FAO Reference Centre for Bivalve Mollusc Sanitation

Generic protocol – Detection of *Salmonella* spp. in bivalve molluscan shellfish (based on ISO 6579-1)

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History of Procedure

Issue	Date	Section	Changes
1	29.06.20	All	First issue
2	28.11.23	All	The temperature range for incubation of non-selective and selective media has been extended from 37 °C ± 1 °C to 34 °C to 38 °C without further tolerance.
		5	Equipment for dissection of gastropods and echinoderms added
		7	Updated Triple sugar iron agar reference strain from *Sphingomonas paucimobilis* to *Pseudomonas aeruginosa* as stated in ISO 6579-1
		8.4	Update to oyster cracker image and information on how to prepare gastropods and echinoderms
		8.5	Procedure for blending and stomaching harmonised with the UK NRL protocol
		13.1 - Appendix 1	Species list updated
3	29.11.24	All	Harmonisation of terminology between different sections
		6	Clarification of method for preparation of TSIA slopes
		8.7	Figure 5 labelling corrected, formatting fixed so that text is not hidden behind figure
		13.1 - Appendix 1	Modification of text to clarify that animal numbers for different species are recommendations only; requirement is fixed at a minimum 10 animals for all species
		13.2 - Appendix 2	Hyperlink updated

Contents

1. Introduction

The risks of infectious human diseases acquired from the consumption of bivalve molluscan shellfish (BMS) are internationally recognised. These health hazards are largely due to the phenomenon of filter feeding whereby BMS concentrate and retain bacterial and viral pathogens, often derived from sewage contamination of their growing waters. The risks of exposure to infectious agents are compounded by the traditional consumption of raw, or only lightly cooked, BMS. The presence of *Salmonella* in ready-to-eat food including BMS is considered significant regardless of the level of contamination. *Salmonella* show little host specificity and can cause gastroenteritis when ingested. Incubation time is usually 12-36 hours although it may be longer as multiplication must occur in the intestines. *Salmonella* belong to the family *Enterobacteriaceae* and are fermentative, facultatively anaerobic, Gram-negative rod-shaped bacteria, which are oxidase negative.

2. Scope

This protocol has been produced with reference to ISO 6579-1 and selected parts of other relevant ISO standards referenced in Section 12. It is suitable for use with raw and cooked shellfish. It describes the method for detecting the presence of *Salmonella* in BMS as an indication of contamination of harvested shellfish and for the end-product testing of the depurated/cooked product. Although not intended for the isolation of *S.* Typhi and *S.* Paratyphi, strains of these serotypes may be incidentally isolated if present in the samples.

Note: In addition to BMS, this method is also applicable to analysis of other marine shellfish species including gastropods and echinoderms. In these cases alternative sample preparation methods should be used – these are detailed in section 8.4.

3. Principle

This protocol for the detection of *Salmonella* in shellfish involves a primary enrichment in buffered peptone water (BPW), followed by selective enrichments in both Muller-Kaufmann tetrathionate-novobiocin broth (MKTTn) and Rappaport-Vassiliadis soya enrichment broth (RVS) or modified semi-solid Rappaport-Vassiliadis soya (MSRV). Following incubation samples are sub-cultured onto plates of xylose lysine deoxycholate (XLD) agar and a second selective medium. Presumptive colonies of *Salmonella* isolated from agar plates are subjected to biochemical and serological tests to confirm their identity. The results are expressed as *Salmonella* detected or not detected in 25g (see ISO 7218).

4. Safety precautions

Standard microbiology safety precautions should be applied throughout. Laboratories should perform a full risk assessment before using this protocol. Risks of cuts and minor physical injury exist when performing this procedure, particularly when using sharp oyster knifes to open shellfish. Appropriate measures to reduce these risks should be taken. Homogenisation of shellfish should be performed in a Class II safety cabinet to reduce the risk of infection from aerosol inhalation. Laboratory procedures should conform to the recommendations given in the WHO Laboratory Biosafety Manual Third edition (WHO, 2004) or relevant national legislation or guidelines. *Salmonella* spp. should be handled in accordance with ACDP category 2 guidelines.

5. Equipment [1]

- Autoclave (or media preparator including an autoclave component)
- Drying cabinet or ventilated oven capable of being maintained between 25±1 °C and 50±1 °C
- Sterile syringe
- Waterbath capable of operating at 47 – 50 °C
- Balance capable of weighing to ±0.1 g and reference weights for calibration
- pH-meter, with automatic or manual temperature compensation and having a resolution of 0.01 pH units and accurate to within ±0.1 pH unit at 25 °C. Reference solutions for calibrating the pH meter
- Local Exhaust Ventilation (LEV; for weighing powders during media preparation)
- Class II safety cabinet
- Thermometer with a resolution of 1 °C or better at the temperature being measured
- Sterile measuring cylinder – 250 ml (a larger cylinder may be needed for some species)
- Sterile glass beaker or flask capable of holding 250 ml volumes and sterile glass tubes or screw-topped bottles capable of holding 20 ml volumes (for liquid media)
- Sterile petri dishes (for solid media): diameter 90 mm; height 15-16 mm; vented
- Shucking knife, oyster cracker or other suitable equipment for opening bivalve molluscs
- Dissecting equipment for gastropods and echinoderms – Sterile tray, forceps, scissors, spatula and hammer or equivalent tools
- Food grade plastic bags
- Absorbent paper towel
- Weighing dish capable of holding the largest size of bivalve mollusc to be tested plus released liquor (intravalvular fluid). Must be capable of being cleaned and sterilized (for use with oyster cracker)
- Safety gloves – for example a chain mail glove (for use with the shucking knife)
- Waring blender and jars or Stomacher and stomacher bags
- Refrigerators at 3±2 ˚C and 5±3 ˚C
- Sterile container of at least 500 ml capacity (A larger capacity may be required when large bivalve molluscs are to be examined. Examples include *Panopea generosa* (geoducks), *Mya arenaria* (soft shell clams; sand gapers) and some types of razor clams)
- Safety Bunsen burner or electric micro incinerator
- Protective gloves – single use
- Membrane filter – 0.22 µm
- Incubator or recirculating water bath at 34 - 38 ˚C and 41.5±1 ˚C [2]
- Platinum or sterile disposable loops - 1 µl and 10 µl
- Pipette filler and graduated pipettes or automatic pipettor and pipette tips of a range of sizes e.g. 1ml and 10ml. For handling mixed shellfish homogenates (1:3 dilution) open-ended pipettes may be necessary due to high concentrations of particulate matter.

