milk products, in ISO 13307[28] for sampling at the primary production stage, in ISO 17604[29] for sampling of carcasses, and in ISO 18593[25] for sampling of surfaces.

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

8 Preparation of test sample

Prepare the test sample from the laboratory 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 diagrams in Annex A)

9.1 Test portion and initial suspension

For preparation of the initial suspension, in the general case, use as diluent the pre-enrichment medium specified in B.2 (buffered peptone water). Pre-warm the BPW to room temperature before use.

In general, an amount of test portion (mass or volume) is added to a quantity of BPW (mass or volume) to yield a tenfold dilution. For this, a 25 g test portion is mixed with 225 ml of BPW. However, for some type of samples (e.g. boot socks, dust), it may be necessary to use another ratio.

For specific products, follow the procedures specified in ISO 6887 (all parts).

This document has been validated for test portions of 25 g. A smaller test portion may be used without the need for additional validation/verification provided that the same ratio between (pre-)enrichment broth and test portion is maintained. A larger test portion than that initially validated may be used if a validation/verification study has shown that there are no negative effects on the detection of *Salmonella* spp.

NOTE 1 Validation can be conducted according to the appropriate parts of ISO 16140. Verification for pooling samples can be conducted according to the protocol described in ISO 6887-1:2017, Annex D[38].

For large quantities (e.g. 1 l or more), it is recommended to pre-warm the BPW to 34 °C to 38 °C before mixing it with the test portion.

NOTE 2 When more than one 25 g test portion from a specified lot of product is to be examined and when evidence is available that combining test portions does not affect the result for that particular food, the test portions can be pooled. More information on pooling of samples as well as a procedure to test the influence of pooling on the sensitivity of the method can be found in ISO 6887-1[38].

9.2 Non-selective pre-enrichment

Incubate the initial suspension (9.1) between 34 °C and 38 °C (6.3) for 18 h ± 2 h.

It is permissible to store the pre-enriched sample after incubation at 5 °C (6.8) for a maximum of 72 h (see References [30] to [34]).

9.3 Selective enrichment

9.3.1 General

Allow the selective enrichment media, RVS broth or MSRV agar (B.3 or B.4), and MKTTn broth (B.5) to equilibrate at room temperature if they were stored at a lower temperature.

Minimize the transfer of particulate material from the pre-enrichment into the selective enrichment media.

After incubation, it is permissible to store the selective enrichment at 5 °C (6.8) for a maximum of 72 h (see References [30] to [34]).

NOTE MSRV agar is intended for the detection of motile *Salmonella* strains and is not appropriate for the detection of non-motile *Salmonella* strains.

9.3.2 Procedure for food, animal feed samples, and environmental samples from the food production area

Transfer 0,1 ml of the culture obtained in 9.2 to a tube containing 10 ml of the RVS broth (B.3) or to the surface of a MSRV agar plate (B.4). Inoculate the MSRV agar with one to three equally spaced spots on the surface of the medium.

Transfer 1 ml of the culture obtained in 9.2 to a tube containing 10 ml of MKTTn broth (B.5).

Incubate the inoculated RVS broth at 41,5 °C (6.4) for 24 h ± 3 h.

Incubate the inoculated MSRV agar plates at 41,5 °C (6.4) for 24 h ± 3 h. **Do not invert the plates**.

Incubate the inoculated MKTTn broth at 37 °C (6.3) for 24 h ± 3 h.

Suspect MSRV plates will show a grey-white, turbid zone extending out from the inoculated drop.

In dried milk products and cheese, *Salmonella* may be sublethally injured. Incubate the selective enrichment media from these products for an additional 24 h ± 3 h (see Reference [35]).

For some other products, e.g. when investigating outbreak samples, this additional incubation time may also be beneficial.

9.3.3 Procedure for samples from the primary production stage

Inoculate the MSRV agar (B.4) with 0,1 ml of the pre-enriched culture (9.2) as one to three equally spaced spots on the surface of the medium.

Incubate the inoculated MSRV plates at 41,5 °C (6.4) for 24 h ± 3 h.

Do not invert the plates.

Suspect MSRV plates will show a grey-white, turbid zone extending out from the inoculated drop.

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

NOTE Sensitivity can be improved by using a second selective enrichment procedure, e.g. MKTTn broth incubated at 41,5 °C for 24 h.[36]

9.4 Plating out

9.4.1 General

From the selective enriched cultures (9.3), inoculate two selective isolation agar media. The first isolation medium is Xylose Lysine Deoxycholate (XLD) agar. The second isolation medium is chosen by the testing laboratory.

Choose a second selective plating medium which is complementary to XLD agar and is based on different diagnostic characteristics to those of XLD agar to facilitate detection of, for instance, lactose positive or H2S-negative *Salmonella.* For examples of isolation media, see Annex E.

Allow the XLD agar (B.6) plates and the second selective plating medium to equilibrate at room temperature if they were stored at a lower temperature. If necessary, dry the surface of the plates before use (see ISO 11133).

9.4.2 Procedure for food, animal feed samples, and environmental samples from the food production area

From the culture obtained in the RVS broth (9.3.2), inoculate by means of a 10 µl loop (6.10) the surface of an XLD plate (B.6) so that well-isolated colonies will be obtained. Proceed in the same way with the second selective plating-out medium.

From the positive growth obtained on the MSRV agar (9.3.2), determine the furthest point of opaque growth from the inoculation points and dip a 1 µl loop (6.10) just inside the border of the opaque growth. Withdraw the loop ensuring that no large lumps of MSRV agar are extracted. Inoculate the surface of an XLD plate (B.6) so that well-isolated colonies will be obtained. Proceed in the same way with the second selective plating-out medium.

From the culture obtained in the MKTTn broth (9.3.2), inoculate by means of a 10 µl loop (6.10) the surface of an XLD plate (B.6) so that well-isolated colonies are obtained. Proceed in the same way with the second selective plating-out medium.

NOTE 1 To obtain well-isolated colonies, large size Petri dishes with plating-out media (diameter approximately 140 mm) or two normal size plates (diameter approximately 90 mm) can be used.

Incubate the XLD plates inverted at 37 °C (6.3) for 24 h ± 3 h.

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

If the selective enrichment media have been incubated for an additional 24 h, follow the same plating-out procedure as described above.

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

NOTE 2 *Salmonella* H2S-negative variants grown on XLD agar are pink with a darker pink centre. Lactose-positive *Salmonella* grown on XLD agar are yellow with or without blackening. The occurrence of these phenotypes is summarized in Table 1.

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*.

9.4.3 Procedure for samples from the primary production stage

From the positive growth obtained on the MSRV agar (9.3.3), determine the furthest point of opaque growth from the inoculation points and dip a 1 µl loop (6.10) just inside the border of the opaque growth. Withdraw the loop ensuring that no large lumps of MSRV agar 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.

Incubate the XLD plates inverted at 37 °C (6.3) 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* 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* H2S-negative variants grown on XLD agar are pink with a darker pink centre. Lactose-positive *Salmonella* grown on XLD agar are yellow with or without blackening. The occurrence of these phenotypes is summarized in Table 1.

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*.

9.5 Confirmation

9.5.1 General

The combination of biochemical and serological test results indicate whether an isolate belongs to the genus *Salmonella*. For characterization of *Salmonella* strains, full serotyping is needed. Guidance for serotyping is described in ISO/TR 6579-3[24].

For some of the confirmation media as specified in 9.5.3 and in B.8 to B.12, alternative (commercial) formulations exist which may also be applicable for biochemical confirmation of *Salmonella*. These alternative formulations are allowed, provided that the performance for the biochemical confirmation of *Salmonella* is verified before use.

