



Public Health
England

Detection of *Salmonella* species

Microbiology Services
Food Water and Environmental
Microbiology
Standard Method

FNES16 [F13]

About Public Health England

Public Health England's mission is to protect and improve the nation's health and to address inequalities through working with national and local government, the NHS, industry and the voluntary and community sector. PHE is an operationally autonomous executive agency of the Department of Health.

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Version number 2 Effective Date 03.03.2014
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Published June 2014
PHE publications gateway number: 2014162

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Status of Microbiology Services Food, Water and Environmental Microbiology Methods

These methods are well referenced and represent a good minimum standard for food, water and environmental microbiology. However, in using Standard Methods, laboratories should take account of local requirements and it may be necessary to undertake additional investigations.

The performance of a standard method depends on the quality of reagents, equipment, commercial and in-house test procedures. Laboratories should ensure that these have been validated and shown to be fit for purpose. Internal and external quality assurance procedures should also be in place.

Whereas every care has been taken in the preparation of this publication, Public Health England (PHE) cannot be responsible for the accuracy of any statement or representation made or the consequences arising from the use of or alteration to any information contained in it. These procedures are intended solely as a general resource for practising professionals in the field, operating in the UK, and specialist advice should be obtained where necessary. If you make any changes to this publication, it must be made clear where changes have been made to the original document. PHE should at all times be acknowledged.

Citation for this document:

Public Health England (2014). Detection of *Salmonella* species. Microbiology Services. Food, Water & Environmental Microbiology Standard Method FNES16 (F13); version 2.

Amendment history

Controlled document reference	FNES16 (F13)
Controlled document title	Standard Method for Detection of <i>Salmonella</i> Species

The amendment history is shown below. On issue of revised or new documents each controlled document should be updated by the copyholder in the laboratory.

Amendment Number/ Date	Version no. Discarded	Insert Version no.	Page	Section(s) involved	Amendment
2/22.01.14	HPA F13 1.0	PHE FNES16 2	All	All	Re-formatted following move to PHE and document renumbered as FNES16 following the implementation of Q-pulse for Document Management.
			5	Background	ISO reference updated and purpose of A1:2007 described. Update of table with justification for not using A1:2007 as part of the method. Table of differences updated to reflect confirmation of up to 20 colonies for outbreak and formal samples.
			7	3.1 General Safety Considerations	Information note added
			13	6.6 Confirmations	Clarification of the number of colonies confirmed and biochemical testing requirements. Inclusion of PCR as an option for confirmation of suspect isolates.
			15	6.7 Public Health Investigations	Addition of requirement to confirm up to 20 colonies and addition of PCR testing.
			16	8.0 Calculation of results	Update to include reference to Starlims added
			17	9.0 Reporting of results	Updated to include reference to Starlims. Reporting per sample changed to per item to reflect options in Starlims
			18	References	Updated
			23	Table 1	Updated to include <i>S. Paratyphi</i> A, B and C

Introduction

Scope

The method described is applicable to the detection of *Salmonella* species in all food types, including milk and dairy products, raw molluscan shellfish, raw shell eggs and to its detection in environmental samples such as swabs and cloths.

Background

Salmonella are a major cause of illness worldwide. The principle sources of these micro-organisms include poultry, eggs, raw meat and raw milk. Cross contamination of ready-to-eat (RTE) foods that are not subsequently cooked or are undercooked can lead to incidents of food poisoning and contamination of RTE foods from the environment or from an infected food handler can occur.

The presence of *Salmonella* species in ready-to-eat food is considered to be unsatisfactory and potentially injurious to health regardless of the level of contamination^{1,2,3}. Isolation of *Salmonella* is therefore carried out by enrichment culture of a defined weight or volume of food, which is normally 25g. For environmental samples such as swabs and dish cloths, the entire sample is usually examined. A pre-enrichment resuscitation stage is incorporated, to allow the recovery of injured cells. The detection of *Salmonella* in food samples can be achieved by a variety of methods, and the method used can vary in success depending on the type of food being examined.

The method described here is based on BS EN ISO 6579:2002 + A1:2007 (Annex D). This uses Rappaport Vassiliadis Soya Peptone Broth (RVS), which is highly effective for recovery of *Salmonella* from foods with a high level of background contamination and Muller Kauffmann Tetrathionate Novobiocin (MKTTn) broth for the isolation of serotypes of *Salmonella* that are inhibited by the constituents of RVS broth. Extensive trials have been performed to demonstrate the efficacy of these media for the recovery of *S. Typhi* and *S. Paratyphi*, and it is recognised that the combination of these two selective media may not recover all strains. This method also includes the use of Selenite Cystine (SC) broth for samples in which *S. Typhi* and *S. Paratyphi* are specifically sought. The amendment (Annex D) to this standard also describes the use of a modified semi-solid Rappaport medium for use with food and feed likely to have high background flora eg raw meats. This this not used in PHE FW&E Laboratories.

