FINAL DRAFT
International Standard

ISO/FDIS 6579-4

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

Part 4: Identification of monophasic *Salmonella* Typhimurium (1,4,[5],12:i:-) by polymerase chain reaction (PCR)

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Introduction

In several international, regional and national laws, regulatory limits are set to ensure the so-called "absence" of *Salmonella* spp. in samples of the food chain. Moreover, several European Commission (EC) regulations also demand the absence of particular *Salmonella* serovars which have shown to cause a relatively high percentage of human salmonellosis. One of these *Salmonella* serovars for which legal criteria are set is *Salmonella* Typhimurium, including its monophasic variant 1,4,[5],12:i:- (e.g. Regulation (EC) No. 1086/2011[10]). Hence, it is important to know that a serovar found with antigenic formula 1,4,[5],12:i:- is indeed the monophasic variant of *Salmonella* Typhimurium (1,4,[5],12:i:1,2) and not the monophasic variant of another *Salmonella* (*S.*) serovar for which no criteria are set, such as *S*. Lagos (1,4,[5],12:i:1,5), *S*. Agama (4,12:i:1,6), *S.* Farsta (4,[5],12:i:e,n,x), *S*. Tsevie (1,4,12:i:e,n,z_{15}), *S.* Gloucester (1,4,12,27:i:l,w) or *S*. Tumodi (1,4,12:i:z_6). Confirmational distinction between *Salmonella* Typhimurium and *Salmonella* non-Typhimurium serovars can be determined using molecular analysis, such as the PCR technique(s) described in this document.

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

Part 4: Identification of monophasic *Salmonella* Typhimurium (1,4,[5],12:i:-) by polymerase chain reaction (PCR)

WARNING — In order to safeguard the health of laboratory personnel, it is essential that tests for detecting, enumerating and (sero)typing *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.

1 Scope

This document specifies a horizontal *in vitro* method for the molecular identification and differentiation of the monophasic variant of *Salmonella enterica* subsp. *enterica* serovar Typhimurium (1,4,[5],12:i:-) lacking the second H phase H:1,2, starting from isolates. The method detects specific DNA sequences of an intergenic region of the first H phase flagellin cluster for identification of *Salmonella enterica* subsp. *enterica* serovar Typhimurium (further called *Salmonella* Typhimurium) and specific DNA sequences of genes associated with second H phase flagellar antigen expression.

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The method is applicable for:

— differentiation of the isolate under analysis between monophasic *Salmonella* Typhimurium and the monophasic variant of another *Salmonella* non-Typhimurium serovar that has the same antigenic formula;

— identification of the isolate under analysis being either monophasic *Salmonella* Typhimurium or (biphasic) *Salmonella* Typhimurium.

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This document is applicable for the analysis of a pure culture belonging to the genus *Salmonella*, isolated from:

— products intended for human consumption;

— products intended for animal feeding;

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

— samples from the primary production stage.

This document can also be applied in other domains for identification of monophasic *Salmonella* Typhimurium (e.g. environmental, human health, animal health).

NOTE This method has been validated in a method evaluation study and in an interlaboratory study with a large set of different strains (target and non-target strains), isolated from different sources (food products, animals, animal feed, primary production samples and humans). For detailed information on the validation, see Annex E.

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.

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ISO 7218, *Microbiology of the food chain — General requirements and guidance for microbiological examinations*

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

ISO 20836, *Microbiology of the food chain — Polymerase chain reaction (PCR) for the detection of microorganisms — Thermal performance testing of thermal cyclers*

ISO 22174, *Microbiology of the food chain — Polymerase chain reaction (PCR) for the detection and quantification of microorganisms — General requirements and definitions*

3 Terms and definitions

For the purposes of this document, the terms and definitions given in ISO 22174 and the following apply.

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

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

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

3.1
monophasic *Salmonella* Typhimurium
variant of *Salmonella enterica* subsp. *enterica* serovar Typhimurium lacking the second H phase H:1,2, having the antigenic formula 1,4,[5],12:i:-

3.2
presumptive monophasic *Salmonella* Typhimurium
pure culture characterized as belonging to the genus *Salmonella*, giving a positive reaction for O-antigen O:4 and H-antigen H:i and with a negative reaction for the second H phase H:1,2

3.3
threshold cycle crossing point
point of the amplification curve at which the fluorescence signal rises above the baseline or crosses a predefined threshold setting

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4 Principle

4.1 General

The identification of the monophasic variant of *Salmonella* Typhimurium comprises the three successive steps described in 4.2 to 4.4, starting with a pure culture characterized as belonging to the genus *Salmonella*.

4.2 Preparation of well-isolated colonies

The culture is streaked onto the surface of a (pre-dried) non-selective agar medium and incubated between 34 °C and 38 °C for 24 h, to obtain well-isolated colonies.

