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Water quality — Detection of *Salmonella* spp.

Qualité de l'eau — Recherche de Salmonella *spp.*

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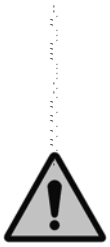

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Foreword

ISO (the International Organization for Standardization) is a worldwide federation of national standards bodies (ISO member bodies). The work of preparing International Standards is normally carried out through ISO technical committees. Each member body interested in a subject for which a technical committee has been established has the right to be represented on that committee. International organizations, governmental and non-governmental, in liaison with ISO, also take part in the work. ISO collaborates closely with the International Electrotechnical Commission (IEC) on all matters of electrotechnical standardization.

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

The main task of technical committees is to prepare International Standards. Draft International Standards adopted by the technical committees are circulated to the member bodies for voting. Publication as an International Standard requires approval by at least 75 % of the member bodies casting a vote.

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

ISO 19250 was prepared by Technical Committee ISO/TC 147, *Water quality*, Subcommittee SC 4, *Microbiological methods*.

This edition cancels and replaces ISO 6340:1995, which has been technically revised.

Introduction

Salmonella species are bacteria which are widely distributed all over the world. They are usually classified as pathogens, although their virulence and pathogenesis vary widely. The natural hosts of *Salmonella* include humans, agricultural and domestic livestock, and wild animals including birds. Humans and animals can excrete these bacteria while carrying them asymptomatically as well as during disease. It is therefore impossible to eliminate them from the environment. Following the infection of humans, the transmission of *Salmonella* can cause severe disease.

Since water is a recognized vehicle of infection, the presence or absence of *Salmonella* is monitored in water where there is perceived to be a risk of infection. *Salmonella* can be present in all types of domestic and agricultural waste water, freshwaters, including ground and drinking waters, as well as sea water.

The detection of *Salmonella* in water usually requires a concentration step. Since *Salmonella* cells can be present in low numbers and injured in the aqueous environment, their detection in water usually requires a pre-enrichment step.

Water quality — Detection of *Salmonella* spp.

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

Persons using this International Standard should be familiar with normal laboratory practice. This standard does not purport to address all of the safety problems, 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.

IMPORTANT — It is absolutely essential that tests conducted according to this International Standard be carried out by suitably trained staff.

1 Scope

This International Standard specifies a method for the detection of *Salmonella* spp. (presumptive or confirmed) in water samples. It is possible that, for epidemiological purposes or during outbreak investigations, other media are also required.

WARNING — It is possible that the method does not recover all *Salmonella* ser. Typhi and ser. Paratyphi.

NOTE For a semi-quantitative approach, most probable number (MPN) tests can be performed using appropriate sample volumes. For these cases, the volume of the buffered peptone water is adjusted accordingly.

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 6579, *Microbiology of food and animal feeding stuffs — Horizontal method for the detection of* Salmonella *spp.*

ISO 6887-1, *Microbiology of food and animal feeding stuffs — Preparation of test samples, initial suspension and decimal dilutions for microbiological examination — Part 1: General rules for the preparation of the initial suspension and decimal dilutions*

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

ISO 7704, *Water quality — Evaluation of membrane filters used for microbiological analyses*

ISO 8199, *Water quality — General guidance on the enumeration of micro-organisms by culture*

ISO 19458, *Water quality — Sampling for microbiological analysis*

3 Terms and definitions

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

3.1
presumptive *Salmonella* spp.
bacteria which grow in the selective enrichment medium specified, and form typical or atypical colonies on the solid selective media

3.2
confirmed *Salmonella* spp.
bacteria which grow in the selective enrichment medium specified, and form typical and suspicious colonies on the solid selective media, and which display specfic biochemical and serological characteristics

NOTE The specific biochemical and serological characteristics are determined by tests specified in this International Standard.

3.3
***Salmonella* detection**
determination of the presence or absence of ***Salmonella*** (3.4)

3.4
***Salmonella* spp.**
Salmonella
microorganisms which form typical or atypical colonies on solid selective media and which display specific biochemical and serological characteristics

4 Principle

4.1 General

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

Pre-enrichment is often necessary to permit detection of low numbers of *Salmonella* or injured *Salmonella*. Some *Salmonella* and those which are sublethally injured may require additional incubation time (4.3). Furthermore, *Salmonella* can be present in small numbers and are often accompanied by considerably larger numbers of other members of Enterobacteriaceae or of other families. Therefore, selective enrichment is necessary.

4.2 Pre-enrichment in non-selective liquid medium

Buffered peptone water (B.1) is inoculated at ambient temperature with a known volume of the sample or its dilutions, then incubated at (36 ± 2) °C for (18 ± 2) h. Larger volumes can be concentrated using membrane filtration and the membrane filter is then added to buffered peptone water.

NOTE For waste water it has been shown that shorter incubation times or direct inoculation of the sample in selective medium (4.3) produce better results.

For a semi-quantitative approach, MPN tests can be performed using appropriate sample volumes. In these cases, adjust the volumes of the buffered peptone water accordingly.

4.3 Enrichment in selective liquid media

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

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

To detect slow-growing *Salmonella* spp., incubate the enrichment broth for a further (24 ± 3) h to a total of (48 ± 4) h at (41,5 ± 1,0) °C.

