



Public Health
England

Protecting and improving the nation's health

Detection of *Salmonella* species

National Infection Service

Food Water and Environmental

Microbiology

Standard Method

FNES16 [F13]

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Status of National Infection Service, 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.

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Public Health England (2017). Detection of *Salmonella* species. National Infection Service, Food, Water & Environmental Microbiology Standard Method FNES16 (F13); version 4.

Amendment history

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

The amendments that have occurred since the previous version of this document are shown below. On issue of revised or new documents each controlled document should be updated by the copyholder in the laboratory.

Page	Section(s) involved	Amendment
All	All	Updated following publication of BS EN ISO 6579- 1:2017 (CR10770)
6	Scope	Update with revised ISO reference and details of deviation associated with the use of MSRV for samples with high background removed.
7	Table of differences	Updated to highlight minor differences between PHE and ISO method
9	4.0 Equipment	Refrigerator and sterile 1 µL and 10µL loops added
10	5.0 Culture media and Reagents	Information notes added to ensure all liquid media are pre-warmed before use. Information note added to ensure MkTTn is stored in the dark and is not used if the pH falls below 7. Weight of Potassium sulphite reduce from 12.5g to 1.25g to give a 0.5% concentration as described in the appendix
13	6.0 Sample processing	Section of sample portion size revised to include pooling
14	6.1 Sample preparation and pre-enrichment	Update to include the need to pre-warm BPW and amendment of the procedure for preparation of raw shell eggs to ensure that shells and contents are tested separately.
15	6.2 Selective Enrichment	Updated to include the need to pre-warm and mix media. Extended incubation included for dried milk and cheese. Information note added allowing rapid screening of BPW by PCR. Extended incubation for dehydrated products has been removed. These products are to be allowed to stand for a minimum of 30 minutes at RT prior to incubation.
16	6.5 Recognition of colonies	Update to include extended incubation period
17	6.6 Confirmatory tests	Updated to include performing confirmation on 1 colony with a further 4 if the first colony is negative Serological confirmation section updated to change time from 30-60 to 5-60 seconds and recommendation to consider manufacturer instruction added. Biochemical confirmation if done can be performed in parallel with the purity check
24	References	Updated
26	Appendix 1- Flowchart for detection of <i>Salmonella</i>	Updated
27	Appendix 2: product additions/adjustments to salmonella enrichment broths	References to BS EN ISO 6887-1:2017 and BS EN ISO 6887-4:2017 added to appropriate products (CR11364)
28	Table 1 Guidance on <i>Salmonella</i> Serology	Updated to include use of PCR as an alternative to API

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. Contamination of RTE foods from the environment or from an infected food handler.

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 6579-1:2017 and includes the method for detection of *Salmonella* Typhi and S.Paratyphi as detailed in Annex D of this standard⁴. 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 assess 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.

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 (modified) 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 6579-1:2017. All differences are considered to be minor.

	PHE method FNES16 (F13)	BS EN ISO 6579-1:2017⁴	Justification for variation
Scope	Includes instructions for the preparation of specific food types including molluscan shellfish and raw eggs and detection of <i>Salmonella</i> from swabs and other environmental samples. Animal faeces is not included	Animal faeces is included	Animal faeces is not tested at PHE FW&E microbiology laboratories.
Media and Reagents	Modified semi-solid Rappaport Medium (MSRV) not used	MSRV recommended as an alternative to RVS	Participation in ISO ring trials have shown comparable results using both RV and MSRV.
Sample	Test method only sampling not included in scope	Sampling section included in ISO	Sampling not dealt with as part of this method. Sampling performed by stakeholders
Enrichment Culture	PCR screening of BPW recommended during outbreak and incident investigations	PCR screening of enrichment cultures not described	Useful tool enabling rapid screening of samples. Sub-culture still performed and full method used for samples to isolate <i>Salmonella</i> bacteria.
Quality control	Control strain <i>Salmonella</i> Nottingham	Control strain Specified in BS EN ISO 11133:2014 ⁵ <i>Salmonella</i> Enteritidis or <i>Salmonella</i> Typhimurium however alternative strains permissible following national rules	Use of alternative unusual control strain to maximise potential to detection of possible cross contamination events within the laboratory.

