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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	01.09.20	All	First issue

Contents

1 INTRODUCTION

The acquisition of infectious human diseases from the consumption of bivalve molluscan shellfish (BMS) is internationally recognised as a food safety risk. This health hazard is largely due to the phenomenon of filter feeding whereby bivalve molluscs 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 bivalve shellfish raw, or only lightly cooked. The presence of salmonellae in ready-to-eat food including BMS is considered significant regardless of the level of contamination. Salmonellae show little host specificity and can cause gastroenteritis when ingested by man. Incubation time is usually 12-36 hours although it may be longer as multiplication must occur in the intestines. Salmonellae 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 11. It is suitable for use with raw and cooked shellfish. It describes the method for detecting the presence of salmonellae in bivalve molluscs 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.

3 PRINCIPLE

This protocol for the enumeration of salmonellae in bivalve molluscs involves an initial selective - 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 to xylose lysine deoxycholate (XLD) agar. Presumptive colonies of salmonellae isolated from XLD 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 using this protocol. Homogenisation of shucked bivalve molluscs 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

- 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, mechanical oyster shucker or other suitable equipment for opening bivalve molluscs
- Weighing dish capable of holding the largest size of bivalve mollusc to be tested plus released intravalvular fluid. Must be capable of being cleaned and sterilized. [For use with oyster cracker]
- Safety gloves – for example a chain mail glove (for the bivalve opening procedure)
- 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
- Timer
- Incubator or recirculating water bath at 34 - 38 ˚C and 41.5±1 ˚C [A]
- Platinum or sterile disposable loops - 1 µl and 10 µl
- Pipettor - automatic or manual for use with 1 ml and 10 ml sterile pipette tips or 1 ml and 10 ml sterile open-ended graduated pipettes.

6 MEDIA AND REAGENTS [B]

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.

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. Dispense into 10±0.2 ml volumes in tubes (or bottles). Cap the tubes (or bottles) and sterilize by autoclaving at 116 °C for 10 minutes. 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 autoclaving at 121 °C for 15 minutes. Allow to set in a sloped position with a 2.5 ml to 5 ml butt depth. 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 5ml - deionised water 5±0.2 ml, 1 x tablet Lysine

[A] 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.

[B] 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.

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 [C]

Table 1: Microbiological reference strains used for media performance testing

Media type	Function	Control strain	WDCM [a]	Characteristic reactions [b]
Buffered peptone water (BPW)	Productivity	*Salmonella* Typhimurium [c d]	00031	Turbid (1-2)
		Salmonella Enteritidis [c d]	00030	
Muller-Kauffmann tetrathionate - novobiocin broth (MKTT)	Productivity	*Salmonella* Typhimurium [c d]	00031	>10 characteristic colonies on XLD agar or other medium of choice
		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	>10 characteristic colonies on XLD agar or other medium of choice
		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
Modified semi-solid Rappaport-Vassiliadis agar (MSRV)	Productivity	*Salmonella* Typhimurium [c d]	00031	Grey-white turbid zone extending out from inoculated drop. Characteristic colonies after subculture on XLD agar
		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	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	

[a] Follow the link http://refs.wdcm.org/species.htm to obtain culture collection numbers for WDCM reference strains

[b] Growth is categorised as 0: no growth, 1: weak growth (partial inhibition), and 2: good growth

[c] Strain choice: 1 – 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

[C] These strains differ from those recommended in ISO TS 11133-2:2003 (as amended 2011) for the quality control of selective enrichment and selective isolation media for *Salmonella* spp.