[1] No validation work has been carried out to assess the impact of using a pulsifier to prepare shellfish samples for *Salmonella* analyses.

[2] In countries where the ambient temperature exceeds the target incubation temperature, an incubator or recirculating water bath with both heating and cooling units may be required to maintain the required temperature range.

6. Media [3] [4]

Media stored under refrigeration should be allowed to equilibrate at room temperature before use (ISO 11133). If necessary, dry the surface of plated media before use. For performance testing of media see Table 1 in Section 7.

Note: ISO 6579-1 contains further details for the preparation of all cultured media and reagents from the basic ingredients. Dehydrated media purchased commercially should be prepared according to the manufacturer's instructions.

- Ethanol
- Buffered peptone water (ISO) (BPW); formula per litre - deionised water 1±0.01 litre, buffered peptone water 20±0.2 g, pH 7.0±0.2. Mix well and dispense into bottles (or flasks) in volumes suitable for use for a set of examinations or a usual working day. Sterilise by autoclaving at 121 °C for 15 minutes. Store at 5±3 °C in the dark.
- Muller-Kauffmann tetrathionate-novobiocin broth base (MKTTn); formula per litre - deionised water 1±0.01 litre, Muller-Kauffmann tetrathionate broth base 89.5±0.2 g, pH 8.0±0.2. Dissolve completely by mixing well and bring to the boil. Avoid overheating. Dispense into 10±0.2 ml volumes in tubes (or bottles). Store at 5±3 °C in the dark. Prior to use add 200±0.5 µl iodine-iodide solution and 50±0.2 µl novobiocin solution for each 10ml volume of broth.
    - Iodine-iodide solution; formula per 100 ml – deionised water 100±1 ml, potassium iodide 25±0.2 g, iodine 20±0.2 g. Dissolve potassium iodide in 10±0.2 ml deionised water. Add iodine then dilute with de-ionised water to a final volume of 100±1 ml. Store in a tightly sealed container in the dark.
    - Novobiocin solution; formula per 5 ml – de-ionised water 5±0.2 ml, novobiocin sodium salt 0.04±0.01 g. Dissolve novobiocin sodium salt in water. Sterilise by filtration using a syringe and filter with a pore size of 0.22 µl. Store at 5±3 °C in the dark.
- Rappaport-Vassiliadis soya enrichment broth (RVS); formula per litre - deionised water 1±0.01 litre, Rappaport-Vassiliadis soya peptone broth 26.75±0.2 g, pH 5.2±0.2. Dissolve completely by gently heating. Dispense into 10±0.2 ml volumes in tubes (or bottles). Cap the tubes (or bottles) and sterilize by autoclaving at 115 °C for 15 minutes. Store at 5±3 °C in the dark.
- Modified semi-solid Rappaport-Vassiliadis agar (MSRV); formula per litre - deionised water 1±0.01 litre, Modified semi-solid Rappaport-Vassiliadis agar (ISO) 31.6±0.2 g, pH 5.2±0.2. Sterilise by bringing to the boil with frequent agitation. DO NOT AUTOCLAVE. Cool to approx. 50 °C before adding 1 x Novobiocin vial (reconstituted as directed) using a syringe and filter with a pore size of 0.22 µl. Dispense aseptically in 15 ml to 20 ml volumes into sterile petri dishes. DO NOT INVERT PLATES. Store at 5±3 °C in the dark.
- Xylose lysine deoxycholate agar (XLD); formula per litre - deionised water 1±0.01 litre, xylose lysine deoxycholate agar 53±0.2 g, pH 7.4±0.2. Sterilise by bringing to the boil with frequent agitation. DO NOT OVERHEAT. Cool to approx. 50 °C before pouring 18 ml to 20 ml volumes into sterile Petri dishes and allow to solidify. Store at 5±3 °C in the dark.
- Triple sugar iron agar (TSIA); formula per litre - deionised water 1±0.01 litre, triple sugar iron agar 65±0.2 g, pH 7.4±0.2. Dissolve completely by gently heating and agitation. Dispense aseptically in 10±0.2 ml volumes in sterile tubes (or bottles). Cap the tubes (or bottles) and sterilize by

[3] For performance testing of media and reagents see Appendix 2.
[4] Formulations provided in this generic protocol are based upon the use of Oxoid Ltd listed products for illustrative purposes, alternative suppliers of media and reagents can be used according to the manufacturers' instructions. Laboratories should determine that alternative products are fit for purpose.

autoclaving at 121 °C for 15 minutes. Allow to set in a sloping position to give a butt of depth 2.5 cm to 5 cm. Store at 5±3 °C in the dark.

- Urea agar (UA); formula per 100 ml - de-ionised water 95±0.5 ml, urea agar base 2.4±0.2 g. pH 6.8±0.2. Dissolve completely by heating. Sterilize by autoclaving at 115 °C for 20 minutes. Cool to approx. 50 °C before adding 5±0.2 ml of sterile 40 % urea solution using a syringe and filter with a pore size of 0.22 µl. Mix well and dispense aseptically in 10±0.2 ml volumes in sterile tubes (or bottles). Allow to set in a sloped position. Store at 5±3 °C in the dark.
- L-Lysine decarboxylation medium (LDC); formula per 5 ml - deionised water 5±0.2 ml, 1 x tablet Lysine decarboxylase broth. Sterilize by autoclaving at 121 °C for 15 minutes.
- Nutrient agar (NA); formula per litre - de-ionised water 1±0.01 litre, Nutrient agar 28±0.2 g, pH 7.4±0.2. Dissolve completely by heating. Sterilize by autoclaving at 121 °C for 15 minutes. Cool to 47 °C to 50 °C before pouring 18 ml to 20 ml volumes into sterile Petri dishes and allow to solidify. Store at 5±3 °C in the dark.
- Saline solution; formula per litre - de-ionised water 1±0.01 litre, Sodium chloride 8.5±0.2 g, pH 7.0±0.2.
- Polyvalent anti-O sera and anti-H sera.