Information Note: *S. Typhi* and *S. Paratyphi* are Hazard Group 3 pathogens. Only laboratories with appropriate expertise, risk assessments, safety procedures and containment facilities should examine samples for these organisms.

Two isolation media are specified; these are Xylose Lysine Deoxycholate (XLD) agar and brilliant green agar (BGA). If *S. Typhi* or *S. Paratyphi* are being sought Hynes, Deoxycholate Citrate Agar (DCA) should also be used. This media does not contain a high concentration of brilliant green, which is thought to inhibit the recovery of some strains.

The table below gives details of the difference between this method and BS EN ISO 6579. All differences are considered to be minor.

	PHE method F13	BS EN ISO 6579:2002 +A1:2007	Justification for variation
Scope	Includes instructions for the preparation of specific food types including molluscan shellfish and raw eggs and to detection of <i>Salmonella</i> from swabs and other environmental samples	Detection of <i>Salmonella</i> in foods and animal feeding stuffs.	Detection of <i>Salmonella</i> in raw molluscan shellfish using ISO 6579:2002 is recommended in EC 2073/2005 (as amended) ^{2,3} . Detection of <i>Salmonella</i> species is considered to be an important tool in the investigation of the food processing environment.
Media and Reagents	Modified semi-solid Rappaport Medium (MSRV) not used	MSRV recommended as an alternative to RVS for products with high background flora	Participation in ISO ring trials have shown comparable results using both RV and MSRV.
Sample processing	Method for isolation of <i>S.Typhi</i> and <i>S.Paratyphi</i> included	<i>S. Typhi</i> and <i>S.Paratyphi</i> not included	Isolation of these organisms is important in the event of public health investigation of cases where there is no history or recent foreign travel.
Sample processing	Information on testing during public health investigations included	No reference to public health investigation	Included to ensure prompt confirmation of presumptive isolates in the event of a public health incident.
Enrichment Culture	Pre-enrichment period extended to 24 h for dehydrated foods	Recommendation to extend the Pre-enrichment period is not included.	Extending the incubation period for these food types maximises recovery of stressed organisms.
Colony confirmation	Recommends the confirmation of 5 colonies for routine samples and up to 20 for some samples.	Recommends further investigation of up to 20 colonies	Further colonies are confirmed if samples have been submitted in connection with and outbreak or are from formal samples only. Not considered to be cost-effective.
Quality control	Control strain <i>Salmonella</i> Nottingham	Control strain <i>Salmonella</i> Poona	Use of alternative unusual control strain to maximise detection of possible cross contamination events within the laboratory.

1.0 Principle

The detection of *Salmonella* species in food and environmental samples from the food production environment involves pre-enrichment in a non-selective liquid medium with adjustments as necessary to enhance recovery from certain food types, enrichment in two selective liquid media, sub-culture onto each of two different selective solid media, and examination for colonies considered to be typical of *Salmonella*.

Confirmation of the colonies as *Salmonella* is performed using serological and biochemical tests. When investigation of samples for the presence of *S. Typhi* and *S. Paratyphi* is indicated and additional selective liquid media and selective solid media are also used.

2.0 Definitions

For the purposes of this method the following definitions apply:

Salmonella species

Micro-organisms which form typical or less typical colonies on solid selective agar media and which display the biochemical and serological characteristics described in this method.

Detection of Salmonella species

Determination of the presence or absence of these micro-organisms in a defined weight or volume of food or in a swab, cloth or other environmental sample.

3.0 Safety considerations

3.1 General safety considerations

Normal microbiology laboratory precautions apply^{5,6}.

All laboratory activities associated with this SOP must be risk assessed to identify hazards^{7,8}. Appropriate controls must be in place to reduce the risk to staff or other groups. Staff must be trained to performed the activities described and must be provided with any personal protective equipment (PPE) specified in this method. Review of this method must also include a review of the associated risk assessment to ensure that controls are still appropriate and effective. Risk assessments are site specific and are managed within safety organiser.

Information Note: Throughout this method hazards are identified using **red text**. Where a means of controlling a hazard has been identified this is shown in **green text**.