NOTE *Salmonella* Typhi and *Salmonella* Paratyphi A are usually not important in routine water quality monitoring, but can be relevant in epidemiological investigations. MKTTn broth is used for enrichment with incubation at (36 ± 2) °C for up to (24 ± 3) h and recovers most strains of *Salmonella*, including some strains of *Salmonella* Paratyphi, but is not thought to be able to recover strains of *Salmonella* Paratyphi C. MKTTn broth is not used if *Salmonella* Typhi is suspected after the use of selenite cystine broth.

4.4 Plating out and recognition

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

a) xylose lysine deoxycholate agar (XLD agar);

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

Incubate the XLD agar at (36 ± 2) °C and examine after (24 ± 3) h to check for the presence of colonies which are considered to be presumptive *Salmonella*. Incubate the second selective agar according to the manufacturer's recommendations.

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

4.5 Confirmation

Subculture colonies of presumptive *Salmonella*, then plate out as described in 4.4 and confirm their identity by means of appropriate biochemical (8.5.3) and serological (8.5.4) tests.

5 Apparatus

Usual microbiological laboratory equipment (see ISO 7218) and, in particular, the following.

5.1 General. Except for disposable glassware which is delivered sterile, sterilize glassware as specified in ISO 8199. Disposable apparatus is an acceptable alternative to reusable glassware if it has suitable specifications.

5.2 Autoclave, capable of being maintained at (121 ± 3) °C and at (115 ± 3) °C.

5.3 Water bath or **incubator**, capable of being maintained at (36 ± 2) °C.

5.4 Water bath or **incubator**, capable of being maintained at (41,5 ± 1,0) °C.

5.5 Water baths, capable of operating at (70 ± 1) °C and at 50 °C to 55 °C.

5.6 Membrane filtration apparatus, as specified in ISO 8199.

5.7 Sterile membrane filters, with a nominal pore size of 0,45 µm.

The quality of membrane filters may vary from brand to brand or even from batch to batch. It is therefore advisable to check the quality on a regular basis, as specified in ISO 7704.

5.8 pH-meter, with an accuracy of calibration of ± 0,1 pH at 20 °C to 25 °C.

5.9 Sterile forceps.

5.10 Sterile loops, approximate diameter 3 mm (10 µl volume), and inoculation needle or wire.

6 Sampling

Sampling is not part of the method specified in this International Standard. Samples should be taken in accordance with ISO 19458.

It is important the laboratory receive a truly representative sample which has not been damaged or changed during transport or storage.

7 Culture media and reagents

NOTE For guidelines on quality assurance and performance testing, see ISO/TS 11133-1[2] and ISO/TS 11133-2[3].

7.1 Basic materials. For uniformity of results, in the preparation of media, either use a dehydrated complete medium or use constituents of uniform quality and reagents of recognized analytical grade.

Other grades of reagents may be used provided they can be shown to produce comparable results.

7.2 Water, ISO 3696[1], grade 3.

7.3 Culture media, prepared in accordance with Annex B.

7.3.1 Buffered peptone water, non-selective pre-enrichment medium buffered peptone water (BPW, B.1).

7.3.2 Rappaport-Vassiliadis broth with soya (RVS broth, B.2), selective enrichment medium.

7.3.3 Xylose lysine deoxycholate agar (XLD agar, B.3).

7.3.4 Second solid selective plating-out medium, whose choice is left to the discretion of the testing laboratory. Follow the manufacturer's instructions precisely regarding its preparation for use.

7.3.5 Nutrient agar (B.4), or other appropriate non-selective agar.

7.3.6 Triple sugar and iron agar (TSI agar, B.5).

As an alternative, iron and two sugar agar may be used.

7.3.7 Urea agar, Christensen (B.6).

7.3.8 L-Lysine decarboxylation medium (B.7).

7.3.9 Selenite cystine broth (B.8).

7.3.10 Muller-Kaufmann tetrathionate-novobiocin broth (MKTTn, B.9).

7.3.11 Filter aid (B.10).

8 Procedure

See Figure A.1.

8.1 Preparation of the sample

For the preparation of the sample, filtration and inoculation on isolation media, follow the instructions as specified in ISO 8199 and ISO 6887-1. Start the examination preferably immediately after taking the samples. If the samples are kept at ambient temperatures, start the examination within 12 h after sampling. Under exceptional circumstances, it is allowable for the samples to be kept at (5 ± 3) °C for up to 24 h prior to examination.

The volume of the sample to be analysed depends on the type of water. Usual volumes for bathing water and drinking water are 1 000 ml to 5 000 ml. For polluted surface waters and waste water, smaller volumes are usually analysed.

If sample dilutions are necessary (e.g. for waste water samples), prepare these dilutions as specified in ISO 8199.

8.2 Non-selective pre-enrichment

8.2.1 Non-selective pre-enrichment for volumes less than 10 ml

Inoculate 50 ml of BPW (B.1) at room temperature with the sample or dilutions thereof and incubate at (36 ± 2) °C for (18 ± 2) h.

8.2.2 Non-selective pre-enrichment for volumes greater than 10 ml

Filter a volume of water appropriate for the water being examined.

Immerse the membrane filter in 50 ml of BPW (B.1).

Alternatively, add the sample to the same volume of double strength BPW.

Note that the latter procedure is not suitable for mineral waters with high salt content or sea water.

Incubate the cultures at (36 ± 2) °C for (18 ± 2) h.

8.2.3 Recommendation for turbid or polluted water

For turbid or polluted waters, sterile filter aid (B.10) can be added and the sample filtered through a sterile absorbent pad acting as a supporting base instead of using the membrane.