7. Microbiological reference cultures for performance testing and controls

The FAORC recommends the use of the positive and negative controls throughout the procedure. A complete list of controls for performance testing of media and reagents recommended in ISO 6579-1 are included as Appendix 2.

Table 1: Microbiological reference strains used for control purposes

Media type	Function	Control strain [a]	WDCM [b]	Characteristic reactions
Buffered peptone water (BPW)	Productivity	*Salmonella* Typhimurium [c d]	00031	Turbid
		Salmonella Enteritidis [c d]	00030	
Muller-Kauffmann tetrathionate - novobiocin broth (MKTT)	Productivity	*Salmonella* Typhimurium [c d]	00031	Growth
		Salmonella Enteritidis [c d]	00030	
		+ *Escherichia coli* [c]	00012 or 00013	
		+ *Pseudomonas aeruginosa*	00025	
	Selectivity	*Escherichia coli* [c]	00012 or 00013	Partial inhibition ≤100 colonies on TSA
		Enterococcus faecalis [c]	00009 or 00087	<10 colonies on TSA
Rappaport-Vassiliadis soya enrichment broth (RVS)	Productivity	*Salmonella* Typhimurium [c d]	00031	Growth
		Salmonella Enteritidis [c d]	00030	
		+ *Escherichia coli* [d]	00012 or 00013	
		+ *Pseudomonas aeruginosa*	00025	
	Selectivity	*Escherichia coli* [c]	00012 or 00013	Partial inhibition ≤100 colonies on TSA
		Enterococcus faecalis [c]	00009 or 00087	<10 colonies on TSA

Media type	Function	Control strain [a]	WDCM [b]	Characteristic reactions
Modified semi-solid Rappaport-Vassiliadis agar (MSRV)	Productivity	*Salmonella* Typhimurium [c d]	00031	Growth. Grey-white turbid zone extending out from inoculated drop.
		Salmonella Enteritidis [c d]	00030	
	Selectivity	*Escherichia coli* [c]	00012 or 00013	Possible growth at inoculation drop without a turbid zone
		Enterococcus faecalis [c]	00009 or 00087	No growth
Xylose lysine deoxycholate agar (XLD)	Productivity	*Salmonella* Typhimurium [c d]	00031	Growth: Black centred colonies with light transparent zone of reddish colour
		Salmonella Enteritidis [c d]	00030	
	Selectivity	*Escherichia coli* [c]	00012 or 00013	Growth or partial inhibition. Yellow colonies if present.
		Enterococcus faecalis [c]	00009 or 00087	Total inhibition
Nutrient agar (NA)	Productivity	*Salmonella* Typhimurium [c d]	00031	Growth
		Salmonella Enteritidis [c d]	00030	
L-Lysine decarboxylase broth (LDC)	Detection of L-Lysine decarboxylase (LDC)	*Salmonella* Typhimurium [c d]	00031	Positive reaction: Growth and colour remains purple
		Salmonella Enteritidis [c d]	00030	
		Proteus mirabilis [c]	00023	Negative reaction: Colour change from purple to yellow
		Escherichia coli [c]	00012 or 00013	
Triple sugar iron agar (TSIA)	Multiple function	*Salmonella* Typhimurium [c d]	00031	Butt – Yellow: glucose fermented. Black: Formation of hydrogen sulphite. Bubbles or cracks: Gas formation. Slant surface – Yellow: Lactose and/or sucrose utilized
		Salmonella Enteritidis [c d]	00030	
	Detection of H2S formation	*Pseudomonas aeruginosa*	00024 00025 or 00026	Butt – Red or unchanged: Glucose not fermented. Slant surface – Red or unchanged: Lactose and sucrose not utilized
Urea agar (UA)	Detection of urea hydrolysis by ammonia production	*Proteus mirabilis* [c]	00023	Positive reaction: Colour change to rose/rose-pink/deep cerise. Liberation of ammonia
		Klebsiella pneumoniae [c]	00097	
		Salmonella Typhimurium [c d]	00031	Negative reaction: No change to colour. No liberation of ammonia
		Salmonella Enteritidis [c d]	00030	
		Escherichia coli [c]	00012 or 00013	

[a] *S*. Nottingham is rarely isolated from the natural environment or food products, it therefore can be used as both a media performance selectively and productivity control and as an assay control in the event a suspected laboratory contamination incident – currently *S.* Nottingham is not listed at WDCM.
[b] Follow the link http://refs.wdcm.org/species.htm to obtain culture collection numbers for WDCM reference strains
[c] Strain choice: Laboratory must select 1 strain as a minimum from the list provided for each media type.
[d] Some national restrictions and directions may require the use of a different serovar. Make reference to national requirements relating to the choice of *Salmonella* serovars

8. Procedures

8.1. Sample transport and receipt

Samples must be placed in an intact food-grade plastic bag, or equivalent, and properly packed in a cool box with ice packs – packed in this manner they should reach a temperature between 0 ˚C and 10 ˚C within 4 hours and then maintain this for at least 24 hours [5]. The specific cool box, ice pack and transport condition combination should be validated to ensure that this can be achieved (https://www.cefas.co.uk/nrl/information-centre/nrl-laboratory-protocols/). For samples where less than 4 hours have elapsed between collection from the growing area and receipt at the laboratory, internal air temperature (or between-shellfish sample temperature) should be less than the temperature recorded at the time of sampling. Samples from harvesting areas should be rinsed, but not immersed, and drained at time of sampling. Samples should be regarded as unsatisfactory if on receipt at the laboratory the sample is frozen, the container is leaking, the shellfish are covered in mud or immersed in water or mud/sand.

The sample transport criteria given here are extracted from ISO 6887-3. The use of alternate sample transport criteria may be acceptable, where verification studies have been undertaken and the results of those studies demonstrate that there is no significant effect on the quality of the test results. For samples being taken in support of a growing area sanitation programme, it is recommended that verification studies supporting the use of sample transport criteria outside of the ranges given in ISO 6887-3 are approved by the responsible authority. (See Section 4.3.6 and Annex 13 of the FAO/WHO technical guidance (https://www.fao.org/3/cb5072en/cb5072en.pdf).