3.2 Specific safety considerations

Salmonellas are pathogenic to man and therefore isolation and identification must be performed by **trained laboratory personnel** in a **properly equipped laboratory** and under the supervision of a qualified microbiologist. **Care must be taken in the disposal and sterilisation of all test materials.** Procedures involving **sub-culturing from pre-enrichment** and **enrichment broths** and **handling of *Salmonella* cultures** during identification procedures must be performed in a **designated area of the laboratory**. **Selenium salts** are used in the preparation of **SC broth**. They are toxic if ingested or inhaled and there is a possible risk of teratogenicity. **Protective Gloves must be worn when handling this medium and it should not be handled by pregnant laboratory workers.**

3.3 Laboratory containment

Most activities can be carried out in a Containment Level 2 (CL2) laboratory. Only laboratories with Containment Level 3 (CL3) facilities should examine samples for **S. Typhi** and **S. Paratyphi**. **All procedures including sample preparation, sub-culture and identification must be performed in a CL3 laboratory** by staff trained in the appropriate CL3 procedures. **Disposable gloves must be worn during all procedures.** Requests for the examination of **S. Typhi** and **S. Paratyphi** will occur rarely. In such cases it is accepted that an ongoing quality control programme is not necessary and that appropriate IQC will be performed after the sample has been tested using the same media and equipment but ensuring complete segregation from the test sample⁹.

4.0 Equipment

Usual laboratory equipment and in addition:

- Top pan balance capable of weighing to 0.1g
- Gravimetric diluter (optional)
- Stomacher
- pH meter (optional)
- pH indicator strips (range .5 – 7.5) and sterile inoculation sticks (optional)
- Vortex mixer
- Incubator: 37 ± 1°C and 41.5 ± 1°C
- Water bath: 37 ± 1°C
- Stomacher bags with closures (sterile)
- Automatic pipettors and associated sterile pipette tips capable of delivering up to 1 mL and 0.1 mL volumes (optional)
- Pipettes (sterile total delivery) 1 mL graduated in 0.1 mL volumes (optional)

Information Note: Due to the risks of cross-contamination single use equipment should be used for all subculture procedures. If disposable pipette tips are used these should contain a filter to prevent contamination of the pipettor, and the pipettor regularly decontaminated.

5.0 Culture media and reagents

Equivalent commercial dehydrated media may be used; follow the manufacturer's instructions.

Buffered peptone water (ISO formulation)

Enzymatic digest of casein	10.0 g
Sodium chloride	5.0 g
Disodium hydrogen phosphate dodecahydrate or anhydrous disodium hydrogen phosphate	9.0 g 3.5 g
Potassium dihydrogen phosphate	1.5 g
Water	1 L

pH 7.0 ± 0.2 at 25°C

Rappaport Vassiliadis Soya Peptone Broth (RVS)

Soya peptone	4.5 g
Sodium chloride	7.2 g
Potassium dihydrogen phosphate	1.26 g
Dipotassium hydrogen phosphate	0.18 g
Magnesium chloride (anhydrous)	13.58 g
Malachite green	36 mg
Water	1 L

pH 5.2 ± 0.2 at 25°C

Muller-Kauffmann Tetrathionate Novobiocin (MKTn)broth

Meat extract	4.3 g
Enzymatic digest of casein	8.6 g
Sodium chloride	2.6 g
Calcium carbonate	38.7 g
Sodium thiosulphate pentahydrate	47.8 g
Ox bile	4.78 g
Brilliant green	9.6 mg
Iodine	4.0 g
Potassium iodide	5.0 g
Novobiocin (sodium salt)	0.04 g

Detection of *Salmonella* species

Water 1 L

pH (of basal broth) 8.2 ± 0.2 at 25°C

Selenite Cystine (SC) broth

Tryptone	5.0 g
Lactose	4.0 g
Disodium phosphate	10.0 g
L-Cystine	10 mg
Sodium biselenite	4.0 g
Water	1 L

pH 7.0 ± 0.2 at 25°C

Xylose lysine Desoxycholate (XLD) Agar

Yeast extract	3.0 g
L-lysine hydrochloride	5.0 g
Xylose	3.75 g
Lactose	7.5 g
Sucrose	7.5 g
Sodium desoxycholate	1.0 g
Sodium chloride	5.0 g
Sodium thiosulphate	6.8 g
Ferric ammonium citrate	0.8 g
Phenol red	80 mg
Agar	12.5 g
Water	1 L

pH 7.4 ± 0.2 at 25°C

Brilliant Green Agar (modified) (BGA)

Meat Extract	5.0 g
Peptone	10.0 g
Yeast extract	3.0 g
Disodium hydrogen phosphate	1.0 g
Sodium dihydrogen phosphate	0.6 g
Lactose	10.0 g
Sucrose	10.0 g
Phenol red	0.09 g
Brilliant green	4.7 mg
Agar	12.0 g
Water	1 L

pH 6.9 ± 0.2 at 25°C

Hynes Deoxycholate Citrate Agar (DCA)

