TECHNICAL REPORT

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

Part 3: Guidelines for serotyping of *Salmonella* spp.

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

Partie 3: Lignes directrices pour la sérotypie des Salmonella *spp.*

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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).

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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 WTO principles in the Technical Barriers to Trade (TBT) see the following URL: Foreword - Supplementary information

The committee responsible for this document is ISO/TC 34, *Food products*, Subcommittee SC 9, *Microbiology*.

ISO 6579 consists of the following parts, under the general title *Microbiology of the food chain — Horizontal method for the detection, enumeration and serotyping of Salmonella*:

— *Part 1: Horizontal method for the detection of* Salmonella *spp.*[1)]

— *Part 2: Enumeration by a miniaturized most probable number technique* [Technical Specification][2)]

— *Part 3: Guidelines for serotyping of* Salmonella *spp.* [Technical Report]

1) Under preparation. (Revision of ISO 6579:2002)

2) The main element of the series title has been changed since Part 2 was published. It is intended that upon revision, the main element of the title will be aligned with Part 3.

Introduction

This part of ISO 6579 gives information on the taxonomy of *Salmonella* spp. and it gives guidance on serotyping of *Salmonella* serovars, based on the White–Kauffmann–Le Minor scheme (see Reference [9]).

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

Part 3: Guidelines for serotyping of *Salmonella* spp.

WARNING — In order to safeguard the health of laboratory personnel, it is essential that tests for detecting and typing *Salmonella*, be undertaken only in properly equipped laboratories, under the control of a skilled microbiologist, and that great care is taken in the disposal of all incubated materials.

IMPORTANT — 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 part of ISO 6579 gives guidance on the procedure for serotyping *Salmonella* serovars and is applicable to the serotyping of pure cultures of *Salmonella* spp., independent of the source from which they are isolated.

2 Normative references

The following documents, in whole or in part, are normatively referenced in this document and are indispensable for its application. For dated references, only the edition cited applies. For undated references, the latest edition of the referenced document (including any amendments) applies.

ISO 6579-1, *Microbiology of the food chain — Horizontal method for the detection, enumeration and serotyping of Salmonella — Part 1: Horizontal method for the detection of Salmonella spp.*

ISO 7218, *Microbiology of food and animal feeding stuffs — 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*

3 Terms and definitions

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

3.1
Salmonella
gram-negative, oxidase-negative, facultatively anaerobic, non-spore-forming, rod-shaped bacteria which generally form colonies of 2 mm to 4 mm in diameter on solid selective media and display biochemical and serological characteristics described when tests are carried out in accordance with this part of ISO 6579

3.2
serotyping of *Salmonella*
determination of the presence or absence of specific O-antigens, H-antigens and Vi-antigens in an isolate confirmed as *Salmonella* (3.1)

3.3
antigenic formula
combination of numbers and letters representing the O-, H-, and Vi-antigens of an isolate confirmed as *Salmonella* (3.1)

4 Principle

For the serotyping of *Salmonella* spp. the following antigens are determined for isolates biochemically confirmed as *Salmonella* spp.:

O-antigens, H-antigens and Vi-antigens.

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

5 Culture media and sera

5.1 General

For current laboratory practice, apply ISO 7218.

For the performance testing of media, follow the recommendations of ISO 11133.

5.2 Culture media and reagents

See Annex A.

5.3 Antisera

O-antisera, H-antisera and Vi-antisera are available from various commercial suppliers. Information on relevant polyvalent antisera and monovalent antisera can be found in Annex B.

6 Apparatus

Disposable supplies are an acceptable alternative to reusable glassware if they have similar specifications.

Usual microbiological laboratory equipment and, in particular, the following.

6.1 Incubator, to grow *Salmonella* isolates, capable of operating in the range 34 °C to 38 °C.

NOTE In this part of ISO 6579, the incubation temperature is not a differential parameter. Isolates are cultured to obtain sufficient material to perform the tests on a pure culture. Therefore, culture step is performed at an optimal growth temperature. For *Salmonella* this is generally a temperature between 34 °C and 38 °C.

6.2 Oven (for dry sterilization) or **autoclave** (for wet sterilization). See ISO 7218.

6.3 Refrigerator (for storage of prepared media), capable of operating at 5 °C ± 3 °C.

6.4 Glass slides.

6.5 Sterile inoculation instrument, e.g. needles, wires, wooden sticks, loops (e.g. of 1 µl).

6.6 Sterile test tubes and **flasks**, of appropriate capacity. Flasks or bottles and test tubes with non-toxic metallic or plastic (screw) caps may be used.