For a clear distinction between positive and negative biochemical reactions, it is helpful to verify the reactions of the media of each biochemical test with well-characterized positive and negative control strains.

NOTE 1 The recognition of colonies of *Salmonella* is, to a large extent, a matter of experience and their appearance can vary somewhat, not only from serovar to serovar, but also from batch to batch of the selective culture medium used.

If shown to be reliable, miniaturized galleries for the biochemical identification of *Salmonella* may be used (see ISO 7218).

NOTE 2 Alternative procedures can be used to confirm the isolate as *Salmonella* spp. providing the suitability of the alternative procedure is verified (see ISO 7218).

9.5.2 Selection of colonies for confirmation

Mark suspect colonies on each plate (9.4). Select at least one typical or suspect colony for subculture and confirmation. If this is negative, select up to four more suspect colonies ensuring that these colonies are subcultured from different selective enrichment/isolation medium combinations showing suspect growth.

Streak the selected colonies onto the surface of a pre-dried non-selective agar medium (B.7) in a manner which will allow well-isolated colonies to develop. Incubate the inoculated plates between 34 °C and 38 °C (6.3) for 24 h ± 3 h.

Alternatively, if well-isolated colonies (of a pure culture) are available on the selective plating media (9.4), the biochemical confirmation can be performed directly on a suspect, well-isolated colony from the selective plating medium. The culture step on the non-selective agar medium can then be performed in parallel with the biochemical tests for purity check of the colony taken from the selective agar medium.

Use pure cultures for biochemical and serological confirmation.

NOTE For epidemiological purposes or during outbreak investigations, confirmation of additional colonies, e.g. five typical or suspect colonies from each selective enrichment/isolation medium combination, can be beneficial.

9.5.3 Biochemical testing

9.5.3.1 General

Inoculate the biochemical confirmation media with each of the cultures obtained from the colonies selected in 9.4 or 9.5.2. For confirmation of *Salmonella* spp., at least the tests specified in 9.5.3.2 to 9.5.3.4 shall be performed. The tests specified in 9.5.3.5 and 9.5.3.6 can also be performed when the results of the other confirmation tests do not give a clear identification.

9.5.3.2 TSI agar (B.8)

Streak the agar slant surface and stab the butt. Incubate at 37 °C (6.3) for 24 h ± 3 h.

Interpret the changes in the medium as follows:

a) butt

- yellow: glucose positive (glucose fermentation);
- red or unchanged: glucose negative (no fermentation of glucose);
- black: formation of hydrogen sulphide;
- bubbles or cracks: gas formation from glucose;

b) slant surface

- yellow: lactose and/or sucrose positive (lactose and/or sucrose fermentation);
- red or unchanged: lactose and sucrose negative (no fermentation of lactose or sucrose).

The majority of the 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) (see Table 1).

When a lactose-positive *Salmonella* is isolated, 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 9.5.3.1).

NOTE Kligler-Hajna medium gives similar results as TSI agar.

9.5.3.3 Urea agar (B.9)

Streak the agar slant surface. Incubate at 37 °C (6.3) for up to 24 h.

If the reaction is positive, urea is hydrolyzed, liberating ammonia. This 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.

Typical *Salmonella* cultures do not hydrolyze urea so that the colour of the urea agar will remain unchanged (see Table 1).

9.5.3.4 L-Lysine decarboxylation medium (LDC, B.10)

Inoculate just below the surface of the liquid medium. Incubate at 37 °C (6.3) for 24 h ± 3 h.

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

The majority of the typical *Salmonella* cultures show a positive reaction in LDC (see Table 1).

9.5.3.5 Detection of *β* -galactosidase (B.11) (optional)

The *β*-galactosidase test can be used to distinguish *Salmonella enterica* subspecies *arizonae* and *diarizonae* and other members of the *Enterobacteriaceae* (all give a positive reaction) from other subspecies of *Salmonella enterica* (in general these give a negative reaction, see Table 1).

Several procedures to perform the *β*-galactosidase test exist. An example is given below.

Suspend a loopful of the suspected colony in a tube containing 0,25 ml of the saline solution (B.13).

Add one drop of toluene and shake the tube. Place the tube in a water bath set at 37 °C (6.6) and leave for several minutes (approximately 5 min). Add 0,25 ml of the reagent for detection of β-galactosidase (B.11) and mix.

Replace the tube in the water bath set at 37 °C (6.6) and leave for up to 24 h.

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

If prepared paper discs are used for the detection of *β*-galactosidase, follow the manufacturer's instructions.

9.5.3.6 Medium for indole reaction (B.12) (optional)

The indole test can be used when there is a need to differentiate *Salmonella* (generally indole negative, see Table 1) from *Escherichia coli* and *Citrobacter* (both indole positive) as these organisms can give typical reactions on some of the *Salmonella* isolation media.

Inoculate a tube containing 5 ml of the tryptone/tryptophan medium (B.12.1) with the suspected colony.

Incubate at 37 °C (6.3) for 24 h ± 3 h. After incubation, add 1 ml of the Kovacs reagent (B.12.2).

The formation of a red ring (surface layer) indicates a positive reaction. A yellow-brown ring (surface layer) indicates a negative reaction.

Table 1 — Interpretation of biochemical tests

Test[a] (9.5.3.2 to 9.5.3.6)	*Salmonella* strain													
	***S.* Typhi**		***S.* Paratyphi A**		***S.* Paratyphi B**		***S.* Paratyphi C**		***S.* Gallinarum biovar gallinarum[b]**		***S.* Gallinarum biovar pullorum[b]**		**Other strains[g]**	
	Reaction	%+[c]	Reaction	%+[c]	Reaction	%+[d]	Reaction	%+[d]	Reaction	%+[c]	Reaction	%+[c]	Reaction	%+[c]
TSI acid from glucose	+	100	+	100	+	100	+	100	+	100	+	100	+	100
TSI gas from glucose	–[e]	0	+	96,1	+	96,1	+	96,1	-	0	+	95,1	+	92
TSI acid from lactose	–	2	–	0	–	0	–	0	–	0	–	0	–	1[h]
TSI acid from sucrose	–	0	–	0,6	–	0,6	–	0,6	–	0,6	–	0,6	–	1
TSI hydrogen sulfide produced	+	97	–	10	+	100	+	100	V[f]		V[f]		+	92
Urea hydrolysis	–	0	–	0	–	0	–	0	–	0	–	0	–	1
Lysine decarboxylation	+	98	–	0	+	95	+	100	+	95	+	95	+	95
β-Galactosidase reaction	–	0	–	0	–		–		–	<10	–	<10	–	2[h]
Production of indole	–	0	–	1,2	–	1,2	–	1,2	–	1,2	–	1,2	–	1

[a] From References [13] and [14].

[b] From References [11],[13] and [14].

[c] Percentages indicate that not all isolates of *Salmonella* serovars show the reactions marked + or –. Reactions may also vary between and within serovars.

[d] Empty cells: Percentages are not known from available literature.

[e] *Salmonella* Typhi is anaerogenic.

[f] V = Variable results.

[g] For further distinction between *Salmonella* species and subspecies, see ISO/TR 6579-3.[24]

[h] *Salmonella enterica* subspecies *arizonae* and *diarizonae* always give a positive *β*-galactosidase reaction. Some strains of subspecies *arizonae* and *diarizonae* can ferment lactose.

