FAO Reference Centre for Bivalve Mollusc Sanitation

Generic protocol – Detection of *Vibrio parahaemolyticus, Vibrio cholerae* and *Vibrio vulnificus* in bivalve molluscan shellfish (based on ISO 21872-1)

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History of Procedure

Issue	Date	Section	Changes
1	29/11/24	All	First issue (replacing protocol originally prepared by the former EURL for monitoring bacteriological and viral contamination of bivalve molluscs)

Contents

1. Introduction 6
2. Scope 6
3. Principle 6
4. Safety precautions 7
5. Equipment 7
6. Media and reagents 8
7. Microbiological reference cultures for media performance testing 10
8. Procedure 11
    8.1. Sample transport and receipt 11
    8.2. Sample storage 12
    8.3. Sample selection 12
    8.4. Sample preparation 12
        8.4.1. Preparation of bivalve molluscs using the oyster cracker 12
        8.4.2. Preparation of bivalve molluscs using a shucking knife 13
        8.4.3. Preparation of gastropods 14
        8.4.4. Preparation of echinoderms (e.g. sea urchins) 14
    8.5. Homogenisation, inoculation and incubation of primary enrichment broth 14
        8.5.1. Homogenisation in a blender 14
        8.5.2. Homogenisation in a stomacher 15
        8.5.3. Process controls 15
        8.5.4. Incubation of primary enrichment broth 15
    8.6. Subculture of primary and secondary enrichments broths 15
        8.6.1. Subculture of primary enrichment and preparation and incubation of secondary enrichment broth 15
        8.6.2. Subculture of secondary enrichment 16
    8.7. Isolation and identification 16
    8.8. *Vibrio* confirmation 17
        8.8.1. Selection of colonies for confirmation 17
        8.8.2. Oxidase test 17
        8.8.3. Biochemical confirmation 18
        8.8.4. PCR confirmation 19
9. Reporting results 24
10. Uncertainty of test results 24

11. Quality control....25
11.1. Internal Quality Controls....25
12. References....26
13. Appendices....27
13.1. Appendix 1: Sample sizes of shellfish for *Vibrio* analysis....27

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 present in their growing waters. The risks of exposure to infectious agents are compounded by the traditional consumption of raw, or only lightly cooked, BMS. Vibrios are Gram-negative, rod-shaped bacteria; the genus *Vibrio* contains over 100 described species, and at least a dozen of these have been demonstrated to cause infections in humans. Typically, *Vibrio* infections are initiated from exposure to seawater or consumption of raw or undercooked seafood including BMS. The species most commonly associated with foodborne infections include *V. parahaemolyticus, V. cholerae* and *V. vulnificus,* all of which are natural inhabitants of warm, low salinity estuarine and marine environments.

Vibrio parahaemolyticus is a halophilic bacterium that is commonly associated with gastroenteritis following the consumption of inadequately cooked, raw or post-process contaminated seafoods. It can be isolated from both fish and shellfish and is not related to sewage contamination. Globally, *V. parahaemolyticus* is the most common bacterial cause of illness associated with consumption of shellfish. *Vibrio cholerae* is often described as non-halophilic as it can grow in the absence of salt. Toxigenic *V. cholerae* (O1/O139) strains are the causative agent of cholera, a severe diarrhoeal illness usually caused by ingestion of contaminated food or water, although person-to-person transmission is also possible. Non-toxigenic *V. cholerae* strains are sometimes associated with consumption of contaminated seafood and can cause mild gastroenteritis or lead to severe infections, including sepsis, in susceptible individuals. *Vibrio vulnificus* is a globally significant pathogen capable of causing acute gastroenteritis following foodborne transmission and, in some cases more severe symptoms including necrotizing wound infections and primary septicaemia (including following foodborne transmission); it is a leading cause of seafood-related mortality. A range of other *Vibrio* species have also been implicated in seafood-related human infections including *V. alginolyticus* and *V. mimicus.*

2. Scope

This protocol has been produced with reference to ISO 21872-1:2017 and selected parts of other relevant ISO standards referenced in Section 12. It describes the method for the detection of potentially enteropathogenic *Vibrio* spp. (*V. parahaemolyticus, V.cholerae* and *V. vulnificus*) in BMS. It includes molecular tests to confirm detection of each species and, for *V. parahaemolyticus,* presence of the thermostable direct haemolysin (*tdh)* and TDH-related haemolysin (*trh)* genes, which are markers for pathogenicity.

3. Principle

This protocol for the enumeration of *V. parahaemolyticus, V.cholerae* and *V. vulnificus* in BMS involves initial two-stage parallel selective enrichments in alkaline saline peptone water (ASPW) at 41.5 °C and 37 °C, followed by direct plating of the enrichments onto thiosulphate citrate bile sucrose agar (TCBS) and a second solid isolation medium. Presumptive colonies of *V. parahaemolyticus, V.cholerae* and *V. vulnificus* isolated from TCBS and the second solid isolation medium plates are

sub-cultured onto saline nutrient agar (SNA) and then subjected to biochemical testing, conventional PCR or real-time PCR 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. 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 shucked BMS 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. *Vibrio* spp. should be handled in accordance with ACDP category 2 guidelines.

Note: the use, storage and transport of *V. cholerae* can be limited by legislation in some countries; laboratories must consider these additional requirements in their safety precautions where relevant.