Peptone	17.0 g
Lactose	10.0 g
Sodium citrate	5.0 g
Sodium thiosulphate	2.5 g
Bile Salts	2.0 g
Ferric ammonium citrate	1.0 g
Neutral red	0.025 g
Agar	14.0 g
Water	1 L

pH 7.2 ± 0.2 at 25°C

MacConkey Agar (MA)

Bile salts	5.0 g
Enzymatic digest of casein	20.0 g
Lactose	10.0 g
Sodium chloride	5.0 g
Neutral red	75 mg
Agar	12.0 g
Water	1 L

pH 7.4 ± 0.2 at 25°C

Nutrient Agar (NA)

Meat extract	1.0 g
Enzymatic digest of animal tissues	5.0 g
Agar	12.0 g
Water	1 L

pH 7.0 ± 0.2 at 25°C

Physiological Saline solution

Sodium chloride	8.5 g
Water	1 L

pH 7.0 ± 0.2 at 25°C

Slopes of nutrient agar

Columbia agar base with 5% horse blood.

Information Note Addition of the substances described below to BPW may be required when testing some foods as described in the Appendix.

Surfactant eg; Tergitol 7, Sorbitan monooleate (Tween 80); Triton 100 (as required)
10% Potassium sulphite solution; add 12.5 mL to 25 g sample homogenate (as required)
Skimmed milk powder; antibiotic free, 100 g per L of BPW (as required)
Casein (not acid casein); final concentration 5% (as required)
Double strength BPW (as required)
1 M Sodium hydroxide solution
1 M Hydrochloric acid solution

Serological reagents for identification of *Salmonella* species to group level (PSO, O4, O6,7, O8, O9, O3,10, O16, Vi, PSH)

Biochemical gallery eg BioMerieux API 20E test kit

PCR testing reagents
Reagents as specified in M2¹⁵ and M3¹⁴ are used

6.0 Sample processing

This method is capable of isolation of very low numbers of *Salmonella* from foods and environmental samples. Procedures must be in place to avoid cross contamination of samples and guidance on how to prevent cross contamination is available¹⁰.

Typically 25 g or sample is examined however an increased amount of sample (eg 100 g) can be examined during investigations of food where the amount of *Salmonella* present is likely to be low or during outbreak investigations.

6.1 Sample preparation and pre-enrichment

Food samples

Prepare the sample using the procedure described in National Standard Method F 2 - Preparation of samples and dilutions¹¹. Using sterile instruments and aseptic technique, weigh a representative 25 g sample of each food into a sterile stomacher bag with wire closures¹². Add nine times that weight or volume of buffered peptone water (BPW). Avoid touching the inside of the bag with the hands.

If the amount of food or dairy product available is less than 25 g or 25 mL maintain the sample : diluent volume at 1:9 (1 in 10). Examination of smaller amounts is also appropriate for low density products such as herbs. Preparation of a 1 in 20 homogenate may be necessary for certain dehydrated products such as dried herbs and vegetables. Ensure that free liquid is present after rehydration. With some food products the pre-enrichment broth requires additions or adjustments as shown in the appendix. For highly acidic or alkaline samples the pH of the sample suspension must be checked and adjusted if necessary to 6.5-7.0 before incubation; the pH of the pre-enrichment broth should not drop below 4.5 during incubation. The use of double strength BPW may assist in preventing this. For very hard products such as dog chews made of hide which cannot be subdivided add sufficient BPW to cover the sample completely.

Information Note; Modification of the sample preparation procedure is required for some food types ie addition or adjustment to the pre-enrichment broth. Details of the adjustments required for particular foods are described in the Appendix.

Homogenise for between 30 seconds and 3 minutes in a stomacher. The homogenisation time required will depend on the manufacturer instructions and the type of sample being examined.

Raw Molluscan Shellfish

For molluscan shellfish follow the sample preparation procedures described in CEFAS Standard Method Enumeration of *Escherichia coli* in bivalve molluscan shellfish by the most probable number (MPN) technique (based on ISO 16649-3)¹³ to obtain a 1 in 3 slurry of shellfish flesh. This 1 in 3 slurry is used for *Salmonella* testing by adding 75 g of slurry to 175 mL of BPW so as to obtain a 10^{-1} dilution of a representative shellfish sample.

Raw Shell Eggs

Raw shell eggs are usually examined in batches (6-10 eggs recommended) including shells without shell disinfection. Whole eggs can be placed in a tared stomacher bag and the eggs broken while in the bag. This minimised the risk of cross contamination. It is advisable to use double bags to prevent leakage due to puncture by the shells. Add an equal weight of BPW. Egg contents and shells can be tested separately if required (with or without prior shell disinfection). Break the contents of the eggs into a tared stomacher bags and transfer the shells to a separate container or bag with closure. Add an equal volume of BPW to the contents and sufficient BPW to cover the shells to give an approximate 10^{-1} dilution. Mix carefully by hand for a minimum of 2 minutes.