In this case, filter an aliquot of filter aid, typically 15 ml, to form an initial layer on the absorbent pad. Mix a second aliquot, typically 15 ml, with the volume of sample and filter. For turbid or dirty waters, additional aliquots may be filtered. When filtration is complete, remove the funnel and carefully transfer the absorbent pad and filter aid to BPW (B.1). If necessary, retain a small volume of BPW to rinse the funnel so that the final volume of BPW is 100 ml. Incubate for presence or absence, or dispense as an MPN series for a semi-quantitative count.

8.3 Selective enrichment

Allow the enrichment broth(s) to equilibrate to room temperature if they were stored at a lower temperature. Transfer 0,1 ml of the culture obtained in 8.2 to a tube containing 10 ml of the RVS broth (B.2). When MKTTn (B.9) is also used, transfer 1 ml of the culture obtained in 8.2 to a tube containing 10 ml of the MKTTn broth.

Incubate the inoculated RVS broth at (41,5 ± 1,0) °C for (24 ± 3) h and, if necessary (see 4.3), for (48 ± 4) h. Care should be taken that the maximum allowed incubation temperature (42,5 °C) is not exceeded. Incubate the inoculated MKTTn broth at (36 ± 2) °C for (24 ± 3) h.

NOTE For RVS broth, the magnesium chloride concentration and incubation temperature have been optimized to yield good recovery without losing selectivity according to Reference [5].

8.4 Plating out

8.4.1 General

Allow the XLD agar plates and the second selective plating-out medium (see ISO 6579:2002, 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.

8.4.2 Plating from RVS broth

Using the culture obtained in the RVS broth, inoculate, after incubation for (24 ± 3) h and, if necessary (see 4.3), for (48 ± 4) h, by means of a sterile loop (5.10), the surface of the following enrichment media so that well-isolated colonies are obtained:

a) XLD agar (B.3);

b) an additional selective medium (7.3.4).

Invert the dishes so that the bottom is uppermost, and place them in the incubator (5.3) set at (36 ± 2) °C for (24 ± 3) h for the XLD agar. The manufacturer's instructions shall be followed for the second selective plating-out medium.

8.4.3 Plating from MKTTn broth

After incubation at (36 ± 2) °C for (24 ± 3) h using the culture obtained, repeat the procedure specified in 8.4.2 with the two selective plating-out media.

8.5 Confirmation

8.5.1 General

If shown to be reliable, commercially available identification kits for the biochemical examination of *Salmonella* may be used. Use these kits according to the manufacturer's instructions.

8.5.2 Selection of colonies for confirmation

For routine monitoring purposes, take, for confirmation, from each Petri dish of each selective medium (8.4), at least one discrete colony considered to be typical or presumptive *Salmonella*. If the first colony is not confirmed as *Salmonella*, then take a further four colonies.

On XLD agar, typical *Salmonella* colonies usually have a black centre and a lightly transparent zone of reddish colour due to the colour change of the indicator. It is recommended that at least five colonies be identified for epidemiological studies. If on one dish there are fewer than five typical or suspect colonies, take all the typical or suspect colonies for confirmation.

NOTE The recognition of *Salmonella* colonies 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 medium used. *Shigella*, *Providencia* and H_2S-negative *Salmonella* spp.(e.g. *Salmonella* Paratyphi A) appear as pink with a darker pink centre; lactose-positive *Salmonella* grown on XLD are yellow with or without blackening; Enterobacteriaceae e.g. *Escherichia coli, Enterobacter*, *Klebsiella*, *Citrobacter*, *Proteus*, and *Serratia* appear as yellow, opaque colonies.

Streak the selected colonies on to the surface of pre-dried “non-selective” agar plates (e.g. nutrient agar, B.4) to allow the development of well-isolated colonies.

Incubate the inoculated plates at (36 ± 2) °C for (24 ± 3) h.

If the plating fails to produce distinct colonies, repeat in a manner which ensures that single discrete colonies are produced. Use single isolated colonies for biochemical and, if appropriate, serological confirmation. If biochemical kits are used for identification, follow the manufacturer's instructions.

8.5.3 Biochemical confirmation

8.5.3.1 General

By means of an inoculating wire, inoculate the media specified in 8.5.3.2 to 8.5.3.4 with each of the cultures obtained from the colonies selected in 8.5.2.

8.5.3.2 TSI agar

Streak the TSI agar (B.5) slant surface and stab the butt. Incubate at (36 ± 2) °C for (24 ± 3) h. Interpret the appearance of the medium as in Table 1.

Table 1 — Interpretation of changes in medium

Appearance	Interpretation
Butt	
Yellow (acid)	Glucose positive (fermentation of glucose)
Red or unchanged (alkaline)	Glucose negative (no fermentation of carbohydrates)
Black	Formation of hydrogen sulfide
Bubbles or cracks	Gas formation from glucose
Slant surface	
Yellow	Lactose or sucrose positive (lactose or sucrose used)
Red or unchanged (alkaline)	Lactose and sucrose negative (neither lactose nor sucrose used)

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

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.

8.5.3.3 Urea agar

Streak the urea agar (B.6) slant surface. Incubate at (36 ± 2) °C for up to 24 h and examine at intervals. If the reaction is positive, hydrolysis of urea liberates ammonia, which changes the colour of phenol red to rose-pink and later to deep cerise. The reaction is often apparent after 2 h to 4 h. Typical *Salmonella* cultures show a negative reaction, i.e. no colour production.

