National Reference Laboratory for bacteriological contamination of bivalve molluscs

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

Note: This generic protocol must be used for official control testing in the UK

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History of Procedure

Issue	Date	Section	Changes
4	10.10.14	8.1 and 8.2	Update to include the extended transport temperatures and times.
		8.4 and 8.4.1	Inclusion of procedure for sterilising shucking implements after every sample.
		8.7.1	Amendment on how colonies are streaked onto BGA and XLD plates
		8.7.2	Amendment to the number of *Salmonella*-like colonies to be tested for confirmation.
		Appendix 1	Updated to include new shellfish species
5	28.01.19	Footnotes	The inclusion of footnotes throughout the protocol.
		2 and 12	Update of ISO 6579-1 publication number and date.
		3, 6, 7, 8.6 and 8.7	Inclusion of Modified semi-solid Rappaport-Vassiliadis agar plate (MSRV) as a selective enrichment medium
		6, 7, 8.9.1	Inclusion of L-Lysine decarboxylation medium for *Salmonella* spp. confirmation.
		7 and 13.2	Amendments to reference cultures used for media performance testing
		7.1, 8.6, 8.7, 8.8 and 8.9.	Update to internal procedure controls (positive and negative). The inclusion of link to identify WDCM numbers.
		8.1	Sample transport temperature should fall between 0˚C and 10˚C. Additional temperature criteria for samples receipted within 4 hours of sample collection. Guidance on verification of alternative transport criteria (ISO 6887-3)
		8.2	Sample should be examined within 24 hours following collection.
		8.6	Change sterile loop from 10µl to 1µl for inoculating control media.
		8.7	Removal of BGA and updated to Second isolation media is the laboratories choice.
		8.8	Update to procedures for confirming *Salmonella* spp. presence. The inclusion of guidance on the use of miniaturised galleries.
		8.8.1	Update to medium used for *Salmonella* confirmation
		8.8.2	Inclusion of serotyping for *Salmonella* confirmation.
		11	Quality control section with Internal quality controls and proficiency testing included.
		13.1 - Appendix 1	Update to species numbers

		13.2 - Appendix 2	List of reference cultures for performance testing of media
6	23.08.19	8.5.2	Footnote added relating to the use of filter bags to minimise the transfer of particulate material
		11.2	Footnote added stating recommended proficiency testing frequency requirements
		12	Update to reference list to included Regulations (Anon 2004, Anon 2004, Anon 2005 and Anon 2017)
7	03.10.23	Front page	Note added to identify that this NRL generic protocol should be used for official testing in the UK
		5	Equipment added to dissect gastropods and echinoderms
		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	Procedures for blending and stomaching harmonised with the NRL *E. coli* protocol
		8.8.2	Presumptive *Salmonella* isolates can be sent to a *Salmonella* Reference centre for confirmation
		11.2	Information added on the use of Internal Quality Controls
		13.1 - Appendix 1	Update to species names etc.

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 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.

In the UK, the criteria for laying down the microbiological standards for BMS and other marine shellfish including gastropods and echinoderms are set out in Retained Regulation (EC) 2017/625 (Anon, 2017) and Retained Regulation (EC) 2073/2005 (Anon, 2005) stipulating conditions for the production and placing on the market of live shellfish.

2. Scope

This protocol has been produced with reference to ISO 6579-1 (Anon, 2017) 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 bivalve molluscs as an indication of contamination of harvested shellfish and for the end-product testing of the depurated/cooked product. Strains of *S.* Typhi and *S.* Paratyphi may be isolated if present in the sample, however this procedure may not detect all *S.* Typhi and *S.* Paratyphi.

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.

4. Safety precautions

Standard microbiology safety precautions should be applied throughout. Laboratories should perform a full risk assessment before performing this protocol. Homogenisation of shellfish should be performed in a Class II safety cabinet to reduce the risk of infection from aerosol inhalation. *Salmonella* spp. should be handled in accordance with ACDP category 2 guidelines.