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
- 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 units at 25 °C. Reference solutions for calibrating the pH meter
- Local Exhaust Ventilation (LEV; for weighing powders during media preparation)
- Class II safety cabinet
- Refrigerators at 3±2 ˚C and 5±3 ˚C
- Thermometer with a resolution of 1 °C or better at the temperature being measured
- Safety Bunsen burner or electric microincinerator
- 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.
- Pipette, single channel, variable volume, 2 – 20 µl, 20 – 200 µl
- Timer
- Protective gloves – single use
- Safety gloves – for example a chain mail glove (for use with the shucking knife)
- 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
- 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)
- Dissecting equipment for gastropods and echinoderms – sterile tray, forceps, scissors, spatula and hammer or equivalent tools
- Food grade plastic bags

- Absorbent paper towel
- Sterile measuring cylinder – 250 ml (a larger cylinder may be needed for some species)
- Blender and 1 litre blending jars or stomacher and stomacher bags
- 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)
- Incubators at 37±1 ˚C and 41.5±1 ˚C [1]
- Platinum or sterile disposable 1 µl and 10 µl loops
- Filter papers
- Micro-centrifuge tubes 0.5 ml and 1.5 ml
- Heating block at 95±2 ˚C
- Equipment for preparing and running agarose gel electrophoresis – microwave or equivalent device for heating agarose gel mixes, mould, comb, tank, powerpack
- Ultraviolet transilluminator
- Vortex
- Micro-centrifuge capable of running at 10.000g
- Conventional PCR machine
- Real-time PCR machine

6. Media and reagents [2] [3]

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.

- Ethanol
- Alkaline salt peptone water (ASPW); formula per litre - de-ionised water 1±0.01 litre, ASPW powder 40±0.5 g, pH 8.6±0.2. 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. May be stored protected from direct sunlight at room temperature after sterilisation.
- Thiosulphate citrate bile sucrose agar (TCBS); - formula per litre - de-ionised water 1±0.01 litre, Cholera medium TCBS powder 88±0.5 g, pH 8.6±0.2. Sterilise by autoclaving at 121 °C for 15 minutes. Pour 15 ml to 18 ml of the molten medium into sterile Petri dishes and allow to solidify. The plates may be stored at 5±3 °C in the dark and protected from desiccation for up to four weeks unless results of the laboratory shelf-life validation indicate a longer shelf-life. Plates should be dried before use to remove excess surface moisture (see ISO 11133 for further considerations in relation to drying). Drying is especially important where TCBS plates are to be subdivided for the inoculation of more than one tube from a single dilution.
- Saline Nutrient agar (SNA); formula per litre - de-ionised water 1±0.01 litre, Meat extract 5±0.1 g, Peptone 3±0.1 g, Sodium chloride 10±0.1 g, Agar-agar 8 g to 18 g (depending on agar- agar strength), pH 7.2±0.2. Sterilise by autoclaving at 121 °C for 15 minutes. Pour 15 ml to 18 ml of the molten medium into sterile Petri dishes and allow to solidify. The plates may be stored at 5±3 °C in the dark and protected from desiccation for up to four weeks unless results of the laboratory

[1] 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.
[2] Allow media to equilibrate at room temperature before use.
[3] 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

shelf-life validation indicate a longer shelf-life. Plates should be dried before use to remove excess surface moisture (see ISO 11133 for further considerations in relation to drying). Drying is especially important where TCBS plates are to be subdivided for the inoculation of more than one tube from a single dilution.

- Oxidase reagent; formula per 100 ml - de-ionised water 100±0.2 ml, N, N, N', N'-Tetramethyl-p-phenylenediamine dihydrochloride 1±0.1 g.
- L-lysine decarboxylase saline medium (LDC); formula per litre - deionised water 1±0.01 litre, L-lysine monohydrochloride 5±0.1 g**,** Yeast extract 3±0.1 g, Glucose 1±0.1 g, Bromocresol purple 0.015±0.05 g, Sodium chloride 10±0.1 g, pH 6.8±0.2. Dispense aseptically in 2 - 5 ml volumes in sterile tubes or bottles. Cap the tubes or bottles after dispensing and sterilize the medium by autoclaving at 121 °C for 15 minutes. If not used immediately after cooling to room temperature, then store in a refrigerator at 5±3 °C.
- Arginine dihydrolase saline medium (ADH); formula per litre - de-ionised water 1±0.01 litre, Arginine monohydrochloride 5±0.1 g**,** Yeast extract 3±0.1 g, Glucose 1±0.1 g, Bromocresol purple 0.015±0.05 g, Sodium chloride 10±0.1 g, pH 6.8±0.2. Dispense aseptically in 2 - 5 ml volumes in sterile tubes or bottles. Cap the tubes or bottles after dispensing and sterilize the medium by autoclaving at 121 °C for 15 minutes. If not used immediately after cooling to room temperature, then store in a refrigerator at 5±3 °C.
- ß-galactosidase detection buffer solution; formula per 50 ml - de-ionised water 45±0.1 ml, Sodium dihydrogen-orthophosphate (NaH_2PO_4) 6.9±0.1 g. Adjust to pH 7.0±0.2 using Sodium hydroxide (0.1mol/L solution), make up to 50 ml using de-ionised water.
- ONPG solution; formula per 15 ml - deionised water 15±0.1 ml, 2-orthonitrophenyl-ß-D-galactosipyranoside 0.08±0.01 g. Dissolve with heating at 50±1 °C then cool to room temperature.
- ß-galactosidase detection reagent; formula per 20 ml – ß-galactosidase detection buffer solution 5±0.1 ml, ONPG solution 15±0.1 ml. Store in a refrigerator at 3±2 °C.
- Tryptophan saline medium; formula per litre - de-ionised water 1±0.01 litre, Enzymatic digest of casein 10±0.1 g, DL-tryptophan 1±0.1 g, Sodium chloride 10±0.1 g, pH 7.0±0.2. Dispense aseptically in 5±0.2 ml volumes in sterile tubes or bottles. Cap the tubes or bottles after dispensing and sterilize the medium by autoclaving at 121 °C for 15 minutes. If not used immediately after cooling to room temperature, store in a refrigerator at 5±3 °C.
- Kovacs reagent; formula per 100 ml – Dimethylamino-4 benzaldehyde 5±0.1 g, Hydrochloric acid, (*p*=1.18 g/ml to 1.19 g/ml) 25±0.1 ml, Methyl-2 butan-2-ol 75±0.1 ml. Dispense into bottles or flasks in volumes suitable for use for a set of examinations or a usual working day. Store in a refrigerator at 5±3 °C.
- Saline peptone waters (0%, 6%, 10%); formula per litre - de-ionised water 1±0.01 litre, Peptone 10±0.5g, Sodium chloride (0 g, 60±0.1 g or 100±0.1 g), pH 7.5±0.2. Dispense aseptically in 10±0.2 ml volumes in sterile tubes or bottles. Cap the tubes or bottles after dispensing and sterilize the medium by autoclaving at 121 °C for 15 minutes. If not used immediately after cooling to room temperature, store in a refrigerator at 5±3 °C.
- Sodium chloride solution (0.85 % NaCl solution); formula per litre - de-ionised water 1±0.01 litre, Sodium chloride 8.5±0.5 g, pH 7.5±0.2. Dispense into bottles or flasks in volumes suitable for use for a set of examinations or a usual working day. Sterilize the medium by autoclaving at 121 °C for 15 minutes. If not used immediately after cooling to room temperature, then store in a refrigerator at 5±3 °C.
- Sterile mineral oil
- Toluene
- Tris acetate EDTA buffer (TAE); formula per litre - de-ionised water 1±0.01 litre, 50X TAE buffer (from commercial sources) 20±0.1 ml. Store in a refrigerator at 3±2 °C.
- Agarose powder