8.2. Sample storage

Upon receipt in the laboratory the temperature of the samples should be recorded. Samples should be examined immediately - if storage in the laboratory is necessary, then samples should be stored at 3±2 ˚C and should be processed within 24 hours of collection. If, due to logistical problems, microbiological analysis of samples taken in support of a growing area programme cannot be started within 24 hours of sample collection, a verification study should be undertaken to show that extended storage does not affect the microbiological content of the sample.

8.3. Sample selection

Choose shellfish that are alive according to the following criteria:

- Reaction or movement of exposed flesh after touching using a sterile shucking knife
- Shellfish open and close of their own accord
- A tap on the shell causes closing or movement
- Tightly closed shellfish

[5] A temperature data logger may be used to monitor the sample temperature during transit. The data logger must be held in a central position within the cool box and not allowed to come into contact with the ice packs. If the logger has not been activated or is found to be in contact with the ice packs on arrival at the laboratory, then the temperature of the shellfish sample itself should be measured using an appropriately calibrated temperature probe.

Discard all dead shellfish and those with obvious signs of damage. Select the appropriate number depending on the species (Appendix 1). More shellfish can be used, if necessary, to produce the required volumes for each analysis.

8.4. Sample preparation

Any mud and sediment adhering to the shellfish should be removed prior to opening the shellfish by rinsing/scrubbing under cold, running tap water of potable quality. Shellfish should not be re-immersed in water as this may cause them to open. Open all selected shellfish as described below [6].

8.4.1. Preparation of bivalve molluscs using the oyster cracker

Sterilise the blade of the oyster cracker before use (see Figure 1 and Figure 2). Place a single animal in a weighing dish on the platform underneath the blade. Lower the lever so that the blade engages the hinge of the shellfish, then fully pull the lever down so that the blade separates the shells. Using a sterilised shucking knife cut the muscle and scrape the flesh of both shells into the sterilised container. Transfer any liquor (intravalvular fluid) collected in the weighing boat into the sterilised container.

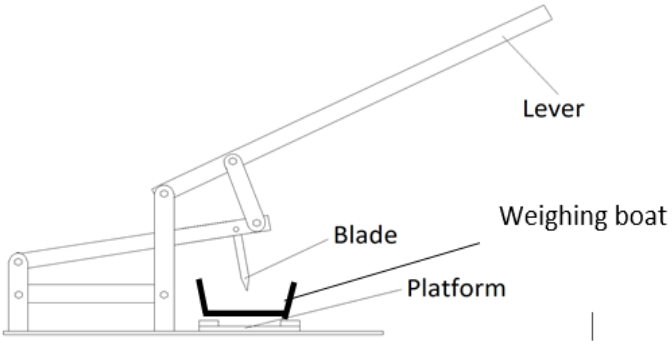

Figure 1: Oyster cracker apparatus

Figure 2: Opening an oyster using an oyster cracker

8.4.2. Preparation of bivalve molluscs using a shucking knife

Open all selected shellfish as described below using a sterilised shucking knife or equivalent and empty flesh and liquor into a sterile container. If sterilised by heating allow the knife to cool before using. When opening shellfish, ensure that the hand holding the shellfish is protected with a heavy-duty safety glove to prevent cuts.

8.4.2.1. Oysters and clams

Insert the knife between the two shells towards the hinge end of the animal. Push the knife further into the animal and prise open the upper shell, allowing any liquor to drain into the sterilised container. Push the blade through the animal and sever the muscle attachments by sliding across the animal. Remove the upper shell and scrape the contents of the lower shell into the sterilised container.

[6] Alternative suitable equipment can be used to open bivalve shellfish.

8.4.2.2. Mussels and cockles

Insert the knife in between the shells of the animal and separate the shells with a twisting motion of the knife. Collect the liquor from the animal in the sterilised container then cut the muscle between the shells and scrape the contents into the sterilised container.

8.4.3. Preparation of gastropods

Place a single animal into the hinge section of the oyster cracker (see Figure 3) and pull the handle down to break open the shell. Transfer the material onto sterile absorbent paper towel on a tray and remove any shell debris using sterile forceps. Repeat the process with the required number of animals (see Appendix 1). Dice the animal flesh using sterile scissors before transferring to a sterile container.

Alternatively, the animals can be opened using a sterile hammer or equivalent tool, without damaging the flesh, by placing the animals in a food grade bag and covering with absorbent paper towel.

Figure 3: Opening a gastropod using an oyster cracker

8.4.4. Preparation of echinoderms (e.g. sea urchins)

Hold the echinoderm with the mouth parts facing upwards. Using sterile forceps and wearing a strong clean glove, cut out the mouth section (Figure 4) with sterile scissors to expose the flesh inside. Transfer the flesh and internal fluid into a sterile container using a sterile spatula. Repeat the process with the required number of animals (see Appendix 1).

Figure 4: Opening an echinoderm using sterile scissors

8.5. Dilution, homogenisation and inoculation and incubation of primary enrichment broth [7, 8]

Weigh the sterilised container and calculate the weight of the contents by subtracting the weight of the pre-weighed container to the nearest gram.

Note: Complete either sections 8.5.1 or 8.5.2.

[7] ISO 6887-3 allows the use of PSS for preparation of the primary dilution. FAORC advice is to use 0.1% P instead at this stage in order to avoid the addition of extra NaCl.

[8] Samples should be examined within 45 minutes after the end of the initial suspension and the end of the inoculum comes into contact with the final culture medium.

8.5.1. Homogenisation in a blender

Measure 2 ml of sterile BPW per 1 g of shellfish (±2 ml) using a measuring cylinder. Place contents of sterilised container into a sterilised 1 litre blender jar with the BPW from the measuring cylinder and homogenise at high speed for approximately 1 minute (4 bursts of 15 seconds with at least 5 seconds between bursts) in a class two microbiological laminar flow cabinet. Decant the contents (mixed shellfish homogenate (1:3 dilution)) back into the sterilised container.

Add 75±1 ml of mixed shellfish homogenate to 175±5 ml of BPW to make a master 10^{-1} dilution (the primary enrichment broth), ensure that this master dilution is thoroughly mixed.

Note: If shellfish are particularly small it may be necessary to use a smaller blender to achieve a consistent homogenate.

Note: it may be necessary to use an open-ended pipette to transfer mixed shellfish homogenate due to high concentrations of particulate matter.

Note: Homogenates prepared in section 8.5 of the FAORC *E. coli* protocol can be used by taking 75 ml of the 1:3 dilution and adding this to 175 ml of BPW [9].