5. Equipment

- Waring blender and jars or Stomacher and Stomacher bags [1]
- Class II safety cabinet
- Electronic top pan balance
- Refrigerator at 5±3˚C and 3±2˚C
- Sterile glassware
- Shucking knife, oyster cracker or other suitable equipment for opening shellfish
- Safety/electric Bunsen system
- Protective gloves – single use
- Safety gloves – for example a chain mail glove
- Incubator at 37±1˚C and 41.5±1˚C
- Loops - sterile, 1µl and10µl
- 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
- Sterile forceps
- Sterile scissors
- Sterile spatula
- Sterile hammer or equivalent tool
- Food grade plastic bags
- Absorbent paper towel

6. Media [2, 3]

- Ethanol
- 0.1% peptone (0.1% P)
- Peptone salt solution (PSS) (referred to as Maximum Recovery Diluent (MRD) in the UK)
- Buffered peptone water (BPW)
- Muller-Kauffmann tetrathionate-novobiocin broth base (MKTTn)
- Rappaport-Vassiliadis soya enrichment broth (RVS)
- Modified semi-solid Rappaport-Vassiliadis agar (MSRV)
- Xylose lysine deoxycholate agar (XLD)
- Triple sugar iron agar (TSIA)
- Urea agar (UA)
- L-Lysine decarboxylation medium (LDC)
- Nutrient agar (NA)
- Saline solution
- Polyvalent anti-O sera and anti-H sera

Note: See ISO 6579-1 for media formulation.

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

[2] Allow media to equilibrate at room temperature before use.

[3] For performance testing of media and reagents see Appendix 2.

7. Microbiological reference cultures for performance testing and controls

The NRL recommends the use of the positive and negative controls throughout the procedure. The strains and criteria included in Table 1 are recommended for use as a minimum, alternative control organisms may be used (e.g. those included in Appendix 2 Performance Testing of Media and Reagents).

Table 1: Microbiological reference strains used for control purposes

Media type	Control strain	WDCM [a]	Criteria
Buffered peptone water (BPW)	*Salmonella* Typhimurium or *Salmonella* Nottingham [b, c]	00031 NCTC 7832	Turbid
Muller-Kauffmann tetrathionate-novobiocin broth (MKTTn)	*Salmonella* Typhimurium or *Salmonella* Nottingham [b, c]	00031 NCTC 7832	Growth
	Escherichia coli	00012 or 00013 [b]	
Modified semi-solid Rappaport-Vassiliadis agar (MSRV)	*Salmonella* Typhimurium or *Salmonella* Nottingham [b, c]	00031 NCTC 7832	Growth
	Escherichia coli	00012 or 00013 [b]	
Rappaport-Vassiliadis soya enrichment broth (RVS)	*Salmonella* Typhimurium or *Salmonella* Nottingham [b, c]	00031 NCTC 7832	Growth
	Escherichia coli	00012 or 00013 [b]	
Xylose lysine deoxycholate agar (XLD)	*Salmonella* Typhimurium or *Salmonella* Nottingham [b, c]	00031 NCTC 7832	Black centred colonies with light transparent zone of reddish colour
	Escherichia coli	00012 or 00013 [b]	Growth / partial inhibition of yellow colonies
Triple sugar iron agar (TSIA)	*Salmonella* Typhimurium or *Salmonella* Nottingham [b, c]	00031 NCTC 7832	Butt – Yellow; Slant surface - Yellow; Black - Formation of hydrogen sulphite; Bubbles or cracks – Gas.
	Pseudomonas aeruginosa [d]	00024, 00025 or 00026	Butt – Red or no colour change; Slant surface – Red or no colour change.
Urea agar (UA)	*Salmonella* Typhimurium or *Salmonella* Nottingham [b, c]	00031 NCTC 7832	No colour change
	Proteus mirabilis [d]	00023	Pink/red colouration
Lysine decarboxylase broth (LDC)	*Salmonella* Typhimurium or *Salmonella* Nottingham [b, c]	00031 NCTC 7832	Turbid with a purple colouration
	Escherichia coli	00012 or 00013 [b]	Yellow colouration

[a] Follow the link (http://www.phe-culturecollections.org.uk/products/bacteria/WDCMStrains.aspx) to obtain NCTC number for WDCM reference strains.