Whole raw chicken

Whole raw chickens can be examined for *Salmonella* species using a combined neck-skin and rinse sample. Using aseptic technique, remove 25 g of neck skin from the chicken and transfer to a tared stomacher bag. Place the whole carcass into a large bag and weigh. Add half this weight of BPW to the bag and use this BPW to wash the surface of the chicken for at least 1 min ensuring the internal and external surfaces are rinsed. Transfer 25 mL of the rinse fluid to the stomacher bag containing the neck skin and add 200 mL of BPW.

Homogenise for between 30 seconds and 3 minutes in a stomacher. The homogenisation time required will depend on the manufacturer instructions and the type of sample being examined.

Swabs and other environmental samples

For swabs and environmental samples including cloths refer to HPA MS Standard Method E1-Detection and Enumeration of Bacteria in Swabs and Other Environmental Samples¹⁴. Ensure ample coverage with BPW even after absorption such that an approximate 10^{-1} dilution is achieved

Place all enrichment cultures in an incubator at $37 \pm 1^\circ\text{C}$ for 18 ± 2 h. For dehydrated foods the incubation period must be extended to 24 ± 2 h.

6.2 Selective enrichment

After incubation pre-enrichment broths must be sub-cultured to selective enrichment broths. This procedure must be carried out so as to avoid cross contamination between samples and should be carried out in a designated laboratory area. Sub-culture 0.1 mL of the pre-enrichment culture to 10 mL of RVS. Place in an incubator at $41.5 \pm 1^\circ\text{C}$ for 24 ± 3 h. For shell eggs re-incubate the RVS broth for a further 24 h. Care should be taken that the maximum incubation temperature of 42.5°C is not exceeded.

Also transfer 1 mL of the pre-enrichment culture to 10 mL of MKTTn. Place in an incubator at $37 \pm 1^\circ\text{C}$ for 24 ± 3 h. For shell eggs re-incubate the MKTTn for a further 24 h.

6.3 Subculture to selective media

Following incubation sub-culture the selective enrichment cultures to XLD and to BGA using a 10 μL loop and using a separate sterile stick or loop streak out to obtain discrete colonies.

Place in an incubator at $37 \pm 1^\circ\text{C}$ for 24 ± 3 h. Re-incubate negative plates from raw shell eggs and samples being tested for public health investigations for a further 24 ± 3 h.

6.4 Procedure for *S. Typhi* and *S Paratyphi*

In addition to the procedure described in 6.2 above, subculture 1 mL of the incubated BPW to 10 mL of selenite cystine broth (SC).

Place in an incubator set at $37 \pm 1^\circ\text{C}$ for 48 ± 3 h. After 24 ± 3 and 48 ± 3 h subculture the SC to XLD, BGA and Hynes DCA.

Place all plates in an incubator at $37 \pm 1^\circ\text{C}$ for 24 ± 3 h.

6.5 Recognition of colonies

After 24 ± 3 h examine the selective agar plates for typical and atypical colonies of *Salmonella*.

XLD

Salmonella ferment xylose, normally decarboxylate lysine, and produce hydrogen sulphide. Characteristic colonies are red with black centres. Isolated colonies may appear yellow with black centres. *Salmonella* species that produce little or no hydrogen sulphide eg: *S. Typhi*, *S. Senftenberg*, *S. Pullorum* grow as red colonies with or without black centres. Red colonies may also be produced by some strains of *Proteus* species and *Pseudomonas* species. Strains of *S. Paratyphi* A do not decarboxylate lysine and so appear as yellow colonies usually with a black centre. Lactose fermenting strains may also appear yellow with or without black centres.

BGA

Salmonella species do not normally ferment sucrose or lactose and produce red colonies surrounded by a bright red medium. Red colonies may also be produced by some strains of *Proteus* species and *Pseudomonas* species.

Hynes DCA

S. Typhi and *S. Paratyphi* produce colourless colonies with or without a black centre.

Information Note: All strains of *S. Typhi* and *S. Paratyphi* other than *S. Paratyphi A* are lysine positive. Production of black colonies due to hydrogen sulphide on XLD and Hynes DCA is variable. *S. Typhi* may not grow on XLD. Strains of *S. Typhi* and *S. Paratyphi* may not grow on BGA.

6.6 Confirmatory tests

Typical colonies (see above) from each plate must be subjected to serological and biochemical confirmation or colony confirmation by PCR. The extent of testing may be reduced once the presence of *Salmonella* is established (see below).