8.5.2. Homogenisation using a stomacher [10]

Place the contents of the sterilised container into at least three stomacher bags (e.g. place three stomacher bags inside each other to avoid small pieces of shell from puncturing the bags). Remove excess air from the bags and operate the stomacher for 2-3 minutes. Transfer 25±0.5 g of homogenised shellfish into another set of 3 stomacher bags, add 50±1 ml of BPW and homogenise for a further 2-3 minutes. After homogenisation decant the contents into a new sterilised container and add 175±5 ml BPW to make a master 10^{-1} dilution, ensure that this master dilution is thoroughly mixed. Label as the 10^{-1} primary enrichment.

8.5.3. Process controls

Using a 1 µl loop, inoculate a 10±1 ml volume of BPW with a *Salmonella* spp. control strain (see Table 1). Leave a second 10±1 ml volume of BPW uninoculated as the negative control.

8.5.4. Incubation of primary enrichment broth

Incubate the sample (the primary enrichment broth) and the process controls at 37±1 ˚C [11] for 18±2 hours.

Note: Following incubation, the primary enrichment (BPW) may be stored at 5±3 ˚C for a maximum of 72 hours.

[9] This approach has been agreed as equivalent at annual meetings of the UK NRL laboratory network. No formal validation of the approach has been undertaken. No significant difference has been seen in proficiency testing (PT) results between laboratories using 25 g of homogenate plus 225 ml BPW and those using 75 ml of 1:3 homogenate and 175 ml BPW.

[10] The FAORC recommends using a blender and not a stomacher when preparing clam and cockle samples.

[11] Incubation temperature range given in ISO 6579-1:2017 for BPW is 34 - 38 ˚C. The temperature range of 37±1˚C in this protocol has been retained in accordance with ISO 6579:2002.

8.6. Inoculation and incubation of selective enrichment media

Following incubation of BPW, inoculate MKTTn broth and either RVS or MSRV using an appropriately sized pipette as described below.

8.6.1. Rappaport-Vassiliadis soya enrichment broth (RVS)

Transfer 100±20 µl of the BPW primary enrichment to 10±1 ml of the RVS and incubate at 41.5±1 ˚C for 24±3 hours.

8.6.2. Modified semi-solid Rappaport-Vassiliadis agar (MSRV)

Drop 100±20 µl of the BPW primary enrichment onto the MSRV plate surface (up to 3 equally spaced drops can placed on MSRV surface). Incubate at 41.5±1 ˚C for 24±3 hours. **Do not invert the plates.**

8.6.3. Muller-Kaufmann tetrathionate-novobiocin (MKTTn) broth

Transfer 1±0.1 ml of the BPW primary enrichment to 10±1 ml of the MKTTn and incubated at 37±1 ˚C for 24±3 hours.

Note: MKTTn made in-house will require Iodine-iodide and Novobiocin reagents to be added before use. Commercial ready-to-use MKTTn broth may contain Iodine-iodide and Novobiocin reagents at the required concentrations already, check before use.

8.6.4. Process control

Using a 10 µl loop, inoculate each selective enrichment broth (MKTTn and RVS or MSRV) with the BPW positive control inoculated with *Salmonella* spp. (prepared in 8.5.3). Repeat the inoculation of MKTT and RVS or MSRV using a negative *E. coli* control (WDCM 00012 or 00013). Incubate the process controls as described in 8.6.1 to 8.6.3.

Note: Following incubation, selective enrichments may be stored at 5±3 ˚C for a maximum of 72 hours.

8.7. Isolation of *Salmonella*

Following incubation of the RVS and MKTTn broths, subculture onto the surface of one 140 mm or two 90 mm XLD plates and one 140 mm or two 90 mm plates of a second isolation medium[12], using a 10 µl loop for each medium to obtain well-isolated colonies.

Note: To obtain well isolated colonies use one 140 mm isolation plate or two 90 mm isolation plates (inoculate without recharging the loop between plates).

Following incubation of the MSRV plate, examine for the presence of a grey-white turbid zone

[12] The second isolation medium selection is the testing laboratory's choice. It should complement XLD agar to facilitate the detection of lactose positive or H2S-negative *Salmonella* strains.

extending out from the inoculation drop. Subculture from just inside the opaque growth of the MSRV plate (without any MSRV agar attached) onto the surface of one XLD plate and one plate of a second isolation medium, using a 1 µl loop for each medium. If no growth is visible after 24 hours, re-incubate for a further 24±3 hours.

Note: If the MSRV media has been incubated for an additional 24 h, follow the same plating out procedure as described above.

Subculture the positive and negative controls onto one XLD plate and one second isolation medium plate.

Incubate XLD plates at 34 - 38 ˚C for 24±3 hours. Incubate the second isolation medium in accordance with the manufacturer's instruction.

After incubation examine all plates for characteristic typical *Salmonella*-like colonies:

XLD: Colonies with black centres and a light transparent zone of reddish colour (Figure 5). [13]

Second isolation medium: Follow the manufacturer's instructions for colony characteristics.

If typical *Salmonella*-like colonies are present in the sample, continue with confirmation as described in 8.8. If no *Salmonella*-like colonies are present, then report the result as '*Salmonella* NOT detected in 25 g.'

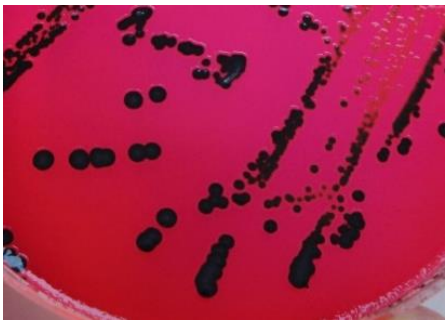

Figure 5: Image of *Salmonella* spp. colonies sub-cultured on to an XLD plate

8.8. *Salmonella* confirmation

Confirmation of presumptive *Salmonella* spp. can be achieved using the procedures identified in 8.8.1. If shown to be reliable, miniaturised galleries for the biochemical identification of *Salmonella* may be used. Additionally, alternate procedures (e.g. agglutination, molecular probe-based approaches, PCR, whole genome sequencing, etc) can be used to confirm the isolate as *Salmonella* spp. providing that the suitability of the alternative procedure is verified (see ISO 7218).