[b] Strain of free choice, one of the strains has to be used as a minimum.

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

[d] Strain to be used as a minimum

8. Procedures

8.1. Sample transport and receipt [4]

Samples must be placed in an intact food grade plastic bag 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] For samples where less than 4 hours have elapsed between collection from the production area and receipt at the laboratory, the internal cool box air temperature (or between-shellfish sample temperature) should be less than the temperature recorded at the time of sampling. Samples from harvesting areas should have been rinsed (but not immersed) and drained at time of sampling. A sample 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 Official Controls, 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 relevant Competent Authority (CA).

Note: The UK NRL has carried out studies on the effect of extended storage time and elevated temperatures on certain species of BMS. The report of this work can be accessed using the following web link https://www.cefas.co.uk/nrl/information-centre/nrl-reports/

8.2. Sample storage [6]

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 microbiological analysis cannot be initiated within 24 hours of sample collection, an absolute limit of 48 hours should be used.

8.3. Sample selection

Choose shellfish that are alive according to the following criteria:

- Movement of any kind if any exposed flesh reacts to touch using a sterile shucking knife.
- Shellfish must open and close of their own accord.
- A tap on the shell causes closing or movement.
- Tightly closed shellfish.

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

[4] This is a combination of NRL advice and UK monitoring programme requirements.
[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.
[6] NRL advice.

required volumes for each analysis.

8.4. Sample preparation

Any mud and sediment adhering to the shell should be removed prior to opening 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[7].

8.4.1. Preparation of BMS 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 meat of both shells into the sterilised container. Transfer any liquor collected in the weighing boat into the sterilised container.

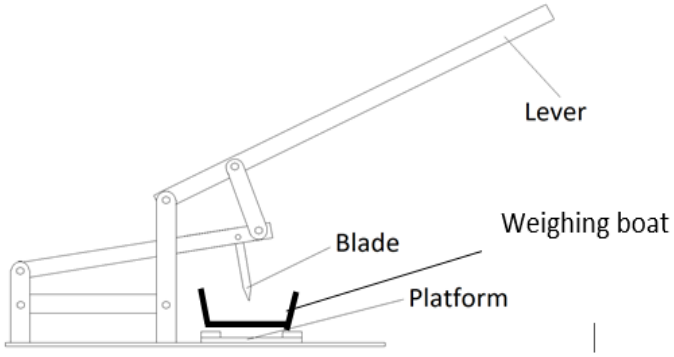

Figure 1: Oyster cracker apparatus

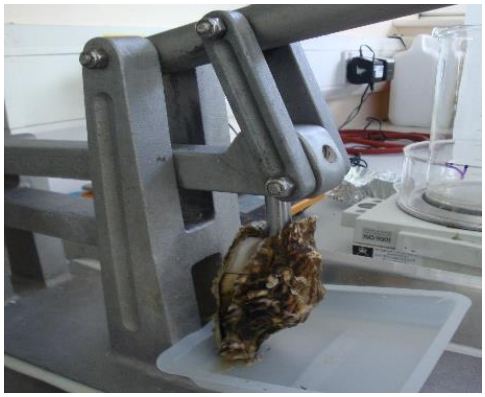

Figure 2: Opening an oyster using an oyster cracker

8.4.2. Preparation of BMS using a shucking knife

Open all selected shellfish as described below using a sterilised shucking knife or equivalent and empty meat 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.

[7] Alternative 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 lever 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.

Figure 3: Opening a gastropod using an oyster cracker

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.