- Ethidium bromide
- 100bp DNA ladder
- Sample loading dye for gel electrophoresis
- Molecular reagents for PCR; (See 8.9.2)

7. Microbiological reference cultures for media performance testing

Microbiological reference cultures shown in Table 1 shall be used for performance testing of media as described in ISO 11333.

Table 1: Microbiological reference strains used for media performance testing

Media type	Function	Control strain	WDCM [a]	Characteristic reaction
Alkaline salt peptone water (ASPW)	Productivity	*Vibrio parahaemolyticus* [b]	00185	>10 colonies on TCBS
		Vibrio vulnificus [b]	00139 or 00187	
Thiosulphate citrate bile sucrose agar (TCBS)	Productivity	*Vibrio parahaemolyticus*	00185 [c]	Good growth of green colonies
		Vibrio furnissii	00186 [c]	Good growth of yellow colonies
	Selectivity	*Escherichia coli* [b]	00012, 00013 or 00090	Total inhibition
Saline nutrient agar (SNA)	Productivity	*Vibrio parahaemolyticus*	00185 [c]	Good growth
		Vibrio vulnificus [b]	00139 or 00187	
Oxidase reagent [d]	Detection of cytochrome oxidase	*Vibrio parahaemolyticus*	00185	Positive reaction: blue to purple colour appears
		Escherichia coli	00012, 00013 or 00090	Negative reaction: no colour change
L-lysine decarboxylase saline medium (LDC) [d]	Detection of L-lysine decarboxylase (LDC)	*Vibrio parahaemolyticus*	00185	Positive reaction: medium remains violet-purple and is turbid
		Enterobacter aerogenes	00175	
		Escherichia coli	00012, 00013 or 00090	Negative reaction: medium changes from purple to yellow
		Proteus mirabilis	00023	
Arginine dihydrolase saline medium (ADH) [d]	Detection of L-arginine dihydrolase	*Vibrio fluvialis*	00137	Positive reaction: turbid with violet-purple colour
		Vibrio parahaemolyticus	00037 or 00185	Negative reaction: yellow colour
ß-galactosidase detection reagent [d]	Detection of β-Galactosidase	*Escherichia coli*	00012, 00013 or 00090	Positive reaction: yellow colour
		Vibrio parahaemolyticus	00185	Negative reaction: no colour change
Tryptophan saline medium with Kovacs reagent [d]	Detection of indole production from tryptophan	*Vibrio parahaemolyticus*	00037 or 00185	Positive reaction: red ring appears within 10 min
		Vibrio vulnificus	00139	
		Enterobacter aerogenes	00175	Negative reaction: yellow-brown ring appears within 10 min
		Citrobacter freundii	00006	

Media type	Function	Control strain	WDCM [a]	Characteristic reaction
Saline peptone water with 0%, 6% or 10% NaCl	Detection of halotolerance 0 % NaCl	*Vibrio mimicus*	11435 [e]	Positive reaction: turbid growth
		Vibrio parahaemolyticus	00185	Negative reaction: no growth
	Detection of halotolerance 6 % NaCl	*Vibrio parahaemolyticus* [b]	00037 or 00185	Positive reaction: turbid growth
		Vibrio vulnificus	00139	
		Vibrio mimicus	11435 [e]	Negative reaction: no growth
	Detection of halotolerance 10 % NaCl	*Staphylococcus aureus* [b]	00032 or 00034	Positive reaction: turbid growth
		Vibrio parahaemolyticus	00185	Negative reaction: no growth
		Vibrio vulnificus	00187	

[a] Follow the link (WDCM reference strains | Culture Collections) to obtain NCTC numbers for WDCM reference strains.
[b] Strain free of choice; one of the strains has to be used as a minimum (see ISO 11133).
[c] Strain to be used as a minimum (see ISO 11133)
[d] Strains provided for guidance; the strain selected must produce the required characteristic.
[e] NCTC reference strain number.

8. Procedure

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 [4]. 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

[4] 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.

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 [5].