For routine samples and using a 1 µL disposable plastic loop, pick a total of 5 suspect *Salmonella* colonies from the selective agar plate/broth combinations (MKTTn/ BGA, MKTTn/ XLD, RVS/BGA or RVS/XLD) and sub-culture onto MacConkey (MA) and nutrient agar (NA) plates. In the case of formal and public health samples, if confirmation tests from the initial 5 picks are negative pick further colonies up to a total of 20 per sample.

If confirming using PCR the loop is then carefully emulsified in 0.5 mL of PCR grade water. All picks (up to 20 colonies) from a single sample should be emulsified in the same tube.

Place NA and MA plates in an incubator at $37 \pm 1^\circ\text{C}$ for 21 ± 3 h.

Colony confirmation using PCR (optional)

Following method M3¹⁴ heat treat the PCR grade water with emulsified colonies at 95°C for 15 minutes allow to cool and add 30 µL of heat-treated bacterial suspension to lyophilised real time PCR assay tubes as described in Standard Method M2¹⁵. The positive control described in Standard Method M4¹⁶ should be included in each real time PCR assays.

Serological confirmation

Identification of specific lipopolysaccharide ('O') and flagella ('H') antigens on the surface of presumptive *Salmonella* isolates using specific antisera is carried out prior to biochemical identification. Live cultures of *Salmonella* are used and care must be taken to avoid infection or cross contamination. Serology must be performed in a designated area of the laboratory. Table 1 and the Appendix provide guidance on serological testing.

Carry out an oxidase test from NA plates on non-lactose fermenting colonies. On MA non-lactose fermenting colonies appear as colourless colonies. *Salmonella* species are oxidase negative. Sub-culture non-lactose fermenting, oxidase negative colonies from NA to slopes of NA. Ensure that some water of condensation is present at the base of the slope; if none is present add a few drops of sterile water to the container prior to inoculation. Inoculate the

colony into the water of condensation and streak up the slope. Incubate at $37 \pm 1^\circ\text{C}$ for 5 ± 1 h, or overnight if insufficient growth is evident.

Auto-agglutination

Wearing gloves and using the growth from the slope and condensate of the NA slope, prepare a saline suspension on a slide using a loop full of saline and a loop full of growth. Rock the slide gently for 30-60 seconds.

If auto-agglutination occurs with any non- lactose fermenting colony type proceed to biochemical confirmation. If auto-agglutination does not occur proceed with further serological testing.

Polyvalent 'O' and 'H' Antigens

Add a loop full of saline, a loop full of polyvalent 'O' and a loop full of polyvalent 'H' antisera to a slide. Using the growth from the slope and the condensate prepare a saline suspension adjacent to loop full of saline. Using growth from the slope prepare a saline suspension adjacent to the 'O' antisera and using growth from the condensate prepare a saline suspension adjacent to the 'H' antisera. Using separate sterile loops mix the saline suspension with the antisera and rock the slide gently for 30-60 seconds.

Grouping

If good agglutination occurs in the specified time with the polyvalent antisera in the absence of auto-agglutination carry out further tests to identify the *Salmonella* to a least group level using specific 'O' antisera as shown in the Table 1.

The first isolate must be serologically typed as comprehensively as possible, determining the 'O' group. All subsequent isolates must be serotyped to determine if they are the same 'O' Group. If the slide agglutination reactions indicate that subsequent isolates differ serologically from the first isolate, then further serological testing must be performed as this may indicate the presence of a mixed population of *Salmonella* serotypes in the sample. Some *Salmonella* species may give agglutination with polyvalent 'H' antisera but not with polyvalent 'O' antisera. This could be due to the presence of antigens not included in the polyvalent 'O' antiserum or to the masking of 'O' antigens by capsular antigens. Such isolates must either be tested with individual 'O' antisera or, Vi antiserum if *S. Typhi* is suspected.

Information note; If isolation of *S. Typhi* or *S. Paratyphi* is suspected based on the serological reactions all cultures and the sample must be transferred immediately to CL3 and all further work carried out in an MSC.

Biochemical confirmation

Biochemical confirmation using API 20 E must be carried out on suspect colonies that auto-agglutinate or give agglutination with any of the polyvalent 'O' or 'H' antisera. If all colonies have the same morphology and exhibit the same serological reactions perform API on one pick only. Where the colony morphology and/ or serology is different perform colony confirmation using PCR or an API on each colony type. In addition PCR or biochemical confirmation may be performed where colonies that are very typical of *Salmonella* fail to cause agglutination with any antisera. Only biochemical results from cultures shown to be pure can be reported and further testing may be necessary if purity is not achieved.

API 20E biochemical kit

Disposable gloves should be worn while carrying out this procedure. Follow the manufacturer's instructions.

After inoculation a purity check must be performed by plating out for single colonies on CBA.

Incubate at $37 \pm 1^\circ\text{C}$ for 21 ± 3 h.