6.7 Sterile Petri dishes, with diameters of approximately 55 mm and 90 mm.

6.8 Water bath, capable of operating at 47-50 °C.

6.9 Water bath (or incubator), capable of operating at 50 °C ± 2 °C.

7 Sample

It is important that the laboratory works with a pure culture which has been biochemically confirmed as *Salmonella* spp.

8 Taxonomy of *Salmonella*

8.1 General

Approximately every 7 years, the WHO Collaborating Centre for Reference and Research on *Salmonella* (Institut Pasteur, Paris) publishes an update of the "Antigenic formulae of the *Salmonella* serovars", which is the basis for assigning serovar names and formulas to isolates of *Salmonella* spp. At the time of publication, the latest version of the White–Kauffmann–Le Minor scheme is that of 2007 (Reference [9]).

NOTE Supplements to the White-Kauffmann-Le Minor scheme are published in *Research in Microbiology*, a publication of the Institut Pasteur (formerly called *Annales de l'Institut Pasteur/Microbiologie*). For instance, supplement no. 47 was published in 2010 and characterises new serovars found between 2003 and 2007 (Reference [10]).

This part of ISO 6579 provides guidance on the serotyping of *Salmonella* serovars.

8.2 Nomenclature

Different nomenclatures have been used (or are still in use) for *Salmonella* strains:

— originally, Kauffmann (Reference [12]) considered each *Salmonella* serovar as a separate species;

— different type species have been used: *S. enterica* vs. *S. choleraesuis*, each having another type strain;

— some "important" *Salmonella* strains (like *Salmonella* Typhi and *Salmonella* Paratyphi) were considered to be species and not being "only" serovars of a species.

The Judicial Commission of the International Committee on Systematics of Procaryotes indicated that many synonyms can be used in *Salmonella* nomenclature (Reference [22]). In this part of ISO 6579, the widely accepted current nomenclature is used, which is also approved by the WHO Collaborating Centre for Reference and Research on *Salmonella* (Reference [9]), the American Society for Microbiology (Reference [20]), the Centers for Disease Control and Prevention (Reference [3]) and *Bergey's manual* (Reference [17]). According to the current nomenclature, the genus *Salmonella* belongs to the family of *Enterobacteriaceae* and consists of only two species: *S. enterica* and *S. bongori*. *S. enterica* is divided into six subspecies: *S. enterica* subsp. *enterica*, *S. enterica* subsp. *salamae*, *S. enterica* subsp. *arizonae*, *S. enterica* subsp. *diarizonae*, *S. enterica* subsp. *houtenae*, and *S. enterica* subsp. *indica.*

Salmonella serovars belonging to *S. enterica* subsp. *enterica* are isolated most frequently (more than 99,5 % of isolated *Salmonella* strains) and they are designated by a name, usually related to the geographical place where the serovar was first isolated. Serovars belonging to other subspecies of *S. enterica* and those of *S. bongori* are designated by their antigenic formula.

Due to combinations of subspecies and many serovars, the full names are long (e.g. *Salmonella enterica* subsp. *enterica* serovar Typhimurium). It has therefore generally been accepted to use a shorter

way to indicate the names of the serovars of subspecies *enterica*. The White–Kauffmann–Le Minor scheme suggests the following shortened names: *S. enterica* serovar Typhimurium or *Salmonella* ser. Typhimurium. According to Reference [3], at the first citation of a serovar in a text the genus name should be given followed by the word "serovar" or the abbreviated term "ser". and then the serovar name. Subsequently, the name may be written with the genus followed directly by the serovar name (e.g. *Salmonella* Typhimurium). This way of indicating *Salmonella* serovars is also accepted in the majority of journals [e.g. journals of the American Society for Microbiology (ASM)] and is also used in this part of ISO 6579.

In summary, the nomenclature of *Salmonella*:

family: *Enterobacteriaceae* (first letter capitalized, italicized)

genus: *Salmonella* (first letter capitalized, italicized)

species: *enterica* (not capitalized, italicized)

subspecies: *enterica* (not capitalized, italicized)

serovar (serotype or ser.): e.g. Typhimurium (first letter capitalized, not italicized)

subspecies: *salamae*
arizonae
diarizonae
houtenae
indica

species: *bongori*

In the 47th Supplement to the White–Kauffmann–Le Minor scheme (Reference [10]) more than 2600 *Salmonella* serovars are mentioned and the numbers increase regularly, as summarized in Table 1.