8.5.3.4 L-Lysine decarboxylation medium

Inoculate well below the surface of the liquid L-lysine decarboxylation medium (B.7). Incubate at (36 ± 2) °C for (24 ± 3) h. Typical *Salmonella* cultures show a purple colour after incubation.

8.5.3.5 Interpretation of the biochemical tests

Salmonella generally show the reactions given in Table 2.

Table 2 — Biochemical reactions of *Salmonella*

Test[a]	Subclause	Reaction	*Salmonella* strains showing the reaction % [b]
TSI glucose (acid formation)	8.5.3.2	+	100,0
TSI glucose (gas formation)	8.5.3.2	+	91,9[c]
TSI lactose	8.5.3.2	–	99,2[d]
TSI sucrose	8.5.3.2	–	99,5
TSI hydrogen sulfide	8.5.3.2	+	91,6[e]
Urea hydrolysis	8.5.3.3	–	99,0
L-Lysine decarboxylation	8.5.3.4	+	94,6[f]

[a] See Reference [8].

[b] These figures indicate only that not all strains of *Salmonella* show the reactions marked + or –. They may vary between geographical areas and from water source to water source.

[c] *Salmonella* Typhi is anaerogenic.

[d] The *Salmonella enterica* subsp. *arizonae* gives positive or negative lactose reactions but is always β-galactosidase positive. The *Salmonella enterica* subsp. *diarizonae* gives a negative lactose reaction, but gives a positive β-galactosidase reaction. In addition, it is possible that these strains do not produce H_2S. For the study of these strains, it can be useful to carry out complimentary biochemical tests (References [9][10]).

[e] Acid formation is sometimes difficult to recognize due to strong blackening.

[f] *Salmonella* Paratyphi A is negative.

The biochemical reactions in Table 3 are typical of *Salmonella* spp.

Table 3 — Typical biochemical reactions of *Salmonella* spp. to tests

Test	Subclause	Reaction
TSI lactose	8.5.3.2	–
TSI glucose	8.5.3.2	+
TSI sucrose	8.5.3.2	–
TSI hydrogen sulfide	8.5.3.2	+
Urea splitting	8.5.3.3	–
L-Lysine decarboxylase	8.5.3.4	+

Isolates which only vary from the typical parameters listed in Table 3 by one or two reactions can still be *Salmonella* and should be further investigated and sent to a recognized reference laboratory for confirmation.

8.5.4 Serological confirmation and serotyping

Isolates which are typical according to the biochemical reactions listed in Table 3 are presumptive *Salmonella* spp. and should, if need be, be investigated further by a reference laboratory. The presence of *Salmonella* O-, Vi-, and H-antigens is detected by slide agglutination as specified in ISO 6579 with the appropriate sera, from pure colonies (8.5.2) and after auto-agglutinating strains have been eliminated.

Isolates which give positive serological reactions are confirmed as *Salmonella* spp.

9 Expression of results

In accordance with the results of the biochemical tests (8.5.3) and serological confirmation (8.5.4), indicate whether presumptive or confirmed *Salmonella* were detected in the test portion examined.

10 Test report

The test report shall contain at least the following information:

a) the test method used, together with a reference to this International Standard (ISO 19250:2010);

b) all the information required for the complete identification of the sample;

c) the result, expressed as presumptive or confirmed *Salmonella* detected or not detected in a test portion of V ml of water;

d) if epidemiological monitoring is performed, the specification of the number of colonies isolated from selective solid media (8.5.2) and the species or serotypes observed (8.5.4);

e) for MPN tests, the estimated number of *Salmonella* per sample volume.

11 Quality assurance

The laboratory shall have a clearly defined quality control system to ensure that the apparatus, reagents, and techniques are suitable for the test. The use of positive controls, negative controls, and blanks is part of the test.

Perform positive controls by introducing reference samples into control flasks of the pre-enrichment medium (see B.1). Proceed with the control flasks as for the test cultures.

Control strains chosen should be easily identifiable and not a strain that is commonly isolated by the testing laboratory. Consultation with the appropriate National Reference Laboratory is advised.

Annex A
(normative)