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

Discard all dead shellfish and those with obvious signs of damage. Select the appropriate number depending on the species – this must be at least 10 individual animals, but more can be used, if necessary, to produce the required quantity (50g) for analysis (recommended numbers of animals for each species are given in Appendix 1).

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 a sterilised container. Transfer any liquor (intravalvular fluid) collected in the weighing boat into the sterilised container. Repeat with the other animals in the sample.

[5] Vibrios can be injured by rapid cooling but grow rapidly in seafood at ambient temperatures.

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

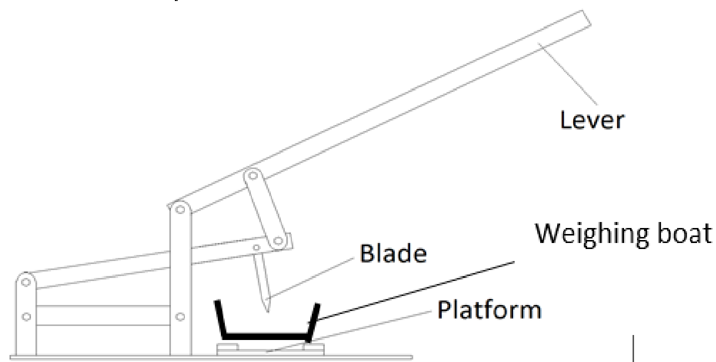

Figure 1: Oyster cracker apparatus

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. For other species of bivalve molluscs a suitable method based on the methods for oysters and clams, or mussels and cockles (below) should be used.

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 a 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.

8.4.2.2. 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 for recommended numbers). Dice the animal flesh using sterile scissors before transferring to a sterilised 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 sterilised container using a sterile spatula. Repeat the process with the required number of animals (see Appendix 1 for recommended numbers).

Figure 4: Opening an echinoderm using sterile scissors

8.5. Homogenisation, inoculation and incubation of primary enrichment broth

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 to obtain the primary suspension.

8.5.1. Homogenisation in a blender

Measure 2ml of sterile ASPW per 1g of shellfish (±2ml) using a measuring cylinder. Place contents of sterilised container into a sterilised 1 litre blender jar with the ASPW 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 two separate 75±1ml volumes of mixed shellfish homogenate to two separate 175±5ml volumes of ASPW to make the parallel primary enrichments (10^{-1} dilution); ensure that these primary enrichments are thoroughly mixed. Label appropriately.

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.

8.5.2. Homogenisation in a stomacher [7]

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 50±0.5g of homogenised shellfish into another set of 3 stomacher bags, add 100±2ml of ASPW and homogenise for a further 2-3 minutes to prepare the mixed shellfish homogenate (1:3 dilution). After homogenisation add two separate 75±1ml volumes of the mixed shellfish homogenate to two separate 175±5ml volumes of ASPW to make to make the parallel primary enrichments (10^{-1} dilution); ensure that these primary enrichments are thoroughly mixed. Label appropriately.

8.5.3. Process controls

For the positive controls inoculate individual bottles containing 10±0.2 ml of ASPW with *V. parahaemolyticus* WDCM 00185, *V. cholerae* WDCM 00136 and *V. vulnificus* WDCM 00187 using a loop. For the negative controls two parallel un-inoculated bottles containing 10±0.2ml of ASPW are included.

8.5.4. Incubation of primary enrichment broth

Incubate one of the parallel primary enrichment broths for all samples and one of the parallel negative process controls at 37±1 °C (for optimal detection of *V. vulnificus*) for 6±1 hours. Incubate the other parallel primary enrichment broth for all samples and the other parallel negative process control at 41.5±1 °C (for optimal detection of *V. parahaemolyticus* and *V. cholerae*) for 6±1 hours. At the same time incubate the positive process controls at 37±1 °C (*V. vulnificus*) and 41.5±1 °C (*V. parahaemolyticus* and *V. cholerae*) for 6±1 hours.

8.6. Subculture of primary and secondary enrichments broths

8.6.1. Subculture of primary enrichment and preparation and incubation of secondary enrichment broth

Following incubation of the ASPW primary enrichment broths, from just below the surface and

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

without mixing, subculture each broth (for both samples and process controls) using a 1 µl loop onto the surface of one TCBS plate and one plate of a second isolation medium [8], streaking with the objective to obtain well-isolated colonies.

Transfer 1±0.1 ml of each primary enrichment broth to 10±0.2 ml of ASPW (secondary enrichment). Incubate each secondary enrichment for 18±1 hours at the same temperature the primary enrichment broth used to prepare it was incubated. For TCBS plates incubate at 37±1 ˚C for 24±3 hours. Incubate the second isolation medium plates in accordance with the manufacturer's instructions.

8.6.2. Subculture of secondary enrichment [9]

Following incubation of the ASPW secondary enrichment broths, from just below the surface and without mixing, subculture each broth (for both samples and process controls) using a 1 µl loop onto the surface of one TCBS plate and one second isolation medium plate, streaking with the objective to obtain well-isolated colonies. Incubate the TCBS plates at 37±1 ˚C for 24±3 hours. Incubate the second isolation medium plates in accordance with the manufacturer's instructions.

8.7. Isolation and identification

Following incubation of the TCBS and second isolation medium plates (sub-cultured from both primary (8.6.1) and secondary enrichments (8.6.2)) examine each plate for the presence of characteristic colonies of *V. parahaemolyticus, V. cholerae* and *V. vulnificus:*

TCBS: *V. parahaemolyticus* and *V. vulnificus* - smooth, green colonies (negative sucrose), 2 - 3 mm in diameter; *V. cholerae* – smooth, yellow colonies (positive sucrose), 1-2 mm in diameter (Figure 5).