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

2. 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. Safety considerations

3.1 General safety considerations

Normal microbiology laboratory precautions apply 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 perform 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

Salmonellae 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 internal quality control (IQC) will be performed after the sample has been tested using the same media and equipment but ensuring complete segregation from the test sample⁸.

4. 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: $37 \pm 1^{\circ}\text{C}$ and $41.5 \pm 1^{\circ}\text{C}$
- water bath: $37 \pm 1^{\circ}\text{C}$
- refrigerator: $5 \pm 3^{\circ}\text{C}$
- 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)
- sterile 1 μ L and 10 μ L loops

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. 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	9.0 g
or anhydrous disodium hydrogen phosphate	3.5 g
Potassium dihydrogen phosphate	1.5 g
Water	1 L

pH 7.0 ± 0.2 at 25°C

Information note: *BPW must be pre-warmed to room temperature before use.*

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

Information note: *RVS must be pre-warmed to room temperature before use.*

Muller-Kauffmann Tetrathionate Novobiocin (MKTn) broth

Meat extract	4.3 g
Enzymatic digest of casein	8.6 g

Detection of *Salmonella* species

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
Water	1 L

pH (of basal broth) 8.2 ± 0.2 at 25°C

Information note: *MKTTn* must be stored in the dark at 5°C. The pH can fall during storage and this product must not be used if the pH has dropped below 7. *MKTTn* must be mixed and pre-warmed before use.

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
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Detection of *Salmonella* species

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

Sterile water

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 1.25 mL to 25 g sample homogenate (as required)

Skimmed milk powder; antibiotic free, 100 g per L of BPW or 2.5g to 25g sample
homogenate to give final concentration 10% (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 and extraction kits

Reagents as specified in FNES42 (M1)⁹, FNES43 (M2)¹⁰ and FNES44 (M3)¹¹ are used

6. 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 available¹².

Typically 25 g of sample is examined, however, smaller or larger amounts of sample (eg 10g or 100 g) can be examined provided the sample to dilute ratio is maintained at 1:9. This may be of particular value during investigations of food where the amount of *Salmonella* present is likely to be low or during outbreak investigations. When more

than one 25g test portion from a specified product is to be examined and when evidence is available that combining test portions does not affect the result for that particular food, the test portions can be pooled²⁰.

6.1 Sample preparation and pre-enrichment

Food samples

Prepare the sample using the procedure described in National Standard Method FNES26 (F2) - Preparation of samples and dilutions, plating and sub-culture¹². Using sterile instruments and aseptic technique, weigh a representative 25 g sample of each food into a sterile stomacher bag with wire closures. Add 9 times that weight or volume of buffered peptone water (BPW) pre-warmed to room temperature. 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 one 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 and in FNES26¹².*

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)¹⁴ 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 of 6 eggs. It is advisable to use double bags to prevent leakage due to risk of bag puncture by egg shells. Egg contents and shells must be tested separately. Aseptically break the eggs and transfer the contents of the eggs into a tared stomacher bag with closures. Place the egg shells in a separate bag with closures. 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 one 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 PHE Standard Method FNES4 (E1) - Detection and Enumeration of Bacteria in Swabs and Other Environmental Samples¹⁵. Ensure ample coverage with pre-warmed 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 allow the sample to stand at room temperature for 30 minutes prior to incubation.

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 pre-warmed to room temperature. Place in an incubator at $41.5 \pm 1^\circ\text{C}$ for 24 ± 3 h. For shell eggs, dried milk and cheese or samples likely to contain environmentally stressed organisms, 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 that has been thoroughly mixed and pre-warmed to room temperature. Place in an incubator at $37 \pm$

1 °C for 24 ± 3 h. For shell eggs, dried milk and cheese or samples likely to contained environmentally stressed organisms, re-incubate the MKTTn for a further 24 h.

Information note: Where rapid screening of the sample is indicated (eg outbreak or incident investigations) PCR screening of the enrichment broth can be performed. Transfer 1mL of the enrichment broth to a nuclease free tube. Heat treat the broth at 95°C for 15 minutes, allow to cool and extract the DNA following method FNES42 (M1)⁹. Following extraction add 30 µL of extracted sample to lyophilised real time PCR assay tubes as described in Standard Method FNES43(M2)¹⁰. The positive control described in Standard Method FNES45 (M4)¹⁶ should be included in each real time PCR assay.