After incubation it will be necessary to add reagents to the API 20E test strip. Disposable gloves must be worn and reagents must be added in a fume hood or MSC.

Acceptable profiles are good, very good or excellent identification with a percentage of identification $\geq 90\%$ and a T index ≥ 0.25 . If a doubtful or unacceptable profile is obtained recheck the purity; if pure and the presence of *Salmonella* remains a possibility, send the strain to the reference laboratory for further identification. If biochemical results exclude the presence of *Salmonella* and the strain is pure no further action is required.

Information Note: *If more than one serotype is present then a representative of each serotype must also be identified biochemically.*

6.7 Public health investigations

If it is known that *Salmonella* is the causative organism of human infection or the clinical symptoms reported in an outbreak situation strongly suggest that this is the cause of illness it is advisable to perform the following additional procedures:

Sub-culture the pre-enrichment culture after incubation directly to selective agar media. Retain the incubated pre-enrichment culture under refrigeration until investigations are complete.

RVS and MKTTn selective enrichment cultures are sub-cultured after 24 h and 48 h incubation and are then retained under refrigeration until investigations are complete.

XLD and BGA plates from these samples are re-incubated if found to be negative after 24 h for a further 24 h.

If confirmation tests from the initial 5 picks from plates are negative pick further colonies up to a total of 20 per sample.

In some instances a rapid response may be required. Provisional recognition of *Salmonella* can be obtained by performing PCR or by agglutination with polyvalent 'O' and 'H' antisera on discrete colonies obtained on the primary isolation media. Alternatively NA slopes may be inoculated directly from XLD and BGA.

If isolated colonies are available, perform biochemical identification using the API 20E biochemical identification kit. After inoculation of the purity check the same suspension must be plated to MA and NA. Incubate all media at $37 \pm 1^\circ\text{C}$ for 21 ± 3 h. Only biochemical results from cultures shown to be pure can be reported and further testing may be necessary if purity is not achieved.

Information note: It is essential that all results are reviewed by a suitably qualified senior microbiologist prior to reporting as reporting errors can have major consequences including reputational damage to PHE¹⁷.

7.0 Quality control

Further quality control of media and internal quality assurance checks should be performed according to in-house procedures using the following test strains:-

Positive control:

Salmonella Nottingham (16:d:enz15) NCTC 7832

Salmonella Poona (13:1,6:z44) NCTC 5792

Negative control

Escherichia coli NCTC 9001

8.0 Calculation of results

No calculations are required as this is a presence absence test only. Results are transferred to the STARLIMS system as describe in Method Q12 Sample processing and result entry in STARLIMS¹⁸.

9.0 Reporting of results

All result are reported using the STARLIMS system as described in method Q13 Technical Validation and release of result in STARLIMS¹⁹. The test report must specify the method used, all details necessary for complete identification of the sample and details of any incidents that may have influenced the result.

The actual weight or volume of sample examined must be reported, for example, 10 g or mL, 25 g or mL, 100 g or mL, unless the product has been examined without reference to weight (shell eggs, hide dog chews). In this case it will be necessary to describe the sample and report per item.

If *Salmonella* species are not found report as follows:

***Salmonella* species Not Detected in 25 g, in 25 mL, per swab or per item.**

If *Salmonella* is recovered from a RTE food the laboratory must review its procedures in accordance with the advice given in: "Procedure for dealing with presumptive pathogens isolated from ready-to-eat foods before reporting the result"¹⁷.

If the presence of *Salmonella* species has been confirmed by biochemical and serological testing or by PCR and the lead microbiologist is satisfied with the procedural review, report as follows:

***Salmonella* species DETECTED in 25 g, in 25 mL, per swab or per item**

If serological testing has been performed also report as follows:

Salmonella species identified as group X eg if O4 group B see Table 1).

A further report from the reference laboratory will give details of the serotype and phage type. If the biochemical or serological results are inconclusive and PCR testing is not possible but the presence of *Salmonella* is still considered to be a strong possibility the result may be reported as a presumptive detection with the comment "confirmation from the Reference Laboratory to follow".

10.0 Reference facilities and referral of cultures

All isolates of *Salmonella* from RTE foods must be referred to the Gastrointestinal Bacteria Reference Unit (GBRU), PHE, Microbiology Services, Colindale for definitive typing. In the event that referral of an isolate of *S.Typhi* or *S.Paratyphi* is required this must be clearly identified as a HG3 organism using the appropriate sample referral forms and the reference laboratory must be notified in advance that an isolate that needs to be handled in CL3 is being sent for confirmation.