Figure 5: TCBS plate showing green (negative sucrose) and yellow (positive sucrose) colonies typical of different *Vibrio spp.*

Second isolation medium plates: Follow the manufacturer's instructions to identify characteristic colonies of *V. parahaemolyticus* and *V. vulnificus*[10].

If characteristic *V. parahaemolyticus, V. cholerae* and/or *V. vulnificus* colonies are present on any isolation plates sub-cultured from primary and/or secondary enrichment broths for a given sample, confirmation must be carried out as described in 8.8.

If no typical *V. parahaemolyticus, V. cholerae* or *V. vulnificus* colonies are present on any isolation

[8] The second isolation medium selection is the testing laboratory's own choice. The FAORC recommends the use of chromID™ Vibrio (VID) agar (bioMerieux).
[9] If shown to be reliable by the user laboratory, conventional PCR using primers for the *tox-R* target may be used to screen for the presence of *V. parahaemolyticus* in secondary enrichment broths (incubated at 41.5 °C) following incubation. Non-detection of the target indicates absence of *V. parahaemolyticus* which may enable a reduction of downstream testing.
[10] For chromID™ Vibrio (VID) agar typical colonies of *V. parahaemolyticus* are pink, typical colonies of *V. cholerae* are bluish-green to green and typical colonies of *V. vulnificus* are bluish-green to blue.

plates sub-cultured from primary and/or secondary enrichment broths for a given sample, then report the result as "not detected" according to section 9.

8.8. *Vibrio* confirmation

Confirmation of presumptive *V. parahaemolyticus, V. cholerae* and *V. vulnificus* colonies from isolation medium plates can be achieved using biochemical and/or molecular (PCR) approaches as described in 8.8.3 – 8.8.4. Test at least one well isolated colony characteristic of each *Vibrio* spp. [11]. If the first isolated colony tested is negative, a further four (where possible) well isolated colonies should be tested to confirm *Vibrio* spp. absence.

8.8.1. Selection of colonies for confirmation

Subculture one well-isolated colony characteristic of each *Vibrio* spp. from each isolation medium plate onto the surface of an SNA plate [12], using a loop to obtain well-isolated colonies. Incubate at 37±1 ˚C for 24±3 hours. Retain all isolation medium plates in the dark at room temperature until confirmation is complete [13].

After incubation check each SNA plate for purity. If pure (that is, all colonies have the same morphology and colour), then continue with identification (8.8.2). If the purity plate shows a mixed culture, re-streak onto more SNA plates to isolate the organisms until pure colonies have been obtained.

8.8.2. Oxidase test [14]

Perform an oxidase test on a colony from each SNA plate giving pure cultures (8.8.1). Using a loop [15], inoculate a filter paper moistened with oxidase reagent with the colony. A positive reaction is indicated by the appearance of a blue to purple colour and a negative reaction will show no colour change [16].

Continue identification by means of biochemical screening tests (8.8.3), conventional PCR (8.8.4.2) or real-time PCR (8.8.4.3) on all SNA plates that give an oxidase positive reaction. Reject any SNA plates that showed an oxidase negative reaction.

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[11] For samples where it is considered important to detect potentially pathogenic *V. parahaemolyticus* using PCR for *tdh* or *trh* targets at least five and preferably all characteristic colonies should be sub-cultured for downstream testing.

[12] Another suitable medium can be used at the laboratory's own choice.

[13] It is recommended to mark the location of colonies showing typical characteristics of *V. parahaemolyticus, V. cholerae* and *V. vulnificus* on the base of the plate as colony colouration may change over time.

[14] ISO 21872-1 allows the use of the Gram stain and motility test on colonies from SNA plates with pure cultures as an alternative to the oxidase test. If these tests are used continue identification with SNA plates giving Gram-negative motility-positive results.

[15] In addition to a loop, a platinum iridium straight wire or rod can be used. It is not recommended to use a nickel-chromium loop or metallic wire as these can give false-positive results.

[16] Commercially available Oxidase test kits can be used as an alternative following the manufacturer's instructions.

8.8.3. Biochemical confirmation [17]

Inoculate a colony from all oxidase positive SNA plates, using a loop and without recharging the loop, into

- 3.5±1.5 ml of LDC
- 3.5±1.5 ml of ADH
- 0.25±0.01 ml of 0.85 % NaCl solution [18]
- 5±0.1 ml of tryptophan saline medium
- 5±0.1 ml of saline peptone waters containing 0 %, 6 % and 10 % NaCl

and finally streak onto an SNA plate [19]. Overlay the surface of inoculated LDC and ADH tubes with about 1 ml of sterile mineral oil. Add 1 drop of toluene to each inoculated 0.85 % NaCl solution tube and mix well before incubating at 37±1 ˚C for approximately 5 minutes. Once incubated add 0.25±0.01 ml of ß-galactosidase detection reagent and mix.

For positive and negative controls, using a loop, inoculate each biochemical screening test as described above, using appropriate microbiological reference cultures given in Table 1. Incubate all inoculated biochemical screening tests for all samples and controls at 37±1 ˚C for 24±3 hours.

After incubation, add 1±0.1 ml of Kovacs reagent to each tube containing tryptophan saline medium. Formation of a red ring in these tubes (indicating indole production) should be recorded as a positive reaction, whereas formation of a yellow-brown ring should be recorded as a negative reaction.

Turbidity (indicating bacterial growth) and a violet-purple colour (indicating decarboxylation of lysine) in LDC tubes after incubation should be recorded as a positive reaction, while a yellow colour after incubation should be recorded as a negative reaction.

Turbidity (indicating bacterial growth) and a violet-purple colour (indicating hydrolysis of arginine) in ADH tubes after incubation should be recorded as a positive reaction, while a yellow colour after incubation should be recorded as a negative reaction.