4.3 Suspension of a colony

A well-isolated colony is selected and suspended in 100 µl saline solution (0,85 % m/v) or in 100 µl PCR grade water.

4.4 Amplification and detection

The suspended bacterial cells are analysed by PCR for detection of the genetic sequences unique to *Salmonella* Typhimurium (1,4,[5],12:i:1,2) and its monophasic variant lacking the second H phase (1,4,[5],12:i:-), as well as for detection of specific genetic sequences of genes associated with the second H phase flagellar antigen expression. Specific PCR assays including primers and probes are described in Annexes B to D.

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5 Culture media and reagents

Follow current laboratory practices in accordance with ISO 7218. For the steps in 4.3 and 4.4, use reagents and consumables of quality suitable for molecular biological applications (see ISO 22174). The composition of culture media and reagents and their preparation are specified in Annex A. For performance testing of culture media, follow the procedures in accordance with ISO 11133 and Clause A.4. The primers and probes for identification of the monophasic variant of *Salmonella* Typhimurium (1,4,[5],12:i:-) are listed in Annexes B to D.

6 Equipment and consumables

Disposable equipment is an acceptable alternative to reusable glassware if it has suitable specifications. The usual microbiological laboratory equipment (see ISO 7218) and molecular biology equipment (see ISO 22174) and, in particular, the following shall be used.

6.1 Incubator, capable of operating in the range of 34 °C to 38 °C.

NOTE The range 34 °C to 38 °C for incubation of culture media includes the use of incubators set at 35 °C ± 1 °C, 36 °C ± 2 °C or 37 °C ± 1 °C.

6.2 Sterile loops, of approximate diameter 3 mm (10 µl) or 0,3 mm (1 µl), or an inoculation needle/wire.

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

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

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

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

6.7 Equipment for suspension of a colony, e.g. (micro)centrifuge tubes.

6.8 Graduated pipettes and **pipette filter tips**, for handling volumes between 0,2 µl and 13,55 µl, depending on the PCR assay used (see Annex B, C or D). For more reactions per mix, larger volumes are needed.

6.9 Mixer.

6.10 Sterile Petri dishes, with a diameter of approximately 90 mm.

6.11 Equipment for PCR and **real-time PCR**, e.g. microcentrifuge or plate spinner.

6.12 Thermal cycler or **real-time PCR thermal cycler**, calibrated in accordance with ISO 20836.

6.13 Associated consumables for conventional or real-time PCR, e.g. PCR tubes, optical plates and seals, optical plate holder, suitable for use with the selected PCR machine (see Annex B, C or D).

6.14 Apparatus for dry sterilization (oven) or **wet sterilization (autoclave)**, as specified in ISO 7218.

7 Presumptive monophasic *Salmonella* Typhimurium

The isolate to be used for further identification shall be a pure culture characterized as belonging to the genus *Salmonella* (see ISO 6579-1). A presumptive monophasic *Salmonella* Typhimurium will show a positive reaction for O-antigen O:4 and H-antigen H:i and a negative reaction for the second H phase (see ISO/TR 6579-3).

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8 Culturing the isolate

Streak the culture of Clause 7 (e.g. with a 10 µl loop; 6.2) on the surface of a non-selective agar medium (e.g. nutrient agar; Clause A.2) to obtain well-isolated colonies. Incubate the plates, inverted, between 34 °C and 38 °C (6.1) for 24 h ± 3 h.

9 Procedure

9.1 Preparation of cell suspension or DNA

By means of an inoculating wire or a sterile loop (6.2), pick and suspend (a portion of) one colony in 100 µl saline solution (0,85 % m/v; Clause A.3) or in 100 µl PCR grade water in an appropriate tube (6.7).

Mix (6.9) for homogenization of the suspension.

An aliquot of 2 µl or 2,5 µl, depending on the specific PCR assay, of this bacterial cell suspension is used (see Table B.2, C.2, D.2, D.3 or D.4).

It is also possible to use a DNA extract for the PCR assay. For DNA extraction, for example, thermal cell lysis can be used, or another appropriate extraction method. If shown to be reliable, commercial kits can also be used for DNA extraction, following the manufacturer's instructions.

9.2 PCR amplification and detection

9.2.1 General

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Different protocols for multiplex probe-based real-time PCR, multiplex PCR followed by agarose gel electrophoresis detection of the amplification products or single target PCR followed by agarose gel electrophoresis detection can be used.

A probe-based multiplex real-time PCR assay is given in Annex B.

An agarose gel-based multiplex PCR assay is given in Annex C.

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An agarose gel-based single target PCR assay is given in Annex D.

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Follow all requirements, including the use of suitable equipment (6.11), for the (real-time) PCR amplification as specified in ISO 22174.