8.8.1. Selection of colonies for confirmation

Mark the location of suspect *Salmonella*-like colonies on the base of the XLD and second isolation plates.

Note: If no isolated colonies are present, streak selected colonies onto a pre-dried non-selective agar plate to obtain well-isolated colonies. Incubate the inoculated plates between 34 - 38 °C for 24 h ± 3 h.

Subculture one well-isolated colony, using a 1 µl loop, into a TSIA slope, UA stab, LDC broth and onto NA plates without recharging the loop. Subculture by first stabbing the main body of the TSIA (the butt) and streak the surface (slant). Next, stab the UA followed by LDC broth

[13] Some H_2S positive colonies may not have black centres, for example younger colonies. However, those showing characteristic *Salmonella*-like phenotypes should be preferentially selected.

before streaking out for single colonies onto a NA plate. Ensure that the TSIA slopes, UA, LDC and NA plates are appropriately labelled, i.e. sample identifier, original culture medium (from RVS, MSRV or MKTTn, XLD, etc.).

For the positive and negative controls, inoculate TSIA, UA and LDC using a 1 µl loop, using microbiological reference cultures given in Table 1. Incubate samples and controls at 34 - 38 ˚C for 24±3 hours.

After incubation check the NA plate for purity. If pure (that is, all colonies have the same morphology and colour), then check the biochemical tests for presumptive *Salmonella* spp. according to Table 2. If the biochemical tests for the selected *Salmonella*-like colony do not confirm presumptive *Salmonella* spp., select a further 4 colonies (marked on the XLD and second enrichment plates) from different selective enrichment/isolation medium combinations and perform biochemical tests as described above to confirm the absence of *Salmonella* spp.

Note: If the purity plate shows a mixed culture, re-streak onto more NA plates to isolate the organisms and repeat step 8.8.1. until pure colonies have been obtained.

Table 2. Differentiation of *Salmonella* spp. using biochemical tests

Salmonella spp.	TSIA agar [14],				UA	LDC agar
	Butt	Slant	Gas	H_2S		
Salmonella spp.	Acid	NC or Alkaline	Positive	Positive	Negative	Positive
Escherichia coli	Acid	Acid	Positive	Negative	Negative	Positive
Proteus mirabilis	Acid	NC or Alkaline	Positive	Positive	Positive	Negative
Salmonella Typhi	Acid	NC or Alkaline	Negative	Positive	Negative	Positive
Salmonella Paratyphi	Acid	NC or Alkaline	Positive	Positive *	Negative	Positive *

* *S.* Paratyphi A is negative for hydrogen sulphide production and Lysine decarboxylation.

Key

TSIA agar - Acid = yellow colouration (Butt – glucose fermentation; Slant – lactose and/or sucrose fermentation); Alkaline = red colouration; NC = no colour change; Gas = Bubbles or cracks; H_2S positive = black colouration; Hydrogen sulphide negative = no black colouration.

UA - Urease positive = Pink/red colouration (Urea is hydrolysed, liberating ammonia); Urease negative = No change.

LDC agar - Lysine decarboxylase positive = Turbid with a purple colouration; Lysine decarboxylase negative = Yellow colouration.

Note: If the TSIA results indicate the presence of presumptive *S.* Typhi or *S.* Paratyphi A no further identification tests should be undertaken, unless the laboratory is equipped with the capability to handle micro-organisms belonging to hazard group 3. Cultures and all associated materials should be transferred to a category 3 exhaust cabinet and placed in formalin overnight. The items must then be sealed in autoclave bags and autoclaved separately from other waste material. All laboratory staff must be informed, and affected areas sterilised with formalin.

[14] In some instances, intense H_2S production in TSIA (shown by a uniform black colouration of the entire media) may mask any acid reaction in the butt and/or alkalinity of the slant. In such cases, and where there is gas production, assume the reaction conforms to that of *Salmonella* spp. (non Typhi or Paratyphi A) and proceed with confirmation. A note should be appended to the effect that hydrogen sulphide production was excessive.

8.8.2. Serological testing

If the biochemical tests indicate the presence of presumptive *Salmonella* spp. (other than *S.* Typhi or *S.* Paratyphi A), serological testing by slide agglutination using polyvalent antisera may be performed. Place one drop of polyvalent anti-O sera on a clear glass slide and inoculate, using a 1 µl loop, a portion of a colony from the NA purity plate and mix until homogenous. Rock the slide gently for approximately 1 minute and observe for any agglutination, i.e. the formation of granules in suspension. Repeat using polyvalent anti-H sera. Table 3 gives the interpretation of the confirmatory tests carried out on the selected colony.

Note: Colonies that confirmed as *Salmonella* spp. can be sent to a recognised *Salmonella* reference centre for definitive typing and to confirm the absence of the microbiological reference cultures. All appropriate local or international rules for transport of infectious materials e.g. UN 3373, should be followed.

Table 3: Interpretation of serological testing

Biochemical reactions	Auto-agglutination	Serological reactions	Interpretation
Typical *	No	O and H antigens positive	Strains considered to be *Salmonella* spp.
Typical *	No	O and H antigens negative	Presumptive *Salmonella* spp.
Typical *	Yes	Not tested because of auto-agglutination	
No typical reactions	-	-	Not considered to be *Salmonella* spp.
* presumptive biochemical identification by TSIA, urease negative result and Lysine decarboxylase positive.			

9. Reporting results

Results should be reported as *Salmonella* detected or not detected in a test portion of 25g (see ISO 7218).

If at least one colony from a sample conforms to the criteria stated in section 8 the sample result is reported as "*Salmonella* spp. detected in 25 g".

If no colonies from a sample conform to the criteria stated in section 8 then the sample result is reported as "*Salmonella* spp. NOT detected in 25 g".

10. Uncertainty of test results

Uncertainty inherent in any test method, i.e. instruments, media, analysts' performance, etc can be assessed by the repeatability and reproducibility of test results. These should be monitored through control tests analysed alongside sample tests, through in-house comparability testing between analysts and through external inter-comparison exercises to highlight any uncertainties within the test methods.

11. Quality control

Quality control systems offer a laboratory a mechanism to control preventable inaccuracies within a procedure.