Diagram of procedure

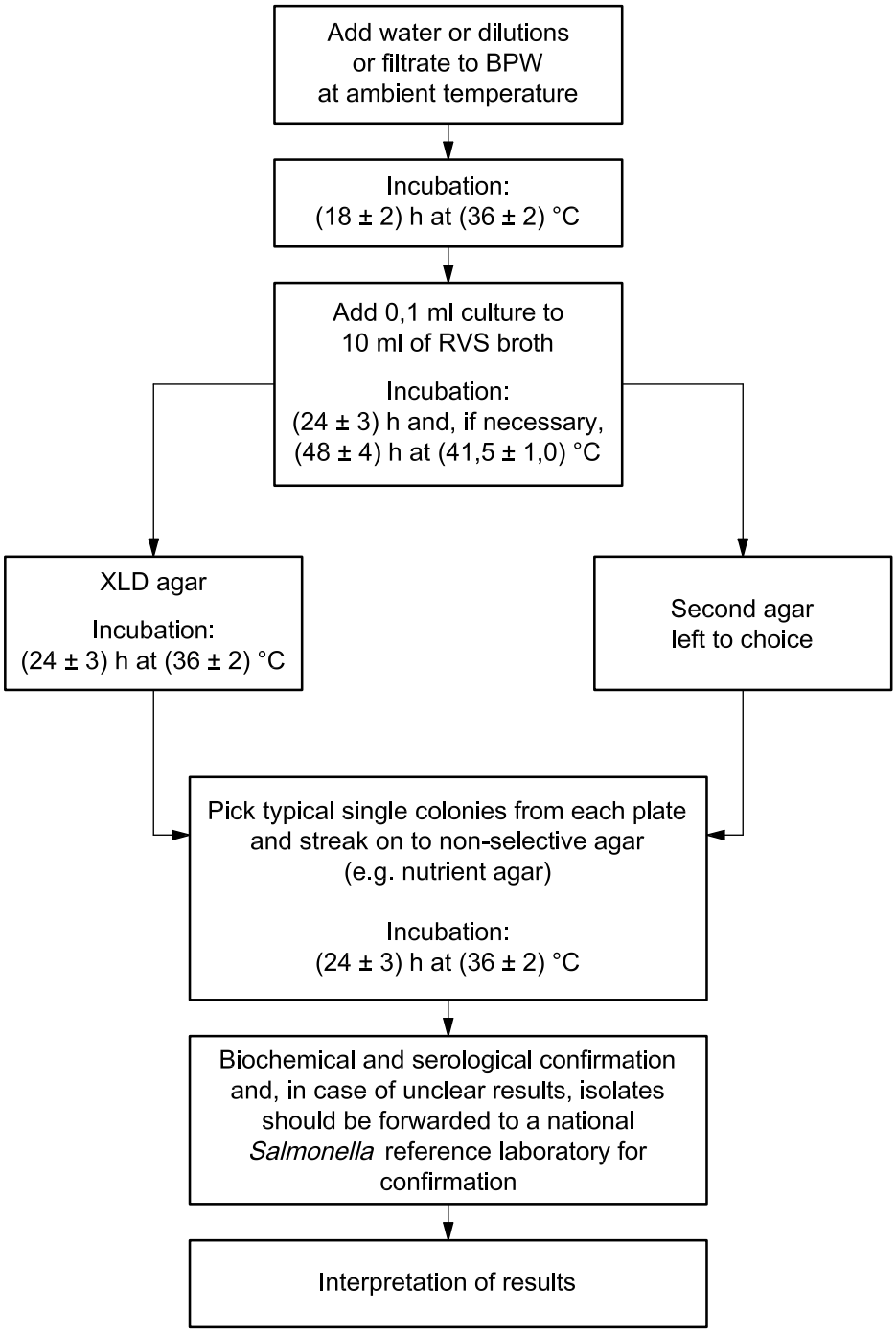

Figure A.1

Annex B
(normative)

Composition and preparation of culture media and reagents

As an alternative to the preparations specified in this annex, dehydrated complete media or diluents may be used. Follow the manufacturer's instructions.

For the preparation of the media, use water (7.2) free from substances that might affect growth of micro-organisms under test conditions.

B.1 Buffered peptone water (BPW)

	Single strength	Double strength
Enzymatic digest of casein	10,0 g	20,0 g
Sodium chloride (NaCl)	5,0 g	10,0 g
Disodium hydrogenphosphate dodecahydrate ($Na_2HPO_4 \cdot 12H_2O$)[a]	9,0 g	18,0 g
Potassium dihydrogenphosphate (KH_2PO_4)	1,5 g	3,0 g
Water (7.2)	1 000 ml	1 000 ml

[a] If anhydrous disodium hydrogenphosphate (Na_2HPO_4) is used, 3,5 g have to be added for single strength BPW and 7,0 g for double strength BPW.

Dissolve the components in the water, if necessary, by heating (without boiling).

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

Dispense the medium into flasks of suitable capacity to obtain the portions necessary for the test.

Sterilize in the autoclave (5.2) set at (121 ± 3) °C for 15 min.

Store at (5 ± 3) °C for up to 3 months.

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

B.2.1 Basic medium, Solution A

Enzymatic digest of soya	5,0 g
Sodium chloride (NaCl)	8,0 g
Potassium dihydrogenphosphate (KH_2PO_4)	1,4 g
Dipotassium hydrogenphosphate (K_2HPO_4)	0,2 g
Water (7.2)	1 000 ml

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

Prepare the solution on the day of preparation of the RVS medium.

B.2.2 Solution B

Magnesium chloride hexahydrate ($MgCl_2 \cdot 6H_2O$)	400 g
Water (7.2)	1 000 ml

Dissolve the magnesium chloride in the water.

In contrast to the usual preparation of reagent solutions, but in accordance with the original formula of References [5][6], add the substance to the volume of water indicated to a total volume of 1,26 l.

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. For instance, add 250 g of $MgCl_2 \cdot 6H_2O$ to 625 ml of water, to a total volume of 788 ml and a mass concentration of about 31,7 g of $MgCl_2 \cdot 6H_2O$ per 100 ml.

The solution is stable for at least 2 years, if stored in a tightly stoppered brown glass bottle at room temperature.

B.2.3 Solution C

Malachite green oxalate	0,4 g
Water (7.2)	100 ml

Dissolve the malachite green oxalate in the water.

The solution is stable for at least 8 months if stored in a brown glass bottle at room temperature.

B.2.4 Complete medium

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

Add 100 ml of solution B and 10 ml of solution C (total volume 1 110 ml) to 1 000 ml of basic medium (solution A).

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

Dispense into test tubes in 10 ml quantities or into flasks in 100 ml quantities.

Sterilize in the autoclave (5.2) at (115 ± 3) °C for 15 min.

Store the medium at (5 ± 3) °C for up to 7 d. The medium is stable for up to 4 months if stored in screw-capped tubes or bottles at (5 ± 3) °C.