9.2.2 PCR controls

Use (process) controls for the PCR assays in accordance with ISO 22174.

For the real-time PCR (see Annex B) and the single target PCR (see Annex D), an internal amplification control (IAC) shall also be used as the targets could all be negative. Since the multiplex PCR (see Annex C) will always result in a PCR fragment (1 000 bp or 250 bp), this procedure does not require an IAC.

10 Expression of results

The results obtained, including controls specified in ISO 22174, shall be unambiguous otherwise the PCR shall be repeated.

The PCR result will be either:

a) positive, if a specific PCR product has been detected and all the controls give expected results, or

b) negative within the limits of detection, if a specific PCR product has not been detected, and controls give expected results.

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If the PCR assay identifies the isolate as monophasic *Salmonella* Typhimurium, report the result preferably by giving the antigenic formula as determined.

It is possible that an isolate is phenotypically identified as monophasic *Salmonella* Typhimurium, but genotypically (with PCR) as biphasic *Salmonella* Typhimurium. This can be caused by the fact that the genes are present, but phenotypically not expressed. For identification of these isolates, the PCR results take precedence over serum agglutination test results.

11 Performance characteristics of the method

11.1 Validation in accordance with ISO 17468

The PCR methods described in Annexes B, C and D were validated in accordance with ISO 17468. All relevant data as obtained in steps 1 to 5 of ISO 17468, as well as the results of the interlaboratory study (step 6 in ISO 17468) were reported in Reference [8].

The performance characteristics of the three PCR methods (inclusivity and exclusivity) were determined in a method(s) evaluation study (described in 11.2.1) and in an interlaboratory study (described in 11.2.2).

11.2 Performance characteristics

11.2.1 Method(s) evaluation study

The three PCR assays described in Annexes B, C and D were tested in a method evaluation study, by analysing 172 different strains (target and non-target strains), isolated from different sources (food products, animals, animal feed, primary production samples and humans), in two different laboratories. For the inclusivity and the exclusivity testing, the typing results of *Salmonella* found by slide agglutination were compared to the typing results found by each PCR method.

All data are given in Annex E and more details can be found in Reference [8].

It depends on the intended specific purpose for which the PCR assay is being applied and its performance evaluated, as to whether only monophasic *Salmonella* Typhimurium is considered as target strain (and thus part of the inclusivity study) or if (biphasic) *Salmonella* Typhimurium is also considered as target strain.

If the intended purpose is to determine if the strain under analysis is the monophasic variant of *Salmonella* Typhimurium and not the monophasic variant of another *Salmonella* non-Typhimurium serovar, then monophasic *Salmonella* Typhimurium as well as (biphasic) *Salmonella* Typhimurium can be considered as target strains and the three PCR assays described in Annexes B, C and D perform equally well for identification of monophasic *Salmonella* Typhimurium strains (see Table E.1).

If the aim is to identify whether the strain under analysis is either monophasic *Salmonella* Typhimurium or (biphasic) *Salmonella* Typhimurium, then *Salmonella* Typhimurium should be considered as non-target strain. For this purpose, the gel-based multiplex PCR (see Annex C) can be less specific for some strains than the other two PCR assays (see Table E.2), as this assay is less suitable to distinguish biphasic from monophasic *Salmonella* Typhimurium.

11.2.2 Interlaboratory study

The performance characteristics of each PCR method (see Annexes B, C and D) were determined in an interlaboratory study (step 6 in ISO 17468) to determine the inclusivity and exclusivity of the three methods, following the procedures described in ISO 16140-6. Details about the interlaboratory study and a summary of the data are given in Clause E.2 for each PCR assay.

A summary of the inclusivity and exclusivity data is given in Table E.4.

In the inclusivity study, pure target strains to be detected by the method were tested. For this interlaboratory study, monophasic *Salmonella* Typhimurium was considered as the only target strain.

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In the exclusivity study, pure non-target strains that are not expected to be detected by the method but can potentially be cross-reactive were tested. For this interlaboratory study, other *Salmonella* serovars than monophasic *Salmonella* Typhimurium, including (biphasic) *Salmonella* Typhimurium and other *Enterobacteriaceae* were considered as non-target strains.

12 Test report

The test report shall specify at least the following:

— the test method used, with reference to this document, i.e. ISO 6579-4;

— all operating conditions not specified in this document, or regarded as optional or informative (including informative annexes), together with details of any incidents which can have influenced the test result(s);

— any deviations from this document;

— all information necessary for the complete identification of the sample;

— the test result(s) obtained;

— the date of the test.

13 Quality assurance

The laboratory should have a quality control system to ensure that the equipment, reagents and techniques are suitable for the method. The use of positive controls, negative controls and blanks are part of the method. Performance testing of culture media is specified in Clause A.4 and described in ISO 11133.

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