6.3 Subculture to selective media

Following incubation sub-culture the selective enrichment cultures to pre-warmed XLD and to BGA media 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± 1°C for 24 ± 3 h. Re-incubate negative plates from raw shell eggs, dried milk and cheese samples that are likely to contained environmentally stressed organisms 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± 1°C for 48 ± 3 h. After 24 ± 3 h and 48 ± 3 h subculture the SC to XLD, BGA and Hynes DCA.

Place all plates in an incubator at 37± 1°C for 24 ± 3 h.

6.5 Recognition of colonies

After 24± 3 h and after 48± 3 h if extended incubation has been applied, 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

A typical colony (see above) from one 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 suspect *Salmonella* colony from any one of 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. If confirmation tests from the initial colony are negative pick further colonies up to a total of 5 per sample.

If confirming using PCR the loop used for sub-culture is carefully emulsified in 0.5 mL of PCR grade water. All picks from a sample (up to 5 colonies) can 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 FNES44 (M3¹¹) 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 FNES43 (M2)¹⁰. The positive control described in Standard Method FNES45 (M4)¹⁶ 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 5-60 seconds or according to the manufacturer instructions.

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 5-60 seconds and according to the manufacturer instructions.

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 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. Some organisms eg *Citrobacter* spp can give rise to positive poly O but negative poly H serology. Further biochemical or PCR confirmations must be performed on these isolates.

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.

If well isolated colonies (of a pure culture) are available on the selective plating media, the biochemical confirmation can be performed in parallel with the purity check of the colony taken from the selective agar medium. 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 colony pick from plates are negative pick further colonies up to a total of 5 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. 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. Calculation of results

No calculations are required as this is a presence absence test only. Results are transferred to the StarLims system as described in Method FNES6 (Q12) Sample processing and result entry in StarLims¹⁷.

9. Reporting of results

All results are reported using the StarLims system as described in method FNES20 (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 detection of pathogens"¹⁶.

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. Reference facilities and referral of cultures

All isolates of *Salmonella* from RTE foods must be referred to the Gastrointestinal Bacteria Reference Unit (GBRU), PHE, National Infection Service, Colindale for definitive typing following the procedure described in method FNES65¹⁹. 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:

https://assets.publishing.service.gov.uk/government/uploads/system/uploads/attachment_data/file/648460/L4_gastrointestinal_bacteria_culture_referral_form.pdf

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 and Plant Health (APHA) and to send isolates derived from the animal feed to the regional APHA laboratory applicable to the production site of the feed.*