A yellow colour (indicating β-galactosidase activity) in the tubes with 0.85 % NaCl solution, toluene and ß-galactosidase detection reagent after incubation should be recorded as a positive reaction, while absence of colour after incubation should be recorded as a negative reaction.

Bacterial growth (turbidity) in each of the saline peptone waters should be recorded as a positive reaction while absence of turbidity is a negative reaction.

Determine the identification of the *Vibrio* spp. from the biochemical screening test results using Table 2.

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[17] Commercially available biochemical kits that have been shown to produce reliable results can be used as an alternative in accordance with the manufacturer's instructions.

[18] Commercially available ONPG disks can be used as an alternative following the manufacturer's instructions.

[19] Following inoculation of saline peptone water with 10% NaCl, it is recommended to subculture onto an SNA plate to ensure a 'no growth' result in the test is not due to a dead culture.

Table 2: Expected results of biochemical confirmation tests

Test		*V. parahaemolyticus*	*V. cholerae*	*V. vulnificus*	*V. mimicus*[20]	*V. alginolyticus*[20]
Oxidase		+	+	+	+	+
LDC		+	+	+	+	+
ADH		-	-	-	-	-
ONPG hydrolysis (ß-galactosidase detection)		-	+	+	+	-
Tryptophan saline medium with Kovacs reagent (indole production)		+	+	+	+	+
Growth in saline peptone water with	0 % NaCl	-	+	-	+	-
	6 % NaCl	+	-	+	-	+
	10 % NaCl	-	-	-	-	+

8.8.4. PCR confirmation [21]

Using a colony from each oxidase positive SNA plate, carry out a DNA extraction as described in 8.8.4.1. The same DNA extract can be used for conventional PCR (8.8.4.3) and/or real-time PCR (8.9.2.3).[22]

8.8.4.1. DNA extraction [23]

Prepare a bacterial suspension using a colony from each SNA plate retained from section 8.8.1. Select one well-isolated colony from the SNA plate and, using a loop, inoculate 500±10 µl of 0.85 % NaCl or nuclease free water in a 1.5 ml micro-centrifuge tube. Heat the inoculated tube in a heating block at 95±2 ˚C for 5±1 minutes, then centrifuge at 10,000 g for 1±0.5 minute. Decant and retain the supernatant (DNA extract) for PCR testing [24].

For each batch of samples tested, run in parallel a positive and negative extraction control. Prepare a positive control for each PCR assay required, using a loop to inoculate 500±10 µl of 0.85 % NaCl or nuclease free water with a suitable reference culture as shown in Tables 5 and 8. For the negative control use 500±10µl of 0.85% NaCl or nuclease free water.

8.8.4.2. Conventional PCR confirmation

For each conventional PCR assay to be performed, prepare a mastermix using the volumes as shown in Table 4, adjusting based on the number of reactions required and using the PCR

[20] Provided for reference purposes
[21] PCR based confirmation can reduce the subjective interpretation of biochemical identification tests and accelerate the identification process.
[22] Note that it is currently only possible to confirm *V. cholerae* using conventional PCR, as suitable real-time PCR primer and probe combinations are not available.
[23] Commercially available DNA extraction kits can be used as an alternative following the manufacturer's instructions.
[24] For long term storage, it is recommended to store extracted DNA at <-15 ˚C. For short term storage (<1 month) extracted DNA can be stored at 5±3 ˚C.

primers appropriate for the PCR assay as shown in Table 5.

Table 4: Composition of conventional PCR mastermix

Reagent	Final concentration (in 50 µl reaction volume)	Volume per reaction (µl)
Reaction buffer (5 x)[a]	1x	10
$MgCl_2$	2.5 mM	as required[b]
dATP	0.25 mM	as required[b]
dTTP	0.25 mM	as required[b]
dCTP	0.25 mM	as required[b]
dGTP	0.25 mM	as required[b]
Forward primer	1 µM	as required[b]
Reverse primer	1 µM	as required[b]
DNA polymerase[a]	-	0.25
Water	-	as required[c]
Total volume	-	47.5

[a] Reagents from GoTaq Flexi DNA Polymerase kit (Promega). Other suitable reagents can be used as an alternative – concentration of other ingredients may need adjusting in this case.
[b] The added volume of each reagent depends on the concentration of the stock and the required final concentration as listed.
[c] Water is added to make the total volume up to 47.5 µl per reaction, taking into account the volume of the other reagents added.

Table 5: Conventional PCR assay details

Target region	Primer sequence [a]		Reference	Product size (bp)	Control strains	WCDM catalogue number
VptoxR [b]	Forward	GTC TTC TGA CGC AAT CGT TG	Kim *et al.*, 1999	368	*Vibrio parahaemolyticus*	00185
	Reverse	ATA CGA GTG GTT GCT GTC ATG				
tdh [c]	Forward	GTA AAG GTC TCT GAC TTT TGG AC	Bej *et al.*, 1999	269	*Vibrio parahaemolyticus*	10884 [g]
	Reverse	TGG AAT AGA ACC TTC ATC TTC ACC				
trh [c]	Forward	TTG GCT TCG ATA TTT TCA GTA TCT		500	*Vibrio parahaemolyticus*	00037
	Reverse	CAT AAC AAA CAT ATG CCC ATT TCC G				
prVC [d]	Forward	TTA AGC STT TTC RCT GAG AAT G [f]	Chun *et al.*, 1999	295 to 310	*Vibrio cholerae*	00136
	Reverse	AGT CAC TTA ACC ATA CAA CCC G				
VVH [e]	Forward	CCG GCG GTA CAG GTT GGC GC	Hill *et al.*, 1991	519	*Vibrio vulnificus*	00139
	Reverse	CGC CAC CCA CTT TCG GGC C				

[a] Primers shown were those used in the ISO 21872-1 validation. Other suitable primers for the same targets can be used as an alternative.
[b] The VptoxR assay is used for species-level detection of *Vibrio parahaemolyticus*.
[c] The tdh and trh assays are used for detection of pathogenic strains of *Vibrio parahaemolyticus*.
[d] The prVC assay is used for species-level detection of *Vibrio cholerae*.
[e] The VVH assay is used for species-level detection of *Vibrio vulnificus*.
[f] S = G or C, R = A or G (degenerate nucleotides)
[g] NCTC reference strain number.