Table 1 continued: Microbiological reference strains used for media performance testing

Media type	Function	Control strain	WDCM [a]	Characteristic reactions [b]
L-Lysine decarboxylase broth (LDC)	Detection of L-Lysine decarboxylase (LDC)	*Salmonella* Typhimurium [c d]	00031	Positive reaction: Turbid growth and Medium remains purple
		Salmonella Enteritidis [c d]	00030	
		Proteus mirabilis [c]	00023	Negative reaction: Medium changes colour 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	*Pseudomonus 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 http://refs.wdcm.org/species.htm to obtain culture collection numbers for WDCM reference strains
[b] Growth is categorised as 0: no growth, 1: weak growth (partial inhibition), and 2: good growth
[c] Strain choice: 1 – 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

Note: These strains may also be used as internal quality controls (see Section 10.1).

8 PROCEDURE

8.1 Sample transport and receipt

Samples must be received 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. 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/) or samples where less than 4 hours have elapsed between collection from the growing area and receipt at the laboratory, internal air temperature should be less than the temperature recorded at the time of sampling. Samples should be regarded as unsatisfactory if on receipt at the laboratory the sample is frozen, the container is leaking, the bivalve molluscs 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 (http://www.fao.org/3/CA1213EN/ca1213en.pdf).

Note: The UK National Reference Laboratory for foodborne viruses and bacteriological contamination of bivalve molluscs has carried out studies on the effect of extended storage time and elevated temperatures on certain species of bivalve molluscs. 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

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 bivalve molluscs that are alive according to the following points:

- Movement of exposed flesh to touch using a sterile shucking knife
- Bivalve molluscs are open and close of their own accord
- A tap on the shell causes closing or movement
- Tightly closed bivalve molluscs

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

8.4 Sample preparation

Any material adhering to the shell should be removed prior to opening by rinsing/scrubbing under cold, running tap water of potable quality. Bivalve molluscs should not be re-immersed in water as this may cause them to open. Open all selected bivalve molluscs as described below.

8.4.1 Mechanical oyster cracker

An example of a mechanical oyster shucker is shown in Figure 1. Sterilise the pin of the mechanical oyster shucker before use. Place a single animal in a sterilized weighing dish underneath the shucking device and rest the shucking lever at the hinge. Pull the handle down to separate the shells. Using a sterilised shucking knife, cut the muscle and scrape the meat of both shell halves into the sterilised container. Transfer any liquor collected in the weighing boat into the sterilised container.

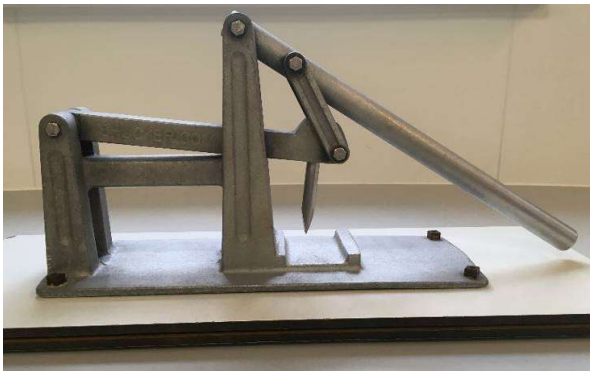

Figure 1. One type of mechanical oyster shucker

8.4.2 Shucking with a knife

Using a sterilised shucking knife, open all selected bivalve molluscs as described below. If sterilised by heating allow the knife to cool before use. When opening bivalve molluscs, ensure that the hand holding the shell is protected with a heavy-duty safety glove to prevent cuts.

8.4.1.1 Oysters and large 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.

8.4.1.2 Mussels, cockles and small clams

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.

8.5 Homogenisation [D]

Place the contents of the sterile container into the inner of at least three stomacher bags, placed inside each other, to avoid small pieces of shell from puncturing the bags. Remove excess air from the bag and operate the stomacher for 2 - 3 minutes.

8.6 Inoculation and incubation of primary enrichment broth

Weigh 25±0.3 g of prepared homogenate and add to a bottle (or flask) containing 225±5 ml of BPW (pre-warmed at room temperature). Inoculate a 10±1 ml volume of BPW with a selected positive control *Salmonella* spp. strain (see Table 1) using a 1 µl loop and leave a second 10±1 ml volume of BPW uninoculated as the negative control. Incubate the sample and controls at 34 - 38 ˚C for 18±2 hours.