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

Hold the echinoderm with the mouth parts facing upwards. Using sterile scissors and wearing a strong clean glove, cut out the mouth section (Figure 4) 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 [8, 9]

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.

8.5.1. Homogenisation in a blender

Measure 2ml of sterile BPW per 1g of shellfish (±2ml) 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±1ml of mixed shellfish homogenate to 175±5ml of BPW to make a master 10^{-1} dilution, 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 NRL *E. coli* protocol can be used by taking 75ml of the 1:3 dilution and adding this to 175ml of BPW [10].

8.5.2. Homogenisation using a stomacher [11]

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±0.5g of homogenised shellfish into another set of 3 stomacher bags, add 50±1ml of BPW and homogenise for a further 2-3 minutes. After homogenisation decant the contents into a new sterilised container and add 175±5ml BPW to make a master 10^{-1} dilution, ensure that this master dilution is thoroughly mixed. Label as the 10^{-1} primary enrichment.

[8] ISO 6887-3 allows the use of PSS for preparation of the primary dilution. NRL advice is to use BPW instead at this stage in order to avoid the addition of extra NaCl.

[9] 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.

[10] This approach has been agreed as equivalent at annual meetings of the 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 prepared using 0.1% P and 175 ml BPW.

[11] Results from proficiency testing distributions of cockles have tended to give lower *E. coli* concentrations following stomaching than following blending. NRL advice is therefore to use blending for samples of cockles and other small species such as clams.

8.5.3. Process controls

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

8.5.4. Incubation of primary enrichment broth

Incubate the sample and the process controls at 37±1˚C [12] for 18±2 hours.

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

8.6. Inoculation and incubation of selective enrichment media

Following incubation of BPW, inoculate and incubate MKTTn broth and either RVS or MSRV as described below.

8.6.1. Rappaport-Vassiliadis soya enrichment broth (RVS)

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

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

Drop 100±20µl 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±0.1ml of the BPW primary enrichment to 10±1ml of the MKTTn and incubate 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 10µl loop, inoculate each selective enrichment medium (MKTTn and RVS or MSRV) with the BPW positive control inoculated with *Salmonella* spp. (prepared in 8.5.3). Repeat the inoculation of MKTTn 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

[12] 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.

hours.

8.7. Isolation of *Salmonella*

Following incubation of the RVS and MKTTn broths, subculture onto the surface of two 90mm XLD plates and two 90mm plates of a second isolation medium. Use a 10µl loop for each medium and inoculate without recharging the loop between duplicate plates to obtain well-isolated colonies [13].

Following incubation of the MSRV plate, examine for the presence of a grey-white turbid zone 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 1µl loop for each medium. If no growth is visible after incubation of the MSRV agar, 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 each control onto one XLD plate and one second isolation medium plate.

Incubate XLD plates at 37±1˚C for 24±3 hours. Incubate the second isolation medium in accordance with the manufacturer's instructions.

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

XLD: Colonies with black centres and a light transparent zone of reddish colour. [14]
Second isolation medium: Follow the manufacturer's instructions for colony characteristics.

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

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 contamination

Mark the location of suspect *Salmonella*-like colonies on the XLD and second isolation medium plates. Subculture one well-isolated colony, using a 1µl 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) then streaking the surface (slant). Next, stab the UA followed by LDC broth

[13] To obtain well isolated colonies, a single 140mm diameter plate can be used.
[14] 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 1µl loop, using microbiological reference cultures given in Table 1. Incubate samples and controls at 37±1˚C for 18-24 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 isolation medium 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.**[15]	**TSIA agar** [16]				**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; H_2S 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.

8.8.2. Serological testing

If the biochemical tests indicate the presence of presumptive *Salmonella* spp. (other than *S.*

[15] If TSIA results indicate the presence of presumptive *S.* Typhi (Risk group 3) no further tests should be undertaken. All infectious waste (all cultures and associated material) must be transported in a sealed, unbreakable and leakproof container prior to autoclaving separately from other waste. All presumptive *S.* Paratyphi A, B or C (Risk group 2) colonies can be disposed of in accordance with biological waste.