NOTE The final medium composition (total volume 1 110 ml) is: 4,5 g/l of enzymatic digest of soya; 7,2 g/l of sodium chloride (NaCl); 1,44 g/l of potassium dihydrogenphosphate (KH_2PO_4) + dipotassium hydrogenphosphate (K_2HPO_4); 28,6 g/l of magnesium chloride hexahydrate ($MgCl_2 \cdot 6H_2O$); 0,036 g/l of malachite green oxalate.

A mass of 28,6 g $MgCl_2 \cdot 6H_2O$ corresponds to 13,4 g $MgCl_2$ (anhydrous).

B.3 Xylose lysine deoxycholate agar (XLD agar)

D(+)-Xylose	3,75 g
L(+)-Lysine hydrochloride	5,0 g
Sodium deoxycholate	1,0 g
Yeast extract	3,0 g
Sucrose	7,5 g
Lactose	7,5 g
Sodium chloride (NaCl)	5,0 g
Sodium thiosulfate ($Na_2S_2O_3$)	6,8 g
Iron(III) ammonium citrate	0,8 g
Phenol red	0,08 g
Agar	9 g to 18 g[a]
Water (7.2)	1 000 ml
[a] Dependent on the gel strength of the agar.	

Dissolve the components in the water and heat with frequent agitation. Do not overheat or boil.

Cool immediately in a water bath to 45 °C to 50 °C. Excessive heating or prolonged cooling may cause precipitation.

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

Pour into Petri dishes and store the cooled medium in sealed plastic bags for up to 2 weeks at (5 ± 3) °C. If necessary, dry the plates before use.

B.4 Nutrient agar

Meat extract	3,0 g
Enzymatic digest of peptone	5,0 g
Agar	9 g to 18 g[a]
Water (7.2)	1 000 ml
[a] Dependent on the gel strength of the agar.	

Dissolve the components in the water by boiling.

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

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

Sterilize in the autoclave (5.2) at (121 ± 3) °C for 15 min.

Transfer approximately 15 ml of the melted medium to sterile Petri dishes.

Store the poured plates for up to 2 months at (5 ± 3) °C. Protect them from drying.

Alternatively, other appropriate non-selective agar media may be used.

B.5 Triple sugar and iron agar (TSI agar)

Meat extract	3,0 g
Yeast extract	3,0 g
Enzymatic digest of 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($Na_2S_2O_3$)	0,3 g
Phenol red	0,024 g
Agar	9 g to 18 g[a]
Water (7.2)	1 000 ml
[a] Dependent on the gel strength of the agar.	

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

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

Dispense the medium in quantities of 10 ml into tubes, preferably with screw caps.

Sterilize in the autoclave (5.2) at (121 ± 3) °C for 15 min. Allow to set in a sloping position to give a butt of depth 25 mm to 50 mm.

Store the tubes for up to 1 month at (5 ± 3) °C.

B.6 Urea agar (Christensen)

B.6.1 Base

Enzymatic digest of peptone	1,0 g
Glucose	1,0 g
Sodium chloride (NaCl)	5,0 g
Potassium dihydrogenphosphate (KH_2PO_4)	2,0 g
Phenol red	0,012 g
Agar	9 g to 18 g[a]
Water (7.2)	1 000 ml
[a] Dependent on the gel strength of the agar.	

Dissolve the components or the dehydrated complete base in the water, if necessary, by heating.

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

Sterilize in the autoclave (5.2) at (121 ± 3) °C for 15 min. After autoclaving, cool it in a water bath to about 50 °C.

Store for up to 1 month at (5 ± 3) °C.

B.6.2 Urea solution

Urea	400 g
Water (7.2) to a final volume of	1 000 ml

Dissolve the urea in the water and sterilize the solution by filtration. Store for up to 1 month at (5 ± 3) °C.

B.6.3 Complete medium

Base (B.6.1)	950 ml
Urea solution (B.6.2)	50 ml

Add, under aseptic conditions, the urea solution to the base, previously melted and then cooled to (45 ± 1) °C.

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

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

Allow to set in a sloping position.

Store the tubes for up to 7 d at (5 ± 3) °C.

B.7 L-Lysine decarboxylation medium

L-Lysine monohydrochloride	5,0 g
Yeast extract	3,0 g
Glucose	1,0 g
Bromocresol purple	0,015 g
Water (7.2)	1 000 ml

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

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

Dispense the medium in quantities of 2 ml to 5 ml into sterile, narrow tubes, preferably with screw caps.

Sterilize in the autoclave (5.2) at (121 ± 3) °C for 15 min. Store the tubes for up to 3 months at (5 ± 3) °C.

B.8 Selenite cystine broth

Tryptone	5,0 g
Lactose	4,0 g
Disodium hydrogenphosphate (Na_2HPO_4)	10,0 g
L-Cystine	0,01 g
Sodium biselenite ($NaHSeO_3$)	4,0 g
Water (7.2)	1 000 ml

Dissolve 4 g of sodium biselenite in 1 000 ml of water and then add the remaining ingredients.

Warm to dissolve and dispense into containers to a depth of at least 60 mm.

Sterilize by placing in free-flowing steam for 15 min.

WARNING — Do not autoclave.

Store the prepared medium at (5 ± 3) °C away from light.

CAUTION — Reference [7] reports miscarriages and possible teratogenic effects on pregnant laboratory workers which may have been caused by the ingestion of sodium biselenite. To minimize any possible risk of teratogenicity to laboratory workers, the sodium biselenite is not added as a dry powder but should be prepared separately as a solution to which the remaining ingredients are added.