Prepare an individual 0.5 ml micro-centrifuge tube for each extracted sample and control by adding 2.5 µl of extracted DNA to 47.5 µl of master mix. Load all micro-centrifuge tubes onto a conventional PCR machine and run with the cycling parameters as given in Table 6 based on the PCR assay to be performed [25].

[25] If alternative primers and/or other reagents are used for the same targets the cycling parameters will need to be adjusted appropriately.

Table 6: Conventional PCR cycling parameters

Step description		Number of cycles	Temperature and time		
			VptoxR and *VVH* assays	*tdh* and *trh* assays	prVC assay
Pre-heating		1	96 °C for 5 min	94 °C for 3 min	94 °C for 2 min
Amplification	Denaturation	30	94 °C for 1 min	94 °C for 1 min	94 °C for 1 min
	Annealing		63 °C for 1.5 min	58 °C for 1 min	50 °C for 1 min
	Extension		72 °C for 1.5 min	72 °C for 1 min	72 °C for 1.5 min
Post amplification		1	72 °C for 7 min	72 °C for 5 min	72 °C for 10 min

After PCR is complete prepare a 2 % agarose gel by mixing 1 g agarose powder with 100 ml 1 xTAE buffer (or the appropriate quantities of agarose and TAE in the same ratio for alternative gel mould sizes), heating in a microwave or equivalent device to dissolve the agarose powder, allowing to cool before adding a few drops of ethidium bromide [26] while still liquid and gently mixing. Pour the gel into a mould with an attached comb to form wells of the appropriate size. Leave to solidify then remove the comb and place the gel in an electrophoresis tank filled with 1 x TAE buffer. Load the gel with 10 µl of 100 bp DNA ladder and 20 µl of each sample and control PCR reaction mix, using an appropriate loading dye as necessary[27]. Run the gel at 130 V for 25 - 30 minutes. Following electrophoresis visualize the gel using an ultraviolet transilluminator. The expected PCR product sizes for each PCR assay are given in Table 5. Presence of a PCR product of the expected size for any sample should be considered a positive for the relevant target species/strains, assuming correct control results.

8.8.4.3. Real-time PCR confirmation

For each real-time PCR assay to be performed, prepare a mastermix using the volumes as shown in Table 7, adjusting based on the number of reactions required and using the PCR primers and hydrolysis probes appropriate for the PCR assay as shown in Table 8.

Prepare a 96 well plate by adding 20 µl of mastermix to individual wells followed by 5 µl of extracted DNA for each extracted sample and control.

[26] Ethidium bromide is highly toxic - when handled appropriate Personal Protective Equipment (PPE) should be worn. Alternatively a different dye enabling visualization of PCR products can be used.
[27] Alternative volumes of PCR reaction mix and DNA ladder may be used according the individual laboratory procedures and manufacturers' recommendations.

Table 7: Composition of real-time PCR mastermix

Reagent	Final concentration (in 25 µl reaction volume)	Volume per reaction (µl)
TaqMan Universal PCR Master Mix (2 x) [a]	1x	12.5
Forward primer	100 nM	as required[b]
Reverse primer	100 nM	as required[b]
Probe	500 nM	as required[b]
Water	-	as required[c]
Total volume	-	20

[a] Reagent from Applied Biosystems. Other suitable reagents can be used as an alternative – concentration of other ingredients may need adjusting in this case.
[b] The added volume of each reagent depends on the concentration of the stock and the required final concentration as listed.
[c] Water is added to make the total volume up to 20 µl per reaction, taking into account the volume of the other reagents added.

Table 8: Real-time PCR assay details

Target region	Primer/probe sequence [a]		Reference	Control strains	WCDM catalogue number
VptoxR [c]	Forward	GAA CCA GAA GCG CCA GTA GT	Taiwo *et al.*, 2016	*Vibrio parahaemolyticus*	00185
	Reverse	AAA CAG CAG TAC GCA AAT CG			
	Probe [b]	TCA CAG CAG AAG CCA CAG GTG C			
Vptdh [d]	Forward	TCC CTT TTC CTG CCC CC	Nordstrom *et al.*, 2007	*Vibrio parahaemolyticus*	10884 [f]
	Reverse	CGC TGC CAT TGT ATA GTC TTT ATC			
	Probe [b]	TGA CAT CCT ACA TGA CTG TG			
VVH [e]	Forward	TGT TTA TGG TGA GAA CGG TGA CA	Campbell & Wright., 2003	*Vibrio vulnificus*	00139
	Reverse	TTC TTT ATC TAG GCC CCA AAC TTG			
	Probe [b]	CCG TTA ACC GAA CCA CCC GCA A			

[a] Primers and probes shown were those used in the ISO 21872-1 validation. Other suitable primers and probes for the same targets can be used as an alternative.
[b] Probes labelled 5’ 6-carboxyfluorescein (FAM), 3’ 6-carboxytetramethylrhodamine (TAMRA).
[c] The VptoxR assay is used for species-level detection of *Vibrio parahaemolyticus*.
[d] The Vptdh assay is used for detection of pathogenic strains of *Vibrio parahaemolyticus*.
[e] The VVH assay is used for species-level detection of *Vibrio vulnificus*.
[f] NCTC reference strain number.