Table 1 — Number of *Salmonella* serovars through the years

Species/subspecies	Supplement		
	1998[a]	2001[b]	2007[c]
	Number of serovars		
Salmonella enterica	2 443	2 502	2 587
subsp. *enterica*	1 454	1 492	1 547
subsp. *salamae*	489	500	513
subsp. *arizonae*	94	95	100
subsp. *diarizonae*	324	331	341
subsp. *houtenae*	70	71	73
subsp. *indica*	12	13	13
Salmonella bongori	20	21	23
Total no. of serovars (genus *Salmonella*)	2 463	2 523	2 610

[a] Reference [18].
[b] Reference [19].
[c] Reference [10], covering 2003–2007.

8.3 Biochemical characteristics

The *Salmonella* species and subspecies are identified based on different biochemical tests. In Table 2, the differential characteristics are listed. See Reference [9] and Annex C for further details.

Table 2 — Biochemical characteristics of *Salmonella* species and subspecies (Reference [9])

Species	*S. enterica*						*S. bongori*
Subspecies	*enterica*	*salamae*	*arizonae*	*diarizonae*	*houtenae*	*indica*	
Dulcitol	+	+	–	–	–	d	+
ONPG[a] (2 h)	–	–	+	+	–	d	+
Malonate	–	+	+	+	–	–	–
Gelatinase	–	+	+	+	+	+	–
Sorbitol	+	+	+	+	+	–	+
Growth with KCN[b]	–	–	–	–	+	–	+
L(+)-tartrate[c]	+	–	–	–	–	–	–
Galacturonate	–	+	–	+	+	+	+
γ-Glutamyltransferase	+ [e]	+	–	+	+	+	+
β-Glucuronidase	d	d	–	+	–	d	–
Mucate	+	+	+	– (70 %)	–	+	+
Salicin	–	–	–	–	+	–	–
Lactose	–	–	– (75 %)	+ (75 %)+	–	d	–
Lysis by phage O1	+	+	–	+	–	+	d

+ = 90 % or more positive reaction

– = 90 % or more negative reaction

d = different reactions given by different serovars

[a] *o*-Nitrophenyl-β-D-galactopyranoside (test for β-galactosidase).

[b] Potassium cyanide.

[c] = D-Tartrate, Paratyphi B: –, Paratyphi B biovar Java: +

[e] = Typhimurium: d, Dublin: –.

8.4 Antigenic characteristics

8.4.1 General

The important antigenic characteristics of *Salmonella* for serological tests are divided into three main types, being:

— the O-antigen, also called the somatic antigen;

— the H-antigen, also called the flagellar antigen;

— the Vi-antigen, also called the capsular antigen.

The antigenic formula of *Salmonella* spp. exists of these three types of antigens, reported in the following way: O-antigens, Vi-antigen (if present): H-antigens of first phase: H-antigens of second phase. For instance, the antigenic formula of *Salmonella* Paratyphi C is: 6,7,[Vi]:c:1,5; with O-antigens O:6 and O:7; with the Vi-antigen, which can be present or absent (indicated by the square brackets); with H-antigen H:c for the first phase; with H-antigens H:1 and H:5 for the second phase.

8.4.2 The O-antigen (somatic antigen)

This antigen consists of a cell wall component and the main substances are polysaccharide, protein, and phospholipid. The O-antigen is very robust and can resist temperatures up to 100 °C for 150 min, treatment with 95 % volume fraction ethanol or dilute acid (Reference [16]).

The reaction of the O-antigen with antisera results in granular agglutination. Historically, the O-antigens were classified in individual O-antigen groups in the Kauffmann–White scheme (Reference [9]). The groups were named with Roman letters beginning with group A, which include antigen O:2, up to group Z which contain antigen O:50. As there were more O-antigens than letters, the remaining antigens were not given as group, but were named by the O-antigens O:51 to O:67. Nowadays it is preferred to designate each O-group using the characteristic O-factor. The letters have been kept and are shown inside brackets, e.g. O:4 (B) (Reference [9]). In Table 3, the old and new designations are summarized.

Table 3 — *Salmonella* serogroups (old designation) and relevant O-antigens (new designation)

Group	O-antigen	Group	O-antigen	Group	O-antigen
A	2	G_1-G_2	13	Q	39
B	4	H	6,14	R	40
C_1(, C_4)[a]	6,7	I	16	S	41
C_2, C_3	8	J	17	T	42
D_1	9	K	18	U	43
D_2	9,46	L	21	V	44
D_3	9,46,27	M	28	W	45
E_1(, E_2, E_3)[b]	3,10	N	30	X	47
E_4	1,3,19	O	35	Y	48
F	11	P	38	Z	50

[a] C_4 has been merged into C_1.

[b] E_2 and E_3 have been merged into E_1.