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

8.7 Inoculation and incubation of selective enrichment broths [E]

Following incubation of BPW, inoculate either RVS or MSRV and MKTTn using a pipette as described below. For positive and negative controls for selective enrichments refer to Table 1, inoculate using a 1 µl loop and incubate according to 8.7.1 to 8.7.3.

8.7.1 Rappaport-Vassiliadis soya enrichment broth (RVS)

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

8.7.2 Modified semi-solid Rappaport-Vassiliadis agar plate (MSRV)

Drop 100±20 µl of the BPW enrichment on to 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.**

8.7.3 Muller-Kaufmann tetrathionate-novobiocin broth (MKTTn)

Transfer 1±0.1ml of the BPW enrichment to 10±1 ml of MKTTn amended with iodine and novobiocin prior to use (See section 6) and incubate at 34 - 38 ˚C for 24±3 hours.

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

8.8 Isolation of *Salmonella* [F]

Following incubation of the RVS and MKTTn broths, subculture onto the surface of one XLD plate and one plate of a second isolation medium, 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 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.

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

[E] MSRV agar is used to detect motile *Salmonella* spp. and is not appropriate for the detection of non-motile *Salmonella* spp.

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

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

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

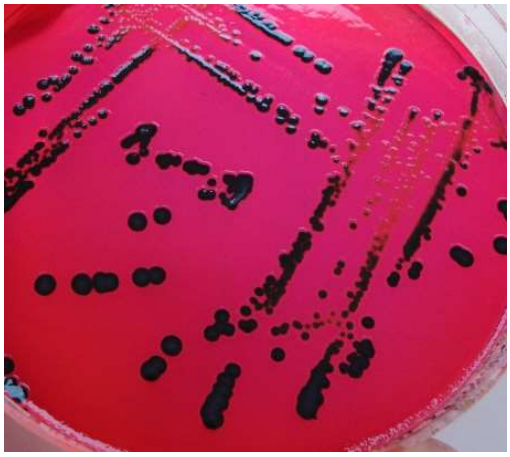

Figure 1: Image of *Salmonella* spp. colonies sub-cultured on to an XLD

If no Salmonella-like colonies are present, then report the result as '*Salmonella* NOT detected in 25g.'
If typical Salmonella-like colonies are present in the sample, continue with confirmation as described in 8.9.

8.9 Salmonella confirmation [H]

Confirmation of presumptive *salmonella* spp. can be achieved using the procedures identified in 8.9.1. If shown to be reliable, miniaturised galleries for the biochemical identification of salmonellae may be used. Additionally, alternate procedures (e.g. agglutination, molecular probe-based approaches) can be used to confirm the isolate as *salmonella* spp. providing that the suitability of the alternative procedure is verified (see ISO 7218).

8.9.1 Colony selection and biochemical inoculation

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 °C - 38 °C for 24 h ± 3 h.

Subculture 1 well-isolated colony, using a 1 µl loop, into a TSIA slopes, 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). Then stab the UA followed by LDC broth 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) 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.

[G] 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.
[H] The use of additional biochemical tests are described in ISO 6579-1. Commercially available kits that have been shown to produce reliable results may be used as an alternative.

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

Salmonella spp.	TSIA agar [I]				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*

**Salmonella* 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.

8.9.2 Serological testing [J] [K]

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. A positive agglutination is 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 recognised Salmonella reference centre for definitive typing and to confirm the absence of the microbiological reference cultures.

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.			

[I] 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.

[J] Auto-agglutination can affect a serological test result. To check for auto-agglutination place one drop of saline solution on a clear glass slide and inoculate with a portion of a colony from an NA purity plate and mix until homogenous. Rock gently for up to 1 minute and observe any auto-agglutination (Agglutination is the formation of granules in suspension).