9.5.4 Serological testing

9.5.4.1 General

The pure colonies (9.5.2) showing typical biochemical reactions for *Salmonella* (9.5.3) are also tested for the presence of *Salmonella* O- and H-antigens (and, in areas where *Salmonella* Typhi is expected in the food supply, also for Vi-antigen) by slide agglutination using polyvalent antisera (B.14). The pure colonies are cultured on a non-selective agar medium (B.7) and tested for auto-agglutination. Strains that are auto-agglutinable cannot be tested for the presence of *Salmonella* antigens. Use the antisera according to the manufacturer's instructions if different from the method described below to detect the presence of *Salmonella* O- and H-antigens (and if necessary, also for Vi-antigen).

The following tests (9.5.4.2 to 9.5.4.5) are the minimum required for serological testing of *Salmonella* spp.

Further guidance on serological confirmation and on serotyping is given in ISO/TR 6579-3[24].

9.5.4.2 Elimination of auto-agglutinable strains

Place one drop of saline solution (B.13) on a clean glass slide. Using a loop, disperse part of the colony to be tested in the saline to obtain a homogeneous and turbid suspension.

Rock the slide gently for 5 s to 60 s (depending on the manufacturer's instructions). Observe the suspension, preferably against a dark background. If the bacteria have formed granules in the suspension, this indicates auto-agglutination and serological confirmation will become complicated. Additional information on the treatment of auto-agglutinating strains can be found in ISO/TR 6579-3[24]).

9.5.4.3 Examination for O-antigens

Using one non-auto-agglutinating pure colony, proceed according to 9.5.4.2 using one drop of polyvalent anti-O sera (B.14) in place of the saline solution.

If agglutination occurs, this is considered a positive reaction.

9.5.4.4 Examination for Vi-antigens (optional)

Using one non-auto-agglutinating pure colony, proceed according to 9.5.4.2 using one drop of anti-Vi sera (B.14) in place of the saline solution.

If agglutination occurs, this is considered a positive reaction.

9.5.4.5 Examination for H-antigens

Using one non-auto-agglutinating pure colony, proceed according to 9.5.4.2 using one drop of polyvalent anti-H sera (B.14) in place of the saline solution.

If agglutination occurs, this is considered a positive reaction.

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- and H-antigens positive (and Vi positive if tested)	Strains considered to be *Salmonella*
Typical	No	O- and/or H-antigens negative	Presumptive *Salmonella*
Typical	Yes	Not tested because of auto-agglutination (see 9.5.4.2)	
No typical reactions	—	—	Not considered to be *Salmonella*

9.5.6 Serotyping

Strains that are confirmed as *Salmonella* spp. (Table 2) can be further typed to serovar level. Guidance for serotyping is described in ISO/TR 6579-3[24].

If required, confirmed strains can be sent to a recognized *Salmonella* reference centre for definitive typing (serotyping, phage typing, molecular typing). If the strain is sent to a reference centre, it should be accompanied by all relevant information such as confirmation results, source from which the strain was isolated, and whether it concerns an isolate from an outbreak.

10 Expression of results

In accordance with the interpretation of the results, indicate *Salmonella* detected or not detected in a test portion of *x* g or *x* ml of product (see ISO 7218), or on the surface area, or in an object (e.g. boot socks).

11 Performance characteristics of the method

11.1 Interlaboratory studies

The performance characteristics of the method were determined in interlaboratory studies to determine the specificity, sensitivity, and the LOD_{50} of the method (see References [6] and [7]). The data are summarized in Annex C. The values derived from the interlaboratory studies may not be applicable to matrix types other than those given in Annex C. Furthermore, the performance characteristics as indicated in Annex C were determined with individual test portions up to 25 g (or ml). When larger size test portions are used, the performance characteristics may be different.

11.2 Sensitivity

The sensitivity is defined as the number of samples found positive divided by the number of samples tested at a given level of contamination. The results are thus dependent on the level of contamination of the sample.

11.3 Specificity

The specificity is defined as the number of samples found negative divided by the number of blank samples tested.

11.4 LOD_{50}

The LOD_{50} (level of detection) is the concentration (cfu/test portion) for which the probability of detection is 50 %.

12 Test report

The test report shall specify the following:

— the sampling method used, if known;

— the size of the test portion and/or the nature of the object examined;

— the test method used, with reference to this document, i.e. ISO 6579-1;

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

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

— the results obtained.

Annex A
(normative)

Diagrams of the procedures

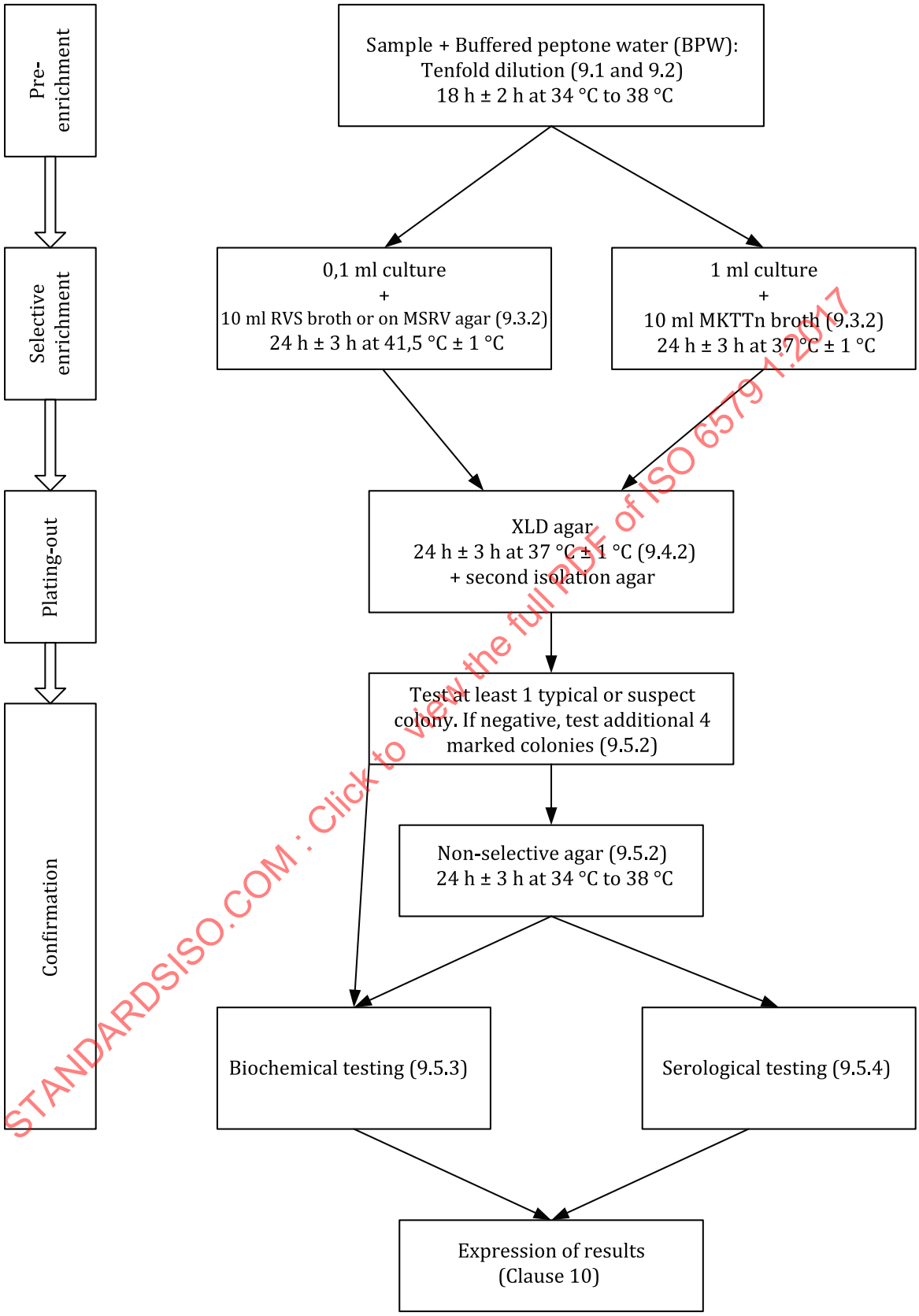

Figure A.1 — Diagram of procedure for detection of *Salmonella* in food, animal feed, and environmental samples from the food production area