8.4.3 The H-antigen (flagellar antigen)

This antigen is located on the flagellum and the main component is protein. It is less robust than O-antigens. It can easily be decomposed by alcohol, acid, and temperature above 60 °C, but it is resistant to a formalin solution with a volume fraction of 0,5 % (Reference [16]).

The reaction of the H-antigen with antisera results in floccular agglutination. Many *Salmonella* spp. possess two phases of the H-antigen, but monophasic and triphasic variants are also known. The first phase is called the specific phase and the second phase is called the non-specific phase. The first phase is indicated by a lower case letter, a to z. However, since the identification of the z-antigen, many new H-antigens have been detected and are named z_1, z_2, z_3 … z_{91}.

Examples of monophasic serovars are:

— *Salmonella* Paratyphi A: 1,2,12:a:[1,5]; with H:a for the first phase and where the square brackets indicate that the second phase (H:1,5) can be present or absent;

— *Salmonella* Typhi: 9,12,[Vi]:d:-; with H:d for the first phase;

— *Salmonella* Derby: 1,4,[5],12:f,g:[1,2]; with H:f,g for the first phase and where the second phase (H:1,2) can be present or absent;

— *Salmonella* Enteritidis: 1,9,12: g,m:-; with H:g,m for the first phase. In addition to factors H:g,m, some strains may have factor H:p, or H:f, or H:t. Exceptional strains can have antigen H:1,7 as second phase;

— *Salmonella* Dublin: 1,9,12,[Vi]:g,p:-; with H:g,p for the first phase.

NOTE 1 Underlined O-factors are determined by phage conversion. They are only present if the culture is lysogenized by the corresponding converting phage (Reference [9]).

NOTE 2 O- or H-factors indicated in square brackets can be present or absent, without relation to phage conversion (Reference [9]).

NOTE 3 Diphasic strains of *Salmonella* Derby and *Salmonella* Enteritidis are very rare. It is possible that phase inversion is required to detect these rare strains. However, this is only necessary for certain (special) cases (e.g. in case of deviating sources and/or in the case of (special) travel-related cases).

8.4.4 Vi-antigen (capsular antigen)

This antigen is a surface (capsular) antigen and can mask the O-antigens so that the bacteria are not agglutinated with O-antisera. The main component of the Vi-antigen is polysaccharide. The *Salmonella* strains which possess a Vi-antigen are more virulent than the strains without Vi-antigen. The Vi-antigen can be present in only three *Salmonella* serovars:

— *Salmonella* Typhi : 9,12,[Vi]:d:-;

— *Salmonella* Paratyphi C : 6,7,[Vi]:c:1,5;

— *Salmonella* Dublin: 1,9,12,[Vi]:g,p:-.

The square brackets indicate that the Vi-antigen can be present or absent.

NOTE The presence of Vi-antigens in *Salmonella* isolates from food or veterinary samples is very rare. If Vi is present, it masks the detection of O-antigens. To detect the O-antigens, it can prove necessary to heat a suspension of the isolate (e.g. in physiological saline solution) at 100 °C for 60 min, or at 120 °C for 15 min.

9 Procedure for *Salmonella* serotyping

9.1 General

Before starting the serotyping, it is important to confirm biochemically that the isolate belongs to the genus *Salmonella* (as specified in ISO 6579-1). Although the H-antigens are specific for *Salmonella*, several O-antigens are common in different genera of the *Enterobacteriaceae* (e.g. *Salmonella, Citrobacter, Hafnia*).

NOTE Alternative procedures can be used to confirm that the isolate belongs to the genus *Salmonella*. provided the suitability of the alternative procedure is verified (see ISO 7218).

Each supplier of antisera produces its own sets of antisera, with its own unique instructions for use. It is therefore not possible to provide here one general set of instructions for serotyping, as it is always important to follow the instructions of the supplier to obtain optimal results. Some manufacturers supply pools of antisera (mixtures of several O-antisera or H-antisera), which are very useful at the beginning of serotyping an unknown type. When the strain agglutinates with an antisera pool, it can be further tested with group antisera and/or single factor antisera relevant to the positive pool. When the focus is on typing only certain serovars and it is sufficient to indicate the other serovars as *Salmonella* spp., the agglutination can immediately be performed with only the specific monofactor antisera of the relevant serovars.

In Annex B, the general procedure is given for serotyping an unknown *Salmonella* isolate.

9.2 Example procedure for serotyping five public health-related *Salmonella* serovars

9.2.1 General

In the following example, the procedure is described for serotyping five important public health-related *Salmonella* serovars (see Annex D). In Table 4 these strains are shown with their antigenic formula.

In the following sections, slide agglutination of *Salmonella* isolates is described, which is the procedure most often performed. However, others also exist, such as the microtitre plate method (see Annex E).