Cover each well with a plastic cap before placing in a real-time PCR machine and subjecting

the plate to cycling as described in Table 9[28]. Analyse the amplification plots using the approach recommended by the manufacturer of the real-time PCR machine[29]. The threshold should ideally be set so that it crosses the area where the amplification plots (logarithmic view) are parallel (the exponential phase).

Table 9: Real-time PCR cycling parameters

Step description	PCR step	Temperature and time	Number of cycles
Preheating	Initial denaturation	95 °C for 10 min	1
Amplification	Denaturation	95 °C for 15 sec	45
	Annealing – extension	60 °C for 1 min	

Check the positive and negative controls. Positive controls should always be positive and negative controls should always be negative; if an unexpected result occurs in a control then affected samples should be retested.

Evidence of logarithmic amplification of the PCR product for any sample should be considered a positive for the relevant target species/strains, assuming correct control results.

9. Reporting results

Depending on the interpretation of the result, if at least one colony from the sample tested indicates that potentially enteropathogenic *Vibrio* spp. (*V. parahaemolyticus, V. cholerae* or *V. vulnificus*) is detected, report as "*Vibrio spp.* detected in 50 g" and specify the name of the *Vibrio* spp. and any identified pathogenicity characteristics if tested.

If no colonies from the sample conform to the expected criteria of *V. parahaemolyticus, V. cholerae* or *V. vulnificus* report the sample as "*V. parahaemolyticus, V. cholerae* and *V. vulnificus* not detected in 50 g"[30].

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.

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[28] If alternative primers and/or other reagents are used for the same targets the cycling parameters will need to be adjusted appropriately.

[29] For real-time PCR machines where the user can set the point of fluorescence measurement, this shall be set at the end of the extension stage.

[30] Depending on the confirmation method used, colonies may be found that conform to the criteria for non-target *Vibrio* species e.g. *V. alginolyticus* or *V. mimicus,* in samples that do not contain *V. parahaemolyticus, V. cholerae* or *V. vulnificus*. In some contexts, it may be appropriate to report detection of these species using the wording for reporting positive samples given in this section.

11. Quality control

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

11.1. 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 reference 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.

12. References

ISO 6887-1:2017+A1:2024. Microbiology of the 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 Standards Organisation, 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 7218:2024. Microbiology of the food chain – General requirements and guidance for microbiological examinations. International Standards Organisation, Geneva.

ISO 11133:2014+A1:2018+A2:2020. Microbiology of food, animal feed and water – Preparation, production, storage and performance testing of culture media. International Standards Organisation, Geneva.

ISO 21872-1:2017+A1:2023. Microbiology of the food chain – Horizontal method for the detection of potentially enteropathogenic *Vibrio parahaemolyticus, Vibrio cholerae* and *Vibrio vulnificus*. International Standards Organisation, Geneva.

ISO 22174:2005. Microbiology of food and animal feeding stuffs – Polymerase chain reaction (PCR) for the detection of food-borne pathogens – General requirements and definitions. International Standards Organisation, Geneva.

Bej A.K., Patterson D.P., Brasher C.W., Vickery M.C.L., Jones D.D., Kaysner C.A. Detection of total and hemolysin-producing *Vibrio parahaemolyticus* in shellfish using multiplex PCR amplification of *tl, tdh* and *trh.* J. Microbiol. Methods. 1999, 36:215–225.

Campbell M.S., Wright A.C. Real-time PCR analysis of *Vibrio vulnificus* from oysters. Appl. Environ. Microbiol. 2003, 69:7137–7144.

Chun J., Huq A., Colwell R.R. Analysis of 16S-23S rRNA intergenic spacer regions of *Vibrio cholerae* and *Vibrio mimicus*. Appl. Environ. Microbiol. 1999, 65:2202–2208.

Hill W.E., Keasler S.P., Trucksess M.W., Feng P., Kaysner C.A., Lampel K.A. Polymerase chain reaction identification of *Vibrio vulnificus* in artificially contaminated oysters. Appl. Environ. Microbiol. 1991, 57:707–711.

Kim Y.B., Okuda J., Matsumoto C., Takahashi N., Hashimoto S., Nishibuchi M. Identification of *Vibrio parahaemolyticus* strains at the species level by PCR targeted to the *toxR* gene. J. Clin. Microbiol. 1999, 37:1173–1177.

Nordstrom J.L., Vickery M.C.L., Blackstone G.M., Murray S.L., DePaola A. Development of a multiplex Real-Time PCR assay with an internal amplification control for the detection of total and pathogenic *Vibrio parahaemolyticus* bacteria in oysters. Appl. Environ. Microbiol. 2007, 73:5840–5847.

Taiwo T., Powell A., Taylor K., Walker D.I., Baker-Austin C. Development and comparison of a *toxR*-based real-time PCR assay for *Vibrio parahaemolyticus.* Food Control. 2016, 77:116-120.

13. Appendices

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

ISO 6887-3 requires that a representative test sample contains a minimum of 10 animals, in addition the protocol for *Vibrio* analysis described here requires a minimum weight of shellfish flesh and liquor of 50 g. 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 [31]	Scientific name [32]	Sample size [33]
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

[31] Species of relevance provided by European NRLs.
[32] 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.
[33] For species not given in the table, sufficient shellfish should be opened to achieve a 50g 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.

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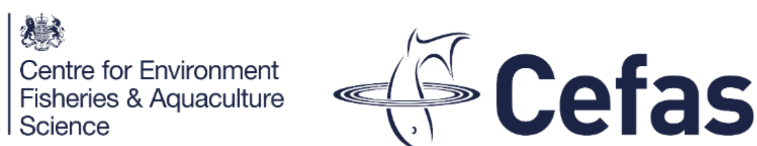

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