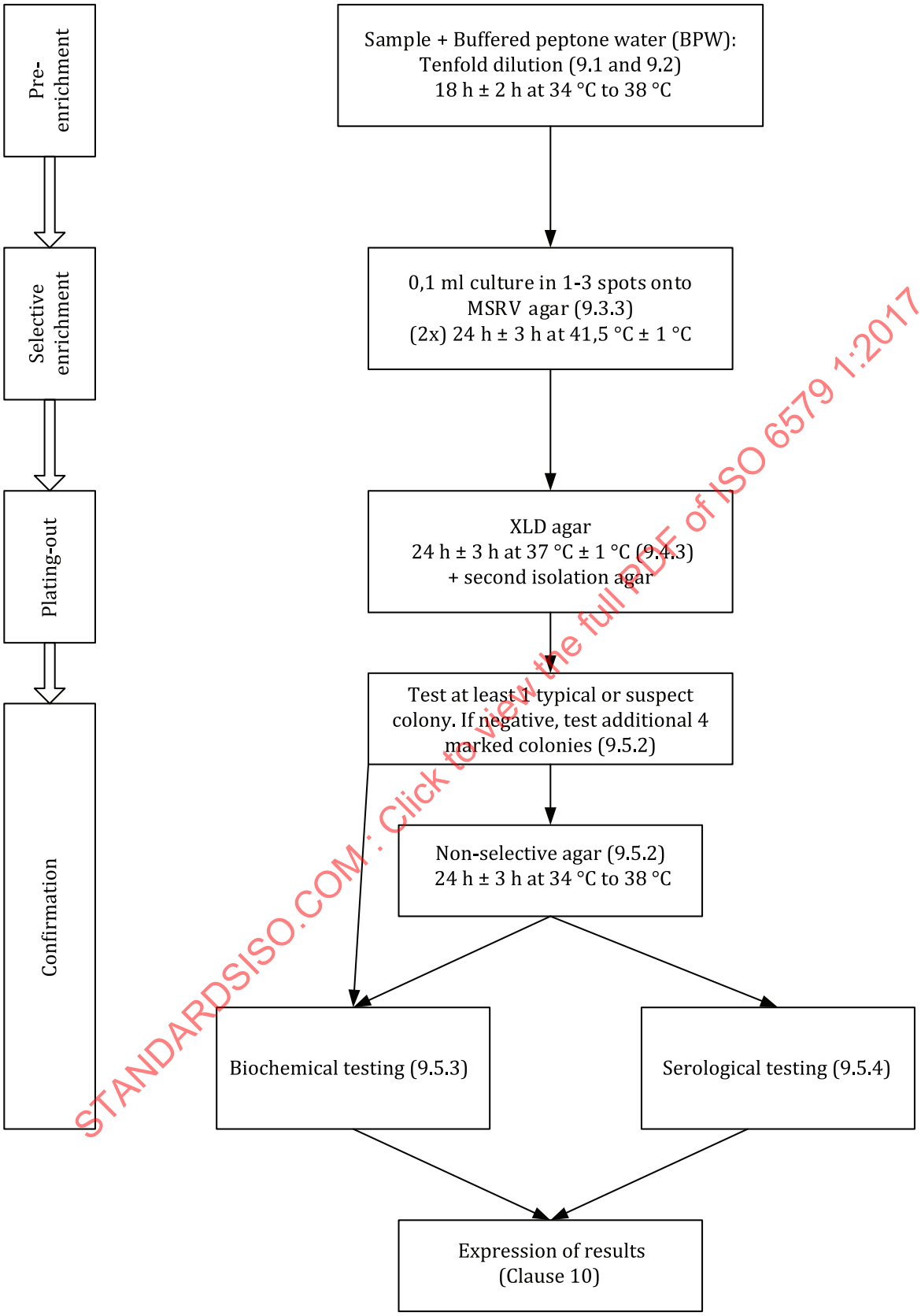

Figure A.2 — Diagram of procedure for detection of *Salmonella* in animal faeces and in environmental samples from the primary production stage

Annex B
(normative)

Culture media and reagents

B.1 General

The general specifications of ISO 11133 are applicable to the preparation and performance testing of the culture media described in this annex. If culture media or reagents are prepared from dehydrated complete media/reagents, or if ready-to-use media/reagents are used, follow the manufacturer's instructions regarding preparation, storage conditions, expiry date, and use.

The shelf life of the media indicated in this annex has been shown in some studies. The user shall verify this under their own storage conditions (as specified in ISO 11133).

Performance testing for the quality assurance of the culture media is described in B.15.

B.2 Buffered peptone water (BPW)

B.2.1 Composition

Peptone[a]	10,0 g
Sodium chloride	5,0 g
Disodium hydrogen phosphate dodecahydrate ($Na_2HPO_4 \cdot 12H_2O$)[b]	9,0 g
Potassium dihydrogen phosphate (KH_2PO_4)	1,5 g
Water	1 000 ml

[a] For example, enzymatic digest of casein.

[b] If disodium hydrogen phosphate with a different water content is used, amend the mass of the ingredient accordingly. For example, in case of anhydrous disodium hydrogen phosphate (Na_2HPO_4), use 3,57 g.

B.2.2 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 (6.12) of suitable capacity to obtain the portions necessary for the test.

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

Store the medium in closed containers (6.12) at 5 °C (6.8) for up to six months.

B.3 Rappaport-Vassiliadis medium with soya (RVS broth)

B.3.1 Solution A

B.3.1.1 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.3.1.2 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.3.2 Solution B

B.3.2.1 Composition

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

B.3.2.2 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 two years.

B.3.3 Solution C

B.3.3.1 Composition

Malachite green oxalate	0,4 g
Water	100 ml

B.3.3.2 Preparation

Dissolve the malachite green oxalate in the water.

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

B.3.4 Complete medium

B.3.4.1 Composition

Solution A (B.3.1)	1 000 ml
Solution B (B.3.2)	100 ml
Solution C (B.3.3)	10 ml

B.3.4.2 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 at 20 °C to 25 °C.

Dispense the medium into tubes or flasks (6.12) of suitable capacity to obtain the portions necessary for the test, e.g. 10 ml quantities dispensed into tubes.

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

Store the complete medium in closed tubes or flasks at 5 °C (6.8) for up to three months.

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, and malachite green oxalate 0,036 g/l.

B.4 Modified semi-solid Rappaport-Vassiliadis (MSRV) agar

NOTE See Reference [12].

B.4.1 Solution A

B.4.1.1 Composition

Enzymatic digest of animal and plant tissue	4,6 g
Acid hydrolysate of casein	4,6 g
Sodium chloride	7,3 g
Potassium dihydrogen phosphate (KH_2PO_4)	1,5 g
Water	890 ml

B.4.1.2 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 MSRV agar.

