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AMENDMENT 1
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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*

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ISO copyright office
Case postale 56 • CH-1211 Geneva 20
Tel. + 41 22 749 01 11
Fax + 41 22 749 09 47
E-mail copyright@iso.org
Web www.iso.org

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Foreword

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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.

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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.