[K] Further guidance on serological confirmation and on serotyping is given in ISO/TR 6579-3.

8.10 REPORTING RESULTS

Results should be reported as *Salmonella* detected or not detected in a test portion of *x* g or *x* ml of product (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 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".

9 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.

The inclusion of the measurement uncertainty (MU) or confidence limits (CL) for the result is not required but it is recommended that laboratories determine, as part of their quality procedure, the MU for the reported results and provide this information on request.

10 QUALITY CONTROL

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

10.1 Internal quality controls

Sterility controls should be set up for each container of diluent (0.1% P or PSS) that is used, at the beginning and end of each set of samples that is examined.

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.

10.2 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.

10.3 Comparative 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 PHE/Cefas Shellfish EQA scheme (for further information contact foodeqa@phe.gov.uk).

11 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.

12 APPENDICES

12.1 Appendix 1: Sub-sample sizes of shellfish required for *Salmonella* spp. analysis

Suggested minimum sample sizes given in this table have been provided by several laboratories. ISO 6887-3 specifies the use of a minimum of ten animals. The weight of bivalve mollusc flesh and liquor should be at least 50 g (for very small species such as the *Donax* spp. a minimum amount of 25 g can be used for practical reasons). For species not given in the table, sufficient bivalve molluscs should be opened to achieve this minimum weight of flesh and liquor, with the provision that a minimum of ten animals should be used even for very large species. In general, the more bivalve molluscs that are included in the initial homogenate, the less the result will be influenced by the inherent animal-to-animal variation.

The following sub-sample sizes are recommended for inclusion in the homogenisation step:

Name	Latin name	Sample size
Saint James Scallop	*Pecten jacobaeus*	10 - 12
King scallops	*Pecten maximus*	10 - 12
Razor clams	*Ensis spp.*	10 - 12
Soft shell clams (Sand Gapers)	*Mya arenaria*	10 - 12
Northern horse mussels	*Modiolus*	10 - 12
Abalone	*Haliotis* spp	10 - 12
Whelks	Buccinum undatum	10 - 15
Variegated scallop	*Chlampys varia*	10 - 18
Oysters	*Crassostrea gigas and Ostrea edulis*	10 - 18
Noah's Ark shells	*Arca noae*	10 - 25
Mediterranean mussels	*Mytilus galloprovincialis*	10 - 30
Smooth clams	*Callista chione*	10 - 30
Purple or green Sea urchins – Adults (juvenile)	*Paracentrotus lividus*	10 – 30 (40 – 80)
Hard clams	*Mercenaria*	12 - 18
Dog winkles	*Thais haemastoma*	15 - 25
Ark clams	*Barbatia barbata*	15 - 25
Bearded horse mussels	*Modiolus barbatus*	15 - 30
Queen scallops	*Aequipecten opercularis or Chlamys opercularis*	15 - 30
Warty venus clams	*Venus verrusosa*	15 - 30
Mussels	*Mytilus spp.*	15 - 30
Manila clams	*Tapes philippinarum*	18 - 35
Palourdes (Grooved carpet shell clams)	*Tapes decussatus* (*Venerupis decussata)*	18 - 35
Rayed artemis	*Dosinia exoleta*	18 - 35
Clam	*Venerupis rhomboides*	20 - 25
Pullet carpet shell	*Venerupis senegalensis*	20 - 25
Cockles	*Cerastoderma edule*	30 - 50
Turbinate monodont	*Phorcus turbinatus*	30 - 50
Atlantic surf clams (Thick trough shells)	*Spisula solida*	30 - 50
Periwinkles	Littorina littorea	30 - 50
Bean clams	*Donax spp.*	30 - 50
Wedge shell clams	*Donax trunculus*	40 - 80
Striped venus clams	*Chamelea gallina*	40 - 80
Cut trough shells	*Spisula subtruncata*	70 - 90

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.