B.4.2 Solution B

B.4.2.1 Composition

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

B.4.2.2 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 two years.

B.4.3 Solution C

B.4.3.1 Composition

Malachite green oxalate	0,4 g
Water	100 ml

B.4.3.2 Preparation

Dissolve the malachite green oxalate in the water.

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

B.4.4 Base medium

B.4.4.1 Composition

Solution A (B.4.1)	890 ml
Solution B (B.4.2)	100 ml
Solution C (B.4.3)	10 ml
Agar[a]	2,7 g

[a] It may be necessary to determine experimentally the concentration of agar needed for the optimal swarming of *Salmonella* (e.g. when using a batch of agar with unknown gel strength).

B.4.4.2 Preparation

Add to 890 ml of solution A, 100 ml of solution B, and 10 ml of solution C and mix by agitation.

Add and suspend the agar.

Adjust the pH, if necessary, so that after sterilization, it is 5,2 (5,1 to 5,4) at 20 °C to 25 °C.

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

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

Cool the medium to 47 °C to 50 °C (6.5).

B.4.5 Novobiocin solution

B.4.5.1 Composition

Novobiocin sodium salt	0,05 g
Water	10 ml

B.4.5.2 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 four weeks at 5 °C (6.8) or in small portions (e.g. of 2 ml) at -20 °C (6.9) for up to one year.

B.4.6 Complete medium

B.4.6.1 Composition

Base medium (B.4.4)	1 000 ml
Novobiocin solution (B.4.5)	2 ml

B.4.6.2 Preparation

Aseptically, add 2 ml of the novobiocin solution (B.4.5) to 1 000 ml of base medium (B.4.4) at 47 °C to 50 °C. Mix carefully. The final concentration of novobiocin in the complete medium is 10 mg/l.

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

Pour the medium into sterile Petri dishes (6.14) up to a volume of 15 ml to 20 ml in dishes with a diameter of 90 mm.

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

Store the plates, **with surface upwards**, and protected from drying for up to two weeks at 5 °C (6.8) 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 visible moisture is apparent, 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.

NOTE 1 The composition of MSRV agar, as described in Reference [12], contains 20 mg/l of novobiocin. However, from a scientific point of view, 10 mg/l novobiocin is preferred. Studies have shown larger migration zones on MSRV agar with a lower concentration of novobiocin[23] and the (negative) influence of novobiocin on bacterial motility.[22]

NOTE 2 The final medium composition is enzymatic digest of animal and plant tissue 4,6 g/l, acid hydrolysate of casein 4,6 g/l, sodium chloride (NaCl) 7,3 g/l, potassium dihydrogen phosphate (KH_2PO_4) 1,5 g/l, anhydrous magnesium chloride ($MgCl_2$) 10,9 g/l, or magnesium chloride hexahydrate ($MgCl_2 \cdot 6H_2O$) 28,6 g/l, malachite green oxalate 0,04 g/l, novobiocin sodium salt 0,01 g/l, and agar 2,7 g/l.

B.5 Muller-Kauffmann tetrathionate-novobiocin (MKTTn) broth

NOTE See Reference [14].

B.5.1 Base medium

B.5.1.1 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.5.1.2 Preparation

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

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

Thoroughly mix the medium.

The base medium may be stored in closed flasks (6.12) at 5 °C (6.8) for up to three months.

B.5.2 Iodine-iodide solution

B.5.2.1 Composition

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

B.5.2.2 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 a (tightly) closed container (6.12) in the dark for up to one year.

B.5.3 Novobiocin solution

B.5.3.1 Composition

Novobiocin sodium salt	0,04 g
Water	5 ml

B.5.3.2 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 four weeks at 5 °C (6.8) or in small portions (e.g. of 5 ml) at -20 °C (6.9) for up to one year.

B.5.4 Complete medium

B.5.4.1 Composition

Base medium (B.5.1)	1 000 ml
Iodine-iodide solution (B.5.2)	20 ml
Novobiocin solution (B.5.3)	5 ml

B.5.4.2 Preparation

Aseptically, add 5 ml of the novobiocin solution (B.5.3) to 1 000 ml of base medium (B.5.1). Mix, then add 20 ml of the iodine-iodide solution (B.5.2). Mix well. The final concentration of novobiocin in the complete medium is 40 mg/l.

Dispense the medium aseptically into containers (6.12) of suitable capacity to obtain the portions necessary for the test, e.g. 10 ml quantities dispensed into tubes. After preparation, the pH of complete MKTTn broth will be approximately 8,0. If the complete medium is not used immediately, store it in the dark at 5 °C (6.8). The pH may drop during storage due to chemical reactions. Do not use the complete medium if the pH drops below 7,0.

B.6 Xylose Lysine Deoxycholate agar (XLD agar)

NOTE See Reference [14].

B.6.1 Composition

Yeast extract	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[a]
Water	1 000 ml

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

B.6.2 Preparation

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

Adjust the pH, if necessary, so that the final pH shall be 7,4 ± 0,2 at 25 °C.

Pour the base medium into tubes or flasks (6.12) of appropriate capacity.

B.6.3 Preparation of the agar plates

Cool the medium to 47 °C to 50 °C in a water bath (6.5), mix, and pour into sterile Petri dishes (6.14). 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 25 °C and 50 °C until the surface of the agar is dry.

Store the poured plates protected from drying, at 5 °C (6.8) for up to four weeks.

B.7 Nutrient agar (example of non-selective medium)

B.7.1 Composition

Meat extract	3,0 g
Peptone	5,0 g
Sodium chloride (NaCl) (optional)	5,0 g
Agar	9 g to 18 g[a]
Water	1 000 ml

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

B.7.2 Preparation

Dissolve the components or the dehydrated complete medium in the water by heating, with frequent agitation.

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 flasks (6.12) of appropriate capacity.

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

B.7.3 Preparation of nutrient agar plates

Cool the medium to 47 °C to 50 °C in a water bath (6.5), mix, and pour into sterile Petri dishes (6.14). 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 25 °C and 50 °C until the surface of the agar is dry.

Store the poured plates protected from drying, at 5 °C (6.8) for up to four weeks.

B.8 Triple sugar/iron agar (TSI agar)

B.8.1 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[a]
Water	1 000 ml

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

B.8.2 Preparation

Dissolve the components or the dehydrated complete medium in the water by heating with frequent agitation.

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

Dispense the medium into tubes or bottles (6.12) 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.

Store the poured tubes protected from drying, at 5 °C (6.8) for up to four weeks.

NOTE As an alternative, a double sugar/iron agar can be used (Kligler-Hajna).

B.9 Urea agar (Christensen)

B.9.1 Base medium

B.9.1.1 Composition

Peptone[a]	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[b]
Water	1 000 ml

[a] For example, enzymatic digest of gelatine.

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

B.9.1.2 Preparation

Dissolve the components or the dehydrated complete base in the water by heating with frequent agitation.

Adjust the pH, if necessary, so that after sterilization, it is 6,8 ± 0,2 at 25 °C.

Pour the base medium into tubes or flasks (6.12) of appropriate capacity.

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

The base medium may be stored in closed tubes or flasks at 5 °C for up to three months.

B.9.2 Urea solution

B.9.2.1 Composition

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

B.9.2.2 Preparation

Dissolve the urea in the water. Sterilize by filtration through a filter with a pore size of 0,22 µm.

See ISO 11133.