[16] 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.

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 1µl 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 are 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 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

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 25g".

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 25g".

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

In order to comply with the requirements of Retained Regulation (EC) No. 2017/625, laboratories undertaking microbiological examination of official control samples of shellfish for *E. coli* and/or *Salmonella* spp. are expected to take part in the UKHSA/Cefas Shellfish EQA scheme (for further information contact foodeqa@ukhsa.gov.uk) and NRL proficiency test distributions [17]. These schemes provide an independent assessment of a laboratory's performance against other participants and can help improve the performance of the laboratory.

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 (e.g. monthly) 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.

[17] It was agreed at the Laboratory network meeting in 2018 that laboratories analysing OC samples should take part in at least 2 EQA (UKHSA/Cefas) schemes and 1 whole animal distribution per year.

12. References

Anon 2004. Retained 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.

Anon 2005. Retained 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.

Anon 2013. 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.

Anon 2017. 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.

Anon 2017. 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.

Anon 2017. 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.

Anon 2020. Regulations 2020: Cross reference Articles 100-101 of Retained Regulation (EU) 2017/625, as amended by The Official Controls for Animals, Feed and Food, Plant Health etc. (Amendment) (EU Exit) Regulations 2020.

Anon 2020. 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.

13. Appendices

13.1. Appendix 1: Sample sizes of shellfish required for *Salmonella* analysis

The following sample sizes are recommended for inclusion in the homogenisation step (the recommended number for sampling is 10% greater to allow for morbidity in a proportion of animals on receipt at the laboratory).

Type	Common name [18]	Scientific name [19]	Sample size [20]
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

[18] Species of relevance provided by European NRLs.
[19] 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.
[20] Number of shellfish to be tested for *Salmonella*. Retained Regulation (EC) No. 2073/2005 specifies a minimum of 10 animals of any species should be examined. Sample sizes given in this table have been provided by European NRLs. The weight of shellfish flesh and liquor should be at least 25g 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 for very large species. 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)

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

Media type	Function	Control strain	WDCM [a]	Criteria [b]
Buffered peptone water (BPW)	Productivity	*Salmonella* Typhimurium [c d]	00031	Turbid
		Salmonella Enteritidis [c d]	00030	Turbid
Muller-Kauffmann tetrathionate-novobiocin broth (MKTTn)	Productivity	*Salmonella* Typhimurium [c d]	00031	Growth
		Salmonella Enteritidis [c d]	00030	
		+ *Escherichia coli* [d]	00012 or 00013	
		+ *Pseudomonas aeruginosa*	00025	
	Selectivity	*Escherichia coli* [d]	00012 or 00013	Partial inhibition
		Enterococcus faecalis [d]	00009 or 00087	Partial inhibition or no growth
Rappaport-Vassiliadis soya enrichment broth (RVS)	Productivity	*Salmonella* Typhimurium [c d]	00031	Good growth
		Salmonella Enteritidis [c d]	00030	
		+ *Escherichia coli* [d]	00012 or 00013	
		+ *Pseudomonas aeruginosa*	00025	
	Selectivity	*Escherichia coli* [d]	00012 or 00013	Partial inhibition
		Enterococcus faecalis [d]	00009 or 00087	Partial inhibition or no growth
Modified semi-solid Rappaport-Vassiliadis agar (RVS)	Productivity	*Salmonella* Typhimurium [c d]	00031	Good growth with grey-white, turbid zone extending out from inoculated drop.
		Salmonella Enteritidis [c d]	00030	
	Selectivity	*Escherichia coli* [d]	00012 or 00013	Possible growth at inoculation drop 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	

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 00013 00090 or 00179	

[a] Follow the link (https://www.culturecollections.org.uk/products/bacteria/WDCMStrains.aspx) to obtain NCTC number 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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