B.9 Muller-Kauffmann tetrathionate-novobiocin broth (MKTTn)

B.9.1 Base medium

Enzymatic digest of 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 (7.2)	1 000 ml

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

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

Thoroughly mix the medium and aseptically dispense into sterile tubes in 10 ml volumes.

The base medium may be stored for up to 4 weeks at (5 ± 3) °C.

B.9.2 Iodine and iodide solution

Iodine (I_2)	20,0 g
Potassium iodide (KI)	25,0 g
Water (7.2)	100 ml

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

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

B.9.3 Novobiocin solution

Novobiocin sodium salt	0,04 g
Water (7.2)	5 ml

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

Store for up to 4 weeks at (3 ± 2) °C.

B.9.4 Complete medium

Base medium (B.9.1)	1 000 ml
Iodine-iodide solution (B.9.2)	20 ml
Novobiocin solution (B.9.3)	5 ml

Aseptically add 5 ml of the novobiocin solution to 1 000 ml of base medium. Mix, then add 20 ml of the iodine and iodide solution. Mix well.

Dispense the medium aseptically in quantities of 10 ml into sterile containers.

Use the complete medium on the day of preparation.

B.10 Filter aid

Diatomaceous earth	1 g
Water (7.2)	15 ml

Weigh out appropriate amounts of diatomaceous earth into suitable bottles and add water.

Sterilize in the autoclave (5.2) at (121 ± 3) °C for 15 min.

Store in the dark at room temperature and use within 12 months.

Annex C
(informative)

Results of the interlaboratory trial

C.1 Introduction

An international interlaboratory trial was organized with 26 laboratories from seven countries participating in the study. Lenticule[1)] discs containing different combinations of *Salmonella* Gold Coast and background organisms (*Escherichia coli*, *Klebsiella aerogenes*, *Enterococcus faecalis*, *Citrobacter* spp. and *Acinetobacter baumanii*) were prepared by the Health Protection Agency, Water External Quality Control Laboratory, Newcastle-upon-Tyne, UK (see Table C.1), together with a negative control (blank), were sent to the participating laboratories and were tested at two different concentration levels.

Laboratories were sent two sets of five different Lenticule[1)] discs coded A to E and requested to test one of each set on separate days to obtain data on reproducibility within laboratories. The Lenticule[1)] discs were dissolved in 1 000 ml of water and five 100 ml test samples were then evaluated for the presence of *Salmonella*. The test samples in which *Salmonella* were detected were then further diluted (1→5) and test portions examined.

The method specifies the use of two selective differential media to include XLD and one other suitable medium. The participants were asked to record details of medium, manufacturer, batch codes and expiry dates of media used.

Results were recorded as “detected” or “not detected” and the completed data sheets were returned to the organizing laboratory.

C.2 Distribution of cells in samples and subsamples

The Lenticule[1)] discs were designed to contain approximately 100 colony forming units (cfu) when reconstituted in 1 000 ml of water to give approximately 10 cfu per 100 ml as the test sample. When diluted (1→5), these should give approximately 2 cfu per 100 ml. If the cells in the sample followed a Poisson distribution, the likelihood that a 100 ml sample did not contain any cells was very small (1/220 26) and the probability of a sample containing at least one cell was very high ($p = 0{,}999\ 95$). With this probability of a positive result, in each of the five replicates, the mean number of positive results would be 4,999 77 with a standard deviation, s, of 0,015 066. A 100 % detection rate would yield five out of five positive results from each laboratory.

In the case of the diluted subsamples, the probability that a sample contained at least one cell was approximately 86 % ($p = 0{,}864\ 66$). Each of the 10 replicate tests would have this probability of success. If it is assumed that the number of true positives followed a binomial distribution with parameters $n = 10$ and $p = 0{,}865$, then the mean number of true positives over 10 tests would be 8,646 65 with $s = 1{,}081\ 76$. However, random tests of the Lenticule[1)] discs indicated that the true cell counts were between 130 cfu/disc to 200 cfu/disc. Therefore, Poisson means were considered to be between 10 cfu/100 ml to 20 cfu/100 ml in the undiluted samples, and of 2 cfu/100 ml and 4 cfu/100 ml for the diluted subsamples when analysing the data using logistic regression [SAS 8.2[1)]].

Results were recorded as “detected” or “not detected” and the completed data sheets were returned to the organizing laboratory.

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1) Product available commercially. This information is given for the convenience of users of this document and does not constitute an endorsement by ISO of the product named.

Table C.1 — Composition of samples

Samples [Lenticule[1)] disc set]	Organisms	Target cfu/disc
A	*E. coli*	100
	K. aerogenes	1 000
	E. faecalis	1 000
	Citrobacter spp.	1 000
B	*Salmonella* Gold Coast	10
C	*Salmonella* Gold Coast	10
	E. coli	100
	K. aerogenes	1 000
	E. faecalis	1 000
D	Blank	
E	*Salmonella* Gold Coast	10
	E. coli	100
	K. aerogenes	1 000
	E. faecalis	1 000
	A. baumanii	1 000

C.2.1 Results

Results were received from 26 participating laboratories and these are summarized in Tables C.2 and C.3.