B.9.3 Complete medium

B.9.3.1 Composition

Base (B.9.1)	950 ml
Urea solution (B.9.2)	50 ml

B.9.3.2 Preparation

Add, under aseptic conditions, the urea solution to the base previously melted and then cooled to 47 °C to 50 °C.

Dispense the complete medium into sterile tubes (6.12) in quantities of 10 ml.

Allow to set in a sloping position.

Store the poured tubes protected from drying, at 5 °C (6.8) for up to four weeks.

B.10 L-Lysine decarboxylation medium (LDC)

B.10.1 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.10.2 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 tubes (6.12) with screw caps.

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

Store the poured tubes at 5 °C (6.8) for up to three months.

B.11 β-Galactosidase reagent (optional)

Additional to the reagent described below, toluene is needed for the β-galactosidase test.

B.11.1 Buffer solution

B.11.1.1 Composition

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

B.11.1.2 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.

The buffer solution may be stored in closed flasks at 5 °C (6.8) for up to six months.

B.11.2 ONPG solution

B.11.2.1 Composition

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

B.11.2.2 Preparation

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

Cool the solution.

B.11.3 Complete reagent

B.11.3.1 Composition

Buffer solution (B.11.1)	5 ml
ONPG solution (B.11.2)	15 ml

B.11.3.2 Preparation

Add the buffer solution to the ONPG solution.

Store the complete reagent in closed flasks (6.12) at 5 °C (6.8) for up to three months. Discard the complete reagent as soon as the colour changes to yellow.

B.12 Medium and reagent for indole reaction (optional)

B.12.1 Tryptone/tryptophan medium

B.12.1.1 Composition

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

B.12.1.2 Preparation

Dissolve the components in the boiling water.

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

Dispense the medium into tubes (6.12) in quantities of 5 ml.

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

Store the poured tubes at 5 °C (6.8) for up to three months.

B.12.2 Kovacs reagent

B.12.2.1 Composition

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

B.12.2.2 Preparation

Mix the components.

Store the complete reagent in closed flasks (6.12) in the dark at 5 °C (6.8) for up to six months.

B.13 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 the solution into flasks or tubes (6.12) of suitable capacity to obtain the portions necessary for the test.

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

Store the solution in closed flasks/tubes at 5 °C (6.8) for up to six months.

B.14 Antisera

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

B.15 Performance testing for the quality assurance of the culture media

The definition of selectivity and productivity is specified in ISO 11133. In general, follow the procedures for performance testing described in ISO 11133. For performance testing of selective liquid media and MSRV agar, use the same inoculum volume as specified in 9.3.2. For MSRV agar the inoculum should contain 10^3 cfu to 10^4 cfu for determining productivity and 10^4 cfu to 10^6 cfu for determining selectivity (see ISO 11133). For the other media, the inoculum levels for the target and the non-target organisms are specified in ISO 11133:2014, 5.4.

Table B.1 — Performance testing for the quality assurance of the culture media

Medium	Function	Incubation	Control strains	WDCM numbers[a]	Criteria[b]
BPW	Productivity	18 h ± 2 h/ 34 °C to 38 °C	*Salmonella* Typhimurium[c,d] *Salmonella* Enteritidis[c,d]	00031 00030	Turbidity (1–2)
MKTTn broth	Productivity	24 h ± 3 h/ 37 °C ± 1 °C	*Salmonella* Typhimurium[c,d] *Salmonella* Enteritidis[c,d] + *Escherichia coli*[d] + *Pseudomonas aeruginosa*	00031 00030 00012 or 00013 00025	>10 characteristic colonies on XLD agar or other medium of choice
	Selectivity		*Escherichia coli*[d]	00012 or 00013	Partial inhibition ≤100 colonies on TSA
			Enterococcus faecalis[d]	00009 or 00087	<10 colonies on TSA
RVS broth	Productivity	24 h ± 3 h/ 41,5 °C ± 1 °C	*Salmonella* Typhimurium[c,d] *Salmonella* Enteritidis[c,d] + *Escherichia coli*[d] + *Pseudomonas aeruginosa*	00031 00030 00012 or 00013 00025	>10 characteristic colonies on XLD agar or other medium of choice
	Selectivity		*Escherichia coli*[d]	00012 or 00013	Partial inhibition ≤100 colonies on TSA
			Enterococcus faecalis[d]	00009 or 00087	<10 colonies on TSA
MSRV agar	Productivity	2x(24 h ± 3 h)/ 41,5 °C ± 1 °C	*Salmonella* Typhimurium[c,d] *Salmonella* Enteritidis[c,d]	00031 00030	Grey-white, turbid zone extending out from inoculated drop(s). After 24 h to 48 h, the turbid zone(s) will be (almost) fully migrated over the plate. Possible extra: characteristic colonies after subculturing on XLD agar
	Selectivity		*Escherichia coli*[d]	00012 or 00013	Possible growth at the place of the inoculated drop(s) without a turbid zone
			Enterococcus faecalis[d]	00009 or 00087	No growth

[a] Refer to the reference strain catalogue at www.wfcc.info for information on culture collection strain numbers and contact details; WDCM: World Data Centre for Microorganisms.

[b] Growth is categorized as 0: no growth, 1: weak growth (partial inhibition), and 2: good growth (see ISO 11133).

[c] Some national restrictions and directions may require the use of a different serovar. Make reference to national requirements relating to the choice of *Salmonella* serovars.

[d] Strain free of choice; one of the strains has to be used as a minimum.

Table B.1 *(continued)*

Medium	Function	Incubation	Control strains	WDCM numbers[a]	Criteria[b]
XLD agar	Productivity	24 h ± 3 h/ 37 °C ± 1 °C	*Salmonella* Typhimurium[c,d] *Salmonella* Enteritidis[c,d]	00031 00030	Good growth (2) of colonies with black centre and a lightly transparent zone of reddish colour due to the colour change of the medium
	Selectivity		*Escherichia coli*[d]	00012 or 00013	Growth or partial inhibition (0–1) of yellow colonies
			Enterococcus faecalis[d]	00009 or 00087	Total inhibition (0)
Nutrient agar	Productivity	24 h ± 3 h/ 34 °C to 38 °C	*Salmonella* Typhimurium[c,d] *Salmonella* Enteritidis[c,d]	00031 00030	Good growth

[a] Refer to the reference strain catalogue at www.wfcc.info for information on culture collection strain numbers and contact details; WDCM: World Data Centre for Microorganisms.

[b] Growth is categorized as 0: no growth, 1: weak growth (partial inhibition), and 2: good growth (see ISO 11133).

[c] Some national restrictions and directions may require the use of a different serovar. Make reference to national requirements relating to the choice of *Salmonella* serovars.

[d] Strain free of choice; one of the strains has to be used as a minimum.

Annex C
(informative)

Method validation studies and performance characteristics

C.1 Performance characteristics RVS broth and MKTTn broth

International interlaboratory studies were organized in 2000 in the frame of the European project SMT CT 96 2098 (see References [8] and [15]). These studies involved 11 laboratories in nine countries in Europe and 10 laboratories in USA and were carried out on fresh cheese curd, dried egg powder, raw poultry meat, and a reference material. The food samples were each tested at low and high levels of contamination, plus a negative control.

The method submitted to the interlaboratory studies was that of ISO 6579:2002 (see Reference [1]), including selective enrichment in RVS broth and MKTTn broth. The procedure for detection of *Salmonella* in food samples as described in ISO 6579:2002 is comparable to the procedure as described in this document.