11.1. Proficiency testing

It is recommended that laboratories undertaking microbiological examination of samples as part of a bivalve mollusc sanitation programme take part in comparative testing (also known as proficiency testing) relevant to the sample type(s) and method(s). Participation may be required under the sanitation programme (by national legislation or official procedures) or specified by importing countries. Comparative testing provides an indication of performance relative to other participating laboratories and can help improve the performance of the laboratory. Examples of comparative testing for bivalve molluscs are the FAO Reference Centre proficiency testing scheme (see https://www.cefas.co.uk/icoe/seafood-safety/services/proficiency-testing-and-quality-assurance/) and the UK HSA/Cefas Shellfish EQA scheme (for further information contact foodeqa@ukhsa.gov.uk).

11.2. Internal Quality Controls

Positive and negative controls should be included with each set of examinations (that is, those processed at approximately the same time) and for each medium type. The control strains listed in Table 1 (Section 7) may be used for this purpose. Controls should be inoculated onto separate plates from the samples. An uninoculated tube or bottle and/or plate should also be incubated with each set as a sterility control. Where more than one batch of medium has been used for the sample examinations, positive, negative and sterility controls should be included for each batch.

11.3. Internal Quality Assurance (IQA)

It is recommended that regular monitoring using known levels of target organism are examined to ensure routine *Salmonella* spp. procedures continue to be efficient and effective. An example for assessing quantitative methods is the use of Lenticule ™ discs.

12. References

ISO 7218:2007/Amd 1:2013. Microbiology of food and animal feeding stuffs – General requirements and guidance for microbiological examinations - Amendment 1. International Standards Organisation, Geneva.

ISO 6887-1:2017. Microbiology of food chain – 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. International Organization for Standardization, Geneva.

ISO 6887-3:2017. Microbiology of the food chain – Preparation of test samples, initial suspension and decimal dilutions for microbiological examination – Part 3: Specific rules for the preparation of fish and fishery products. International Standards Organisation, Geneva.

ISO 6579:2017. ‘Microbiology of food chain – Horizontal method for the detection, enumeration and serotyping of *Salmonella* – Part 1: Detection of *Salmonella* spp’. International Standards Organisation, Geneva.

ISO 6579-1:2017+A1:2020. ‘Microbiology of food chain – Horizontal method for the detection, enumeration and serotyping of *Salmonella* – Part 1: Detection of *Salmonella* spp. Amendment 1: Broader range of incubation temperatures, amendment to the status of Annex D, and correction of the composition of MSRV and SC’. International Standards Organisation, Geneva.

ISO 11133. Microbiology of food, animal feed and water — Preparation, production, storage and performance testing of culture media. International Organization for Standardization: Geneva, Switzerland.

Regulation (EC) No 854/2004 of the European parliament and the council, 29 April 2004 laying down specific rules for the organisation of official controls on products of animal origin intended for human consumption. Off. J. Eur. Communities L 226, 25.6.04 : 83-127.

Regulation (EC) No 882/2004 of the European Parliament and of the Council of 29 April 2004 on official controls performed to ensure the verification of compliance with feed and food law, animal health and animal welfare rules. Off. J. Eur. Communities L 165, 30.4.04 : 1-141.

Commission Regulation (EC) No 2073/2005 of the European Parliament and the Council, 15 November 2005 on microbiological criteria for foodstuffs. *Off. J. Eur. Communities* L338, 22.12.05 : 1-26.

13. Appendices

13.1. Appendix 1: Numbers of shellfish recommended for *Salmonella* analysis

ISO 6887-3 requires that a representative test sample contains a minimum of 10 animals, with a minimum weight of shellfish flesh and liquor of 50 g (25 g for smaller species). The following numbers of animals are recommended for inclusion in the homogenisation step to ensure the required minimum weight is obtained (the recommended number of animals for collection at the sampling stage is 10 % greater to allow for morbidity in a proportion of animals on receipt at the laboratory):

Type	Common name [15]	Scientific name [16]	Sample size [17]
Scallops	Mediterranean scallop	*Pecten jacobaeus*	10 - 12
	King (Great Atlantic) scallop	*Pecten maximus*	10 - 12
	Queen scallop	*Aequipecten (Chlamys) opercularis*	15 - 30
	Variegated scallop	*Mimachlamys (Chlamys) varia*	10 - 18
Oysters	Pacific oyster	*Magallana (Crassostrea) gigas*	10 - 18
	Portuguese oyster	*Magallana (Crassostrea) angulata*	10 - 18
	European flat oyster	*Ostrea edulis*	10 - 18
Mussels	Blue or common mussel	*Mytilus edulis*	15 - 30
	Mediterranean mussel	*Mytilus galloprovincialis*	10 - 30
	Northern horse mussel	*Modiolus modiolus*	10 - 12
	Bearded horse mussel	*Modiolus barbatus*	15 - 30
Ark Clams	Ark clam	*Barbatia barbata*	15 - 25
	Noah's ark shell	*Arca noae*	15 - 30
Clams	Smooth clam	*Callista chione*	10 - 30
	Striped venus clam	*Chamelea gallina*	40 - 80
	Rayed artemis	*Dosinia exoleta*	18 - 35
	Hard clam	*Mercenaria mercenaria*	12 - 18
	Palourde (Grooved carpet shell)	*Ruditapes (Tapes/Venerupis) decussatus*	18 - 35
	Manila clam	*Ruditapes (Tapes) philippinarum*	18 - 35
	Banded carpet shell	*Polititapes (Venerupis) rhomboides*	20 - 25
	Pullet carpet shell	*Venerupis corrugata (senegalensis)*	20 - 25
	Warty venus clam	*Venus verrucosa*	15 - 30
	Atlantic surf clam (Thick trough shell)	*Spisula solida*	30 - 50
	Cut trough shell	*Spisula subtruncata*	70 - 90
	Bean clam	*Donax spp.*	30 - 50
	Wedge shell clam	*Donax trunculus*	40 - 80
	Common cockle	*Cerastoderma edule*	30 - 50
	Soft shell clam (Sand gaper)	*Mya arenaria*	10 - 12

[15] Species of relevance provided by European NRLs.
[16] Scientific names are those used by the World Register of Marine Species at the time of writing, however commonly used alternatives for genus or species names are shown in brackets.
[17] Sample sizes given in this table have been provided by European NRLs. The weight of shellfish flesh and liquor must be at least 25 g for the *Salmonella* method. For species not given in the table, sufficient shellfish should be opened to achieve this minimum weight of flesh and liquor, with the provision that a minimum of ten animals should be used in all cases. In general, the more shellfish that are included in the initial homogenate, the less the final result will be influenced by the inherent animal-to-animal variation.