A request form for referral to reference facilities can be obtained using the following link:

http://hpanet/webc/HPAnetFile/HPAnet_C/1266227946713

Information Note: *It is a statutory requirement to report isolations of Salmonella from live animals, animal by-products not intended for human consumption, and animal/poultry feedstuffs and ingredients to the Animal Health and Veterinary Laboratories Agency (AHVLA) and to send isolates derived from the animal feed to the regional VLA laboratory applicable to the production site of the feed.*

11.0 Acknowledgements and contacts

This Standard Method has been developed, reviewed and revised by Microbiology Services, Food, Water and Environmental Microbiology Methods Working Group.

The contributions of many individuals in Food, Water and Environmental laboratories, reference laboratories and specialist organisations who have provided information and comment during the development of this document are acknowledged.

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Appendix 1: Flowchart for detection of *Salmonella* species

In CL2 or CL3 if *S Typhi* or *S Paratyphi* are sought, weigh the require amount of sample (eg 25g or 75mL or 10 eggs or whole sample) and dilute to 10^{-1} in BPW (with additions if required). For environmental samples immerse in BPW to give an approximate 10^{-1} dilution



Homogenise by stomaching.
If high or low pH product check and adjust pH if necessary



Incubate at 37°C for 18 h (24 h for dehydrated foods)



Inoculated 0.1 mL to
10 mL of RVS broth

Inoculated 1 mL to 10 mL
of MKTTn broth

Incubate at 37°C for
24 h re-incubate if required



Incubate at 41.5°C for 24 h
re-incubate if required

Incubate at 37°C for
24 h re-incubate if required

In CL3 inoculated 1 mL to
10 mL of SC broth (If *S. Typhi* or *S. Paratyphi* is sought



Sub-culture onto
XLD and BGA
If outbreak samples re-
incubate broth at
41.5°C for a further 24
h and sub-culture again
to XLD and BGA

Subculture onto
XLD and BGA
If outbreak samples
re-incubate broth at
37°C for a further 24 h
and sub-culture again
to XLD and BGA

Subculture onto
XLD, BGA and DCA.
Re-incubate broth at
37°C for a further 24
h and sub-culture
again to XLD, BGA
and DCA



If present subculture five colonies with typical *Salmonella* morphology including at least one typical colony from each plate to MA and NA



Incubate plates at 37°C for 24 ± 3 h. Re-incubate negative plates for a further 24 ± 3 h if outbreak or shell egg samples



Incubate at 37°C for 21 ± 3 h Sub-culture discrete colonies to NA slopes



Perform serology and API 20 E on non-lactose fermenting (NLF) colonies



Review procedures, report result and send to LGP for definitive typing

Appendix 2: Product additions/adjustments to *Salmonella* enrichment broths

Product	Additions	Purpose
High fat foods eg cheese	Add surfactant (eg tergitol 7; final concentration of 0.22%, Triton 100, Tween 80; final concentration 1.0%)	Aids food dispersion (must be pre-warmed to 45+1°C)
Onion & garlic	Add potassium sulphite to give a final concentration of 0.5%	Reduces natural bactericidal properties
Cocoa powder & chocolate confectionery	Add skimmed milk powder to give a final concentration of 10% or casein (not acid casein) to give a final concentration of 5%	Reduces bactericidal properties
High salt/sugar	Increase sample to broth ratio to obtain final concentration below 2%	Maintains salt or sugar concentration below 2%
Oregano, cinnamon, cloves, All spice	Increase sample to broth ratio eg: 1/100 for allspice, cinnamon, oregano, 1/1000 for cloves	Reduces inhibitory properties
High pH foods (eg egg albumen) and low pH foods eg cheeses, mayonnaise, vinegar based marinades, fruits	Adjust pH to 6.5-7.0 prior to incubation.	Neutralises the antibacterial effect of acid and alkali
Some low pH foods eg: freeze dried berry fruits	Suspend in double strength BPW	Prevents pH dropping below 4.5 during incubation

Table 1: Guidance on *Salmonella* serology

Reagent	Result	Further tests		
Saline (auto-agglutination)	+	Perform API20E		
	-	Test with PSO, PSH and other specific antiserum as shown below		
Polyvalent O antigens		Specific O antiserum (Group)	Further tests	
Polyvalent 'O'	+	2(A)	Possible S Paratyphi (A)	Perform API 20E
		4 (B)	Possible S Typhi (Hb and Vi) Possible S Paratyphi (B)	
		6,7 (C)	Possible S Paratyphi (C)	
		8 (C)		
		9 (D)		
		3,10 (E)		
		16 (I)	Suggests presence of S. Nottingham ie IQC strain. Further investigation required.	
		Vi	Only perform if S Typhi or S Paratyphi is suspected.	
	-	If Polyvalent 'H' antisera is also -ve and colony morphology is not typical of <i>Salmonella</i> report as Not detected. If colony morphology is typical of <i>Salmonella</i> perform API20E.		