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

Parameter	Fresh cheese curd (blank)	Fresh cheese curd (low level contamination)[a]	Fresh cheese curd (high level contamination)[a]
Number of participating collaborators	23	23	23
Number of samples per collaborator	5	5	5
Number of collaborators retained after evaluation of the data	21	21	21
Number of samples retained after evaluation of the data	105	105	105
Test portion size, in g	25	25	25
Specificity, in %	100	—	—
Sensitivity, in %	—	74,3	83,8
LOD_{50} (95 % confidence interval), in cfu/test portion	—	5,7 (4,0 to 8,1)	

[a] Cheese samples were artificially contaminated with *Salmonella* Montevideo (lactose positive strain).

Most probable number (MPN) results of the artificially contaminated samples were the following:

	MPN/25 g
Low level	0,7 (0,2 to 2,4)
High level	37,2 (7,5 to 95,0)

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

Parameter	Trial I dried egg powder (blank)	Trial I dried egg powder (low level contamination)[a]	Trial I dried egg powder (high level contamination)[a]	Trial II dried egg powder (low level contamination)[a]
Number of participating collaborators	26	26	26	9
Number of samples per collaborator	5	5	5	5
Number of collaborators retained after evaluation of the data	21	21	21	8
Number of samples retained after evaluation of the data	105	105	104	40
Test portion size, in g	25	25	25	25
Specificity, in %	100	—	—	nd
Sensitivity, in %	—	98,1	99	nd
LOD_{50} (95 % confidence interval), in cfu/test portion	—	6,0 (4,7 to 7,7)		

nd = not determined.

[a] Egg powder samples were artificially contaminated with *Salmonella* Panama.

MPN results of the artificially contaminated samples were the following:

	MPN/25 g
Trial I low level	9,6 (2,2 to 26)
Trial I high level	115 (22,5 to 495)
Trial II low level	0,7 (0,2 to 2,3)

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

Parameter	Trial I raw poultry meat (blank)	Trial I raw poultry meat (low level contamination)[a]	Trial I raw poultry meat (high level contamination)[a]	Trial II raw poultry meat (low level contamination)[a]	Trial II raw poultry meat (high level contamination)[a]
Number of participating collaborators	25	25	25	13	13
Number of samples per collaborator	5	5	5	6	6
Number of collaborators retained after evaluation of the data	20	20	20	13	13
Number of samples retained after evaluation of the data	100	99	100	78	78
Test portion size, in g	25	25	25	25	25
Specificity, in %	100	—	—	nd	nd

Table C.3 *(continued)*

Parameter	Trial I raw poultry meat (blank)	Trial I raw poultry meat (low level contamination)[a]	Trial I raw poultry meat (high level contamination)[a]	Trial II raw poultry meat (low level contamination)[a]	Trial II raw poultry meat (high level contamination)[a]
Sensitivity, in %	—	98	100	nd	nd
LOD_{50} (95 % confidence interval), in cfu/test portion	—	nd	nd	2,2 (1,5 to 3,2)	

nd = not determined.

[a] Poultry meat samples were artificially contaminated with *Salmonella* Typimurium in Trial I and were naturally contaminated with *Salmonella* spp. in Trial II.

MPN results of the contaminated samples were:

	MPN/25 g
Trial I low level	3,7 (1 to 9,5)
Trial I high level	5,8 (1 to 25)
Trial II low level	0,2 (0,04 to 0,9)
Trial II high level	1,0 (2,2 to 4,5)

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

Parameter	Reference material (capsules containing approx. 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
Specificity, in %	—
Sensitivity, in %	94,4

C.2 Performance characteristics of MSRV agar for detection of *Salmonella* spp. in food and animal feed

In 2003, a validation study conforming to ISO 16140:2003[5] was performed (see Reference [9]) to compare recovery using MSRV agar alone with the method described in ISO 6579:2002[1] (using the selective enrichment media, RVS broth and MKTTn broth). The results of the method comparison part of this study are summarized in Tables C.5 and C.6. As part of the study, an interlaboratory comparison study was also performed of which results are summarized below Tables C.5 and C.6.

Table C.5 — Number of tested samples in the method comparison study on validation of MSRV agar

Food categories	Positive			Negative	Total
	n.c.	a.c.	total		
Meat and meat products	26	13	39	40	79
Dairy products	13	18	31	36	67
Fish, seafood, vegetables	4	28	32	32	64
Egg products, pastry	3	29	32	34	66
Environmental samples	0	30	30	31	61
Total	46	118	164	173	337
n.c. = naturally contaminated; a.c. = artificially contaminated.					

Table C.6 — Results of method comparison study of the validation study of MSRV agar

Food categories	AC (%)	SP (%)	SE (%)
Meat and meat products	96	98	95
Dairy products	100	100	100
Fish, seafood, vegetables	95	94	97
Egg products, pastry	98	100	97
Environmental samples	98	100	97
All samples	98	98	97
AC = relative accuracy; SP = relative specificity; SE = relative sensitivity.			

Other results of the method comparison study are as follows:

— eight deviating results (five negative deviations and three positive deviations), not significant;

— relative detection level for the five matrices was 0,49 to 0,78 *Salmonella* cells per 25 g or ml;

— inclusivity tested on 55 *Salmonella* food isolates (10 cfu/ml to 90 cfu/ml in BPW);

— exclusivity tested on 48 non-*Salmonella* strains interfering with *Salmonella* detection methods (10^4 to 10^6 cfu/ml in BPW);

— results inclusivity/exclusivity study showed method specificity. Three *Salmonella* strains were not detected (2x *S.* Paratyphi A, 1x *S.* Enteritidis) and two strains of *Enterobacter cloacae* gave presumptive positive results.

Results of the interlaboratory comparison study are as follows:

— 29 participating laboratories from 10 different countries;

— each lab tested 24 artificially contaminated skim milk powder samples at three different contamination levels (0 cfu/25 g, 10 cfu/25 g, 30 cfu/25 g). Eight replicate samples per contamination level were tested;

— duplicate testing with MSRV agar and ISO 6579:2002[1];

— 240 results per method;

— no outliers;

— for all contamination levels: AC, SE, SP: 100 %, and confidence interval: 98 %;

— two false-positive results, most likely due to cross-contamination.

C.3 Performance characteristics of MSRV agar for detection of *Salmonella* spp. in animal faeces and in environmental samples from the primary production stage

The precision data of MSRV agar for detection of *Salmonella* spp. in animal faeces and in environmental samples from the primary production stage were calculated from three different interlaboratory studies organized by the EURL-*Salmonella*, RIVM, The Netherlands. This concerned studies organized in 2008[16], 2012[17] and 2013.[21] The samples tested in the three studies were, respectively, chicken faeces, pig faeces, and boot socks. The samples were each tested at two different levels of contamination, plus a negative control. All studies were funded by the European Commission and the latter study was also performed as part of the CEN mandate M381.

The method submitted to the interlaboratory studies was that of ISO 6579:2002/Amd 1:2007[2] for the detection of *Salmonella* in samples from the primary production stage including selective enrichment on MSRV agar. This method has been incorporated in this document.

The values of the performance characteristics derived from the interlaboratory studies are shown per type of sample in Tables C.7 to C.9. Data obtained by some collaborators have been excluded from the calculations only on the basis of clearly identified technical reasons (deviations to the protocol).