Razor Clams and Geoducks	Pacific geoduck	*Panopea generosa*	10 - 12
	Sword razor	*Ensis ensis*	10 - 12
	Razor shell	*Ensis magnus (arcuatus)*	10 - 12
	Atlantic razor clam (Jacknife clam)	*Ensis leei*	10 - 12
	Pod razor	*Ensis siliqua*	10 - 12
	Grooved razor shell	*Solen marginatus*	10 - 12
Gastropods	Abalone	*Haliotis spp.*	10 - 12
	Turbinate monodont	*Phorcus turbinatus*	30 - 50
	Whelk	*Buccinum undatum*	10 - 15
	Dog winkle	*Stramonita (Thais) haemastoma*	15 -25
	Common periwinkle	*Littorina littorea*	30 - 50
Echinoderms	Purple sea urchin	*Paracentrotus lividus*	10 - 30 (adults), 40 - 80 (juveniles)

Note: Laboratories are encouraged to submit suggestions on minimum sample sizes for other species to the FAORC. Please note that these recommendations must conform to the minimum requirement of 10 animals given in ISO 6887-3.

13.2. Appendix 2: Microbiological reference cultures for performance testing of media[18]

Media type	Function	Control strain	WDCM [a]	Criteria [b]
Buffered peptone water	Productivity	*Salmonella* Typhimurium [c d]	00031	Turbid (1-2)
		Salmonella Enteritidis [c d]	00030	
Muller-Kauffmann tetrathionate-novobiocin broth	Productivity	*Salmonella* Typhimurium [c d]	00031	>10 characteristic colonies on XLD agar or other medium of choice
		Salmonella Enteritidis [c d]	00030	
		+ *Escherichia coli* [d]	00012 or 00013	
		+ *Pseudomonas aeruginosa*	00025	
	Selectivity	*Escherichia coli* [d]	00012 or 00013	Partial inhibition ≤100 colonies on TSA
		Enterococcus faecalis [d]	00009 or 00087	<10 colonies on TSA
Rappaport-Vassiliadis soya enrichment broth	Productivity	*Salmonella* Typhimurium [c d]	00031	>10 characteristic colonies on XLD agar or other medium of choice
		Salmonella Enteritidis [c d]	00030	
		+ *Escherichia coli* [d]	00012 or 00013	
		+ *Pseudomonas aeruginosa*	00025	
	Selectivity	*Escherichia coli* [d]	00012 or 00013	Partial inhibition ≤100 colonies on TSA
		Enterococcus faecalis [d]	00009 or 00087	<10 colonies on TSA
Modified semi-solid Rappaport-Vassiliadis agar	Productivity	*Salmonella* Typhimurium [c d]	00031	Grey-white, turbid zone extending out from inoculated drop(s). After 24 h to 48 h, the turbid zone(s) will be (almost) fully migrated over the plate. Possible extra: characteristic colonies after subculturing on XLD agar
		Salmonella Enteritidis [c d]	00030	
	Selectivity	*Escherichia coli* [d]	00012 or 00013	Possible growth at the place of the inoculated drop(s) without a turbid zone
		Enterococcus faecalis [d]	00009 or 00087	No growth
Xylose lysine deoxycholate agar (XLD)	Productivity	*Salmonella* Typhimurium [c d]	00031	Good growth (2). Black centred colonies with light transparent zone of reddish colour
		Salmonella Enteritidis [c d]	00030	
	Selectivity	*Escherichia coli* [c]	00012 or 00013	Growth or partial inhibition (0-1) Yellow colonies if present.
		Enterococcus faecalis [c]	00009 or 00087	Total inhibition (0)
Nutrient agar (NA)	Productivity	*Salmonella* Typhimurium [c d]	00031	Good growth (2)
		Salmonella Enteritidis [c d]	00030	
L-Lysine decarboxylase broth (LDC)	Detection of L-Lysine decarboxylase (LDC)	*Salmonella* Typhimurium [c d]	00031	Positive reaction: Turbid growth and colour remains purple
		Salmonella Enteritidis [c d]	00030	
		Proteus mirabilis [c]	00023	Negative reaction: Colour change from purple to yellow
		Escherichia coli [c]	00012 or 00013	

[18] *S*. Nottingham is rarely isolated from the natural environment or food products, it therefore can be used as both a media performance selectively and productivity control and as an assay control in the event of a suspected laboratory contamination incident – currently *S.* Nottingham is not listed at WDCM**.**

Media type	Function	Control strain	WDCM [a]	Criteria [b]
Triple sugar iron agar (TSIA)	Multiple function	*Salmonella* Typhimurium [c d]	00031	Butt – Yellow: glucose fermented. Black: Formation of hydrogen sulphite. Bubbles or cracks: Gas formation Slant surface – Yellow: Lactose and/or sucrose utilized
		Salmonella Enteritidis [c d]	00030	
	Detection of H2S formation	*Pseudomonas aeruginosa*	00024 00025 or 00026	Butt – Red or unchanged: Glucose not fermented. Slant surface – Red or unchanged: Lactose and sucrose not utilized
Urea agar (UA)	Detection of urea hydrolysis by ammonia production	*Proteus mirabilis* [c]	00023	Positive reaction: Colour change to rose/rose-pink/deep cerise. Liberation of ammonia
		Klebsiella pneumoniae [c]	00097	
		Salmonella Typhimurium [c d]	00031	Negative reaction: No change of colour. No liberation of ammonia
		Salmonella Enteritidis [c d]	00030	
		Escherichia coli [c]	00012 00013 00090 or 00179	

[a] Follow the link (WDCM reference strains | Culture Collections) to obtain NCTC numbers for WDCM reference strains.
[b] Growth categorised as 0: no growth, 1: weak growth (partial inhibition), and 2: good growth.
[c] Some national restrictions and directions may require the use of a different serovar. Make reference to national requirements relating to the choice of *Salmonella* serovars.
[d] Strain of free choice, one of the strains has to be used as a minimum.

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