Table C.2 — Summary of results for undiluted samples

Parameter	Blank	Background organisms only	*Salmonella* Gold Coast only	*Salmonella* Gold Coast plus background organisms
No. laboratories returning results	26	26	26	26
Total No. results	245	252	246	611
No. results excluded	5	0	0	15
No. accepted results	240	252	246	596

Table C.3 — Summary of results for diluted samples

Parameter	*Salmonella* Gold Coast only	*Salmonella* Gold Coast plus background organisms
No. laboratories returning results	26	26
Total No. results	485	935
No. results excluded	0	30
No. accepted results	485	905

Logistic regression [SAS 8.2[1)]] showed no significant difference between the samples containing *Salmonella* ($p = 0{,}98$) or between days ($p = 0{,}83$) for diluted samples. There was little variation in the results from undiluted samples ($p = 0{,}98$) and there was no difference between test samples B, C, and E or between A and D. Therefore, the data were summarized over laboratories, test samples, and days on which samples were tested. All data returned by one laboratory (laboratory 14) were excluded, as there was clear evidence of technical problems in that laboratory. Data were also excluded from the statistical analysis where there was a clear case of mislabelling or transcription errors. See Table C.4.

Table C.4 — Summary of results for samples and diluted subsamples with number and percentage of positives

Sample	Organisms	No. positive /No. results	Positive %
A1	*E. coli, K. aerogenes*	0/130	0
A2	*E. faecalis, Citrobacter* spp.	0/130	0
B1	*Salmonella* Gold Coast	130/130	100
B1 diluted 1→5		236/250	94,4
B2		126/126	100
B2 diluted 1→5		252/255	98,9
C1	*Salmonella* Gold Coast, *E. coli*	129/130	99,2
C1 diluted 1→5		243/250	97,2
C2	*K. aerogenes, E. faecalis*	126/126	100
C2 diluted 1→5		236/240	98,3
D1	Blank	0/130	0
D2		0/125	0
E1P	*Salmonella* Gold Coast, *E. coli*	128/130	98,5
E1P diluted 1→5		236/240	98,3
E2P	*K. aerogenes, E. faecalis* *A. baumanii*	120/121	99,2
E2P diluted 1→5		239/245	97,6

The method specifies the use of two selective solid media to include XLD agar and one other suitable medium, to be decided by the laboratory. The different secondary media used are summarized in Table C.5.

Table C.5 — Secondary media

Medium	No. laboratories
Brilliant green agar (BGA)	8
AES *Salmonella* Plate (ASAP)	2
Rambach agar	10
Brilliant green phenol red lactose sucrose agar (BPLS)	2
Salmonella detection and identification medium (SMID)	2
Önöz medium	2

C.2.2 Undiluted samples A1 to E1

On each Lenticule[1)] disc, 130 replicate tests were performed. The total number of valid results was 763 of which 759 were positive (Table C.3). The expected number was 762,965 with a standard deviation of 0,186. This suggests that either the detection rate was less than 100 % or the binomial distribution was overdispersed. As it appears that the number of cells was >10 per disc, overdispersion is the most likely explanation of the result, which is not unexpected in microbiological examinations.

C.2.3 Diluted samples (B1P, C1P, and E1P) all at 1→5 dilution

Of 1 480 valid tests performed, 1 442 were positive. Assuming a mean count of 2 cfu and a 100 % detection rate, the expected number of positives would be 1 279,6 ($s = 5{,}52$). The 99 % tolerance interval ranges from 1 254 to 1 304 positive results. The results indicate that the mean number of cfu per subsample was >2. The high number of positive results indicates that the binominal distribution was underdispersed, but it is more likely that the mean cell count was higher than anticipated.

C.2.4 Sensitivity and specificity

For the undiluted test samples, the 99 % tolerance interval is limited to $k = 763$. Therefore, the total number of true positives from the undiluted samples was 763 and the number of false negatives was four. From the diluted test portions, the total number of positives obtained and the respective laboratories' "gold standard" were counted as true positives. Therefore, there were 1,442 true positives and three false negatives. The number of true negatives was 130 and no false positives were reported. Therefore the sensitivity of the method for neat samples was 0,995 and for the diluted subsamples was 0,998; the specificity was therefore close to 100 %.

C.2.5 Conclusions

The results from the 26 participating laboratories show remarkable uniformity, with the exception of mislabelling in one laboratory and operational problems in a second. No differences were observed between the different media used (Table C.5) or between samples tested on different days. The lack of false positive results shows that the method is very specific and it appears to be sufficiently sensitive to detect very low numbers of bacteria even in the presence of high levels of background organisms. This method has been successfully used to detect *Salmonella* from sewage-contaminated river water samples over a period of months with a wide range of salmonellae detected (Table C.6).

Table C.6 — *Salmonella* strains from naturally contaminated river water samples isolated by the organizing laboratory using this method (2005)

***Salmonella* strain**	**No. isolates**
Salmonella Typhimurium	3
Salmonella Enteritidis	2
Salmonella Kentucky	1
Salmonella Kottbus	4
Salmonella Newport	4
Salmonella Chester	3
Salmonella Fresno	1
Salmonella Colindale	1
Salmonella Agona	4
Salmonella Virchow	3
Salmonella Falkensee	2
Salmonella Virginia	3
Salmonella Oakland	2
Salmonella Adane	1
Salmonella Muenchen	1
Salmonella Weltevreden	1
Salmonella Derby	1
Salmonella Bareilly	1
Salmonella Mbandka	2
Salmonella Saint-Paul	1
Salmonella Thompson	1
Salmonella Stanley	1
Salmonella Oranienburg	1
Salmonella Braenderup	1
Salmonella Unnamed	6

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