11. Acknowledgements and contacts

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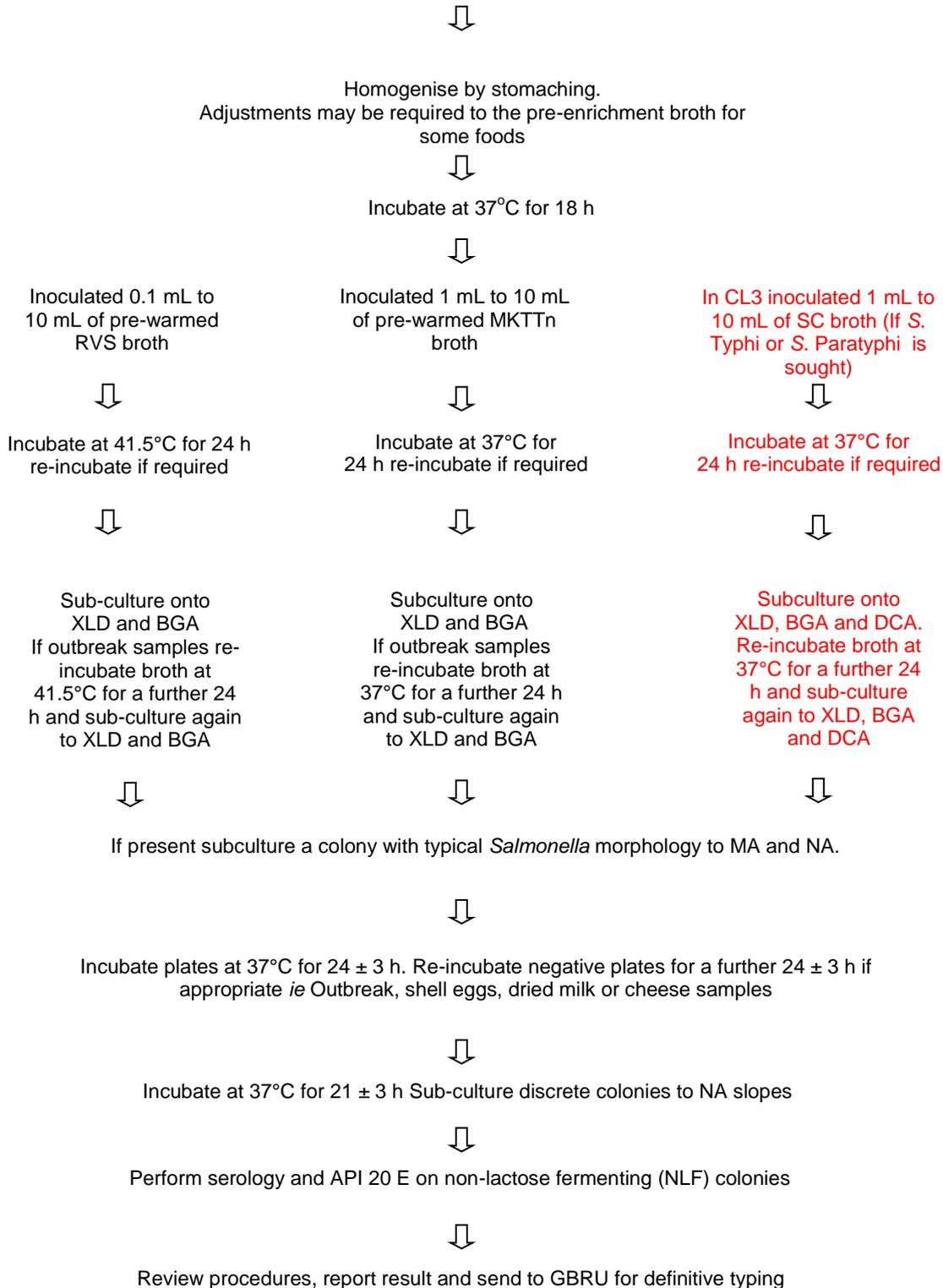
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Appendix 1: Flowchart for detection of *Salmonella* species

In CL2 (or CL3 if *S Typhi* or *S Paratyphi* are sought), weigh the required amount of sample (eg 10g, 25g, 75mL or 6-10 eggs or whole sample) and dilute to 10^{-1} in pre-warmed BPW (with additions if required). For environmental samples immerse in pre-warmed BPW to give an approximate 10^{-1} dilution.



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

Product	Additions	Purpose
High fat foods eg cheese ²¹	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 and garlic	Add potassium sulphite to give a final concentration of 0.5%	Reduces natural bactericidal properties
Cocoa powder & chocolate confectionery ²¹	Add skimmed milk powder (antibiotic free) 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 ²¹	Increase sample to broth ratio to obtain final concentration below 2%	Maintains salt or sugar concentration below 2%
Oregano, cinnamon, cloves, All spice ²¹	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 ²⁰	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 or PCR		
	-	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 <i>S</i> Paratyphi (A)	Perform API 20E or PCR
		4 (B)	Possible <i>S</i> Typhi (Hb and Vi) Possible <i>S</i> Paratyphi (B) <i>S. Typhimurium</i> (Hi)	
		6,7 (C)	Possible <i>S</i> Paratyphi (C)	
		8 (C)		
		9 (D)	<i>S. Enteritidis</i> (Hg)	
		3,10 (E)		
		16 (I)	Suggests presence of <i>S. Nottingham</i> ie IQC strain. Further investigation required.	
		Vi	Only perform if <i>S</i> Typhi or <i>S</i> 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 or PCR		