Table C.7 — Results of data analysis obtained with chicken faeces samples

Parameter	Chicken faeces +				
	Blank	STM5[a]	STM44[a]	SE7[a]	SE91[a]
Number of participating collaborators	32	32	32	32	32
Number of samples per collaborator	5	5	5	5	5
Number of collaborators retained after evaluation of the data	19	19	19	19	19
Number of samples retained after evaluation of the data	95	95	95	95	95
Test portion size, in g	10	10	10	10	10
Specificity, in %	100	—	—	—	—
Sensitivity per serovar and level, in %	—	96,8	100	67,4	100
LOD_{50} per serovar (95 % confidence interval), in cfu/test portion	—	1,0 (0,7 to 1,4)		4,3 (3,3 to 5,6)	
LOD_{50} overall (95 % confidence interval), in cfu/test portion	—	2,5 (2,1 to 3,0)			

[a] Chicken faeces samples were artificially contaminated with reference materials with the following strains and levels:

Salmonella Typhimurium (STM) at a level of 5 cfu/test portion and a level of 44 cfu/test portion;

Salmonella Enteritidis (SE) at a level of 7 cfu/test portion and a level of 91 cfu/test portion.

Table C.8 — Results of data analysis obtained with pig faeces samples

Parameter	Pig faeces +				
	Blank	SD6[a]	SD37[a]	STM10[a]	STM58[a]
Number of participating collaborators	33	33	33	33	33
Number of samples per collaborator	5	5	5	5	5
Number of collaborators retained after evaluation of the data	26	26	26	26	26
Number of samples retained after evaluation of the data	130	130	130	130	130
Test portion size, in g	25	25	25	25	25
Specificity, in %	99,2	—	—	—	—
Sensitivity per serovar and level, in %	—	88,5	97,7	91,5	98,5
LOD_{50} per serovar (95 % confidence interval), in cfu/test portion	—	2,8 (2,2 to 3,5)		3,8 (3,0 to 4,7)	
LOD_{50} overall (95 % confidence interval), in cfu/test portion	—	3,2 (2,8 to 3,8)			

[a] Pig faeces samples were artificially contaminated with reference materials with the following strains and levels:

Salmonella Derby (SD) at a level of 6 cfu/test portion and a level of 37 cfu/test portion;

Salmonella Typhimurium (STM) at a level of 10 cfu/test portion and a level of 58 cfu/test portion.

Table C.9 — Results of data analysis obtained with boot sock samples

Parameter	Boot socks +10 g laying hen environmental material +		
	Blank	STM9[a]	STM81[a]
Number of participating collaborators	36	36	36
Number of samples per collaborator	8	8	8
Number of laboratories retained after evaluation of the data	33	33	33
Number of samples retained after evaluation of the data	264	264	264
Sample size	Boot socks	Boot socks	
Specificity, in %	99,6	—	—
Sensitivity per level, in %	—	94,7	98,1
LOD_{50} (95 % confidence interval), in cfu/sample	—	3,8 (3,2 to 4,4)	

[a] The boot sock samples were artificially contaminated with a diluted culture of *Salmonella* Typhimurium (STM) at a level of 9 cfu/sample and a level of 81 cfu/sample.

Annex D
(normative)

Detection of *Salmonella enterica* subspecies *enterica* serovars Typhi and Paratyphi

D.1 General

Certain serovars of *Salmonella* are not consistently detected by the method described in this document. This annex specifies additional steps to be taken when the detection of *Salmonella enterica* subspecies *enterica* serovars Typhi and Paratyphi is of specific concern. The full method described in this document shall also be performed.

NOTE Strains of serovar Gallinarum (biovars gallinarum and pullorum) are of no significance to human health and so their isolation is not included in this document (see Reference [19]). However, if these biovars are specifically sought, Selenite Cystine medium (SC) can be used in addition to RVS broth and MKTTn broth as described in this annex.

D.2 Detection of *Salmonella* Typhi and *Salmonella* Paratyphi

D.2.1 Principle

WARNING — *Salmonella* Typhi and *Salmonella* Paratyphi are Hazard Group 3 organisms. Appropriate containment facilities should be used when handling these strains.

D.2.1.1 General

See 4.1.

D.2.1.2 Pre-enrichment in non-selective liquid medium

See 4.2.

D.2.1.3 Enrichment in selective liquid media

See 4.3. Selenite cystine medium (SC) is inoculated with the culture obtained in 4.2 in addition to inoculation of RVS broth and MKTTn broth.

The SC medium is incubated at 37 °C (6.3) for 24 h and 48 h.

D.2.1.4 Plating out and identification

See 4.4. Bismuth sulphite agar (BS) is inoculated with the cultures obtained in 4.3 and D.2.1.3 in addition to XLD agar.

The BS agar is incubated at 37 °C (6.3) and examined after 24 h, and again, if necessary, after 48 h.

D.2.1.5 Confirmation of identity

See 4.5.

D.3 Culture media

D.3.1 Selenite cystine medium (SC)

WARNING — Sodium hydrogen selenite is potentially teratogenic and may harm the unborn child. Appropriate protective precautions should be taken when preparing and handling this medium.

D.3.1.1 Base medium

D.3.1.1.1 Composition

Peptone[a]	5,0 g
Lactose	4,0 g
Disodium hydrogen phosphate dodecahydrate ($Na_2HPO_4.12H_2O$)	10,0 g
Sodium hydrogen selenite	4,0 g
Water	1 000 ml

[a] For example, enzymatic digest of casein.

D.3.1.1.2 Preparation

Dissolve the sodium hydrogen selenite in the water, then add the remaining ingredients. Heat to boiling to dissolve.

D.3.1.2 L-Cystine solution

D.3.1.2.1 Composition

L-Cystine	0,1 g
Sodium hydroxide solution, c (NaOH) = 1 mol/l	15 ml
Sterile water	Approx. 85 ml

D.3.1.2.2 Preparation

Add the components to a sterile 100 ml one-mark volumetric flask (D.4.2). Dilute to the mark with sterile water. Do not sterilize.

D.3.1.3 Complete medium

D.3.1.3.1 Composition

Base (D.3.1.1)	1 000 ml
L-Cystine solution (D.3.1.2)	100 ml

D.3.1.3.2 Preparation

Add the L-cystine solution aseptically to the base. Adjust the pH, if necessary, so that the final pH will be 7,0 ± 0,2 at 25 °C. Dispense the medium aseptically into sterile tubes or flasks (6.12) to achieve a depth of at least 5 cm. Sterilize by steaming for 15 min. Do not autoclave.

Store the poured tubes at 5 °C (6.8). The medium may be used until a red precipitate occurs.

D.3.2 Bismuth sulfite agar (BS)

D.3.2.1 Composition

Enzymatic digest of animal tissues	10,0 g
Meat extract	5,0 g
Dextrose	5,0 g
Disodium hydrogen phosphate (anhydrous) (Na_2HPO_4)	4,0 g
Ferrous sulfate (anhydrous)	0,3 g
Bismuth sulfite (indicator)	8,0 g
Brilliant green	0,025 g
Agar	20,0 g
Water	1 000 ml

D.3.2.2 Preparation

Add the components to the water and heat with frequent agitation until boiling. Continue to boil gently for 30 s to 60 s to dissolve the agar and obtain a uniform suspension (precipitate will not dissolve). Cool to 47 °C to 50 °C, then gently agitate to suspend the precipitate.

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

Pour 20 ml to 25 ml into Petri dishes (6.14) and allow to set. Correctly prepared plates will be a pale straw colour with a smooth cream-like opacity.

Prepare the plates the day before use and store in the dark at ambient temperature.

Dry the plates, if necessary, before use. Do not overdry.

D.3.3 Performance testing

The definition of selectivity and productivity is specified in ISO 11133. The inoculum volume should be the same as that used in the method for that medium and should contain the number of target or non-target organisms specified in ISO 11133:2014, 5.4.