Cefas

European Union Reference Laboratory for monitoring bacteriological and viral contamination of bivalve molluscs

Generic protocol

Detection of *Vibrio parahaemolyticus* and *Vibrio vulnificus* in bivalve molluscan shellfish (based on ISO 21872)

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13 pages

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Document prepared by:	Louise Stockley	Location	

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History of Procedure

Issue	Date	Section	Changes
1	17.11.07		ALL
2	14.05.18	1	Update to include information on V. vulnificus
		2, 3	Update to ISO 21872, inclusion of *V. vulnificus* and use of PCR in confirmation.
		5 and 6	Updated of equipment, media and reagents for biochemical and PCR (conventional and real-time).
		7, 8.5	Amendments to reference strains to be consistent with ISO 21872. The inclusion of link to identify WDCM numbers.
		8.1	Update to transport conditions
		8.4	Updated to include the use of the Florida cracker and to be in line with other EURL protocols
		8.5	Removal of dilution series for enumeration. Inclusion of table for ASPW enrichment temperatures
		8.6	Amended to include the inoculation of the second ASPW enrichment and the second plated media Laboratories own choice
		8.8	Update on confirmation to be performed on 1 presumptive colony.
		8.9	Inclusion of conventional PCR (8.9.2.2) and real-time PCR (8.9.2.3)
		9	Inclusion of reporting for *V. vulnificus*
		Tables	Included throughout where required
		Footnotes	Included throughout the protocol

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Contents

1. Introduction ... 3
2. Scope ... 3
3. Principle ... 3
4. Safety precautions ... 3
5. Equipment ... 3
6. Media and reagents ... 4
7. Microbiological reference strains for control purposes ... 5
8. Procedure ... 6
8.1 Sample transport and receipt ... 6
8.2 Sample storage ... 6
8.3 Sample selection ... 6
8.4 Sample preparation ... 6
8.4.1 The oyster cracker ... 6
8.4.2 Shucking with a knife ... 6
8.5 Homogenisation, inoculation and incubation of primary enrichment broth ... 6
8.6 Subculture of primary and secondary enrichment broths ... 7
8.6.1 Subculture of primary enrichment ... 7
8.6.2 Subculture of secondary enrichment ... 7
8.7 Isolation and identification ... 7
8.8 *Vibrio* confirmation ... 8
8.8.1 Selection of colonies for confirmation ... 8
8.9 Presumptive identification ... 8
8.9.1 Biochemical screening testing ... 8
8.9.2 PCR confirmation ... 9
9. Results and reporting ... 12
10. Uncertainty of test results ... 12
11. References ... 12
12 Appendices ... 13
12.1 Appendix 1: sub-sample sizes of shellfish required for *Vibrio* spp. analysis ... 13

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1. Introduction

Infectious human diseases acquired from the consumption of bivalve molluscan shellfish are internationally recognised. These health hazards are largely due to the phenomenon of filter feeding where-by bivalve molluscs concentrate and retain bacterial and viral pathogens often derived from the contamination of their surrounding waters. The risks of exposure to infectious agents are compounded by the traditional consumption of bivalve shellfish raw, or only lightly cooked. Vibrios are Gram-negative rod-shaped bacteria that are natural inhabitants of estuarine and marine environments. The genus *Vibrio* contains over 100 described species, and around 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 produce. The species most commonly associated with foodborne infections include *V. parahaemolyticus, V. vulnificus* and *V. cholerae.* *Vibrio parahaemolyticus* is a halophilic, oxidase positive, Gram-negative, rod-shaped 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 originating from warm and temperate coastal waters and is not related to sewage contamination. Globally, *V. parahaemolyticus* is the most common bacterial cause of illness associated with consumption of shellfish. *Vibrio vulnificus* is a common inhabitant of estuarine environments. Globally, *V. vulnificus* is a significant foodborne pathogen capable of causing necrotizing wound infections and primary septicaemia and 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, V. mimicus* and non-toxigenic strains of *V. cholerae.*

2. Scope

This procedure has been produced with reference to ISO 21872 (Anon 2017) [1] and describes the method for the detection of potentially enteropathogenic *Vibrio* spp. (*V. parahaemolyticus* and *V. vulnificus*) in bivalve molluscan shellfish. It includes molecular tests to determine both species identification and, for *V. parahaemolyticus* the presence of the putative pathogenicity markers (thermostable direct and thermostable direct related haemolysins (*tdh* and *trh* genes))

3. Principle

The method used to enumerate *V. parahaemolyticus* and *V. vulnificus* in bivalve molluscan shellfish involves an initial selective-enrichment in alkaline salt peptone water (ASPW) at 41.5±1°C and/or 37±1°C for 18±1h, followed by direct plating of the sample onto thiosulphate citrate bile sucrose agar (TCBS). Presumptive colonies of *V. parahaemolyticus* and *V. vulnificus* isolated from TCBS 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. 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. *Vibrio* spp. should be handled in accordance with ACDP category 2 guidelines

5. Equipment

- Stomacher and Stomacher bags
- Timer
- Laminar air flow cabinet (class II)
- Refrigerator at 3±2˚C and 5±3˚C
- Incubator 30±1˚C, 37±1˚C and 41.5±1˚C
- Balance
- Shucking knives
- Safety/electric Bunsen system
- Latex gloves
- Safety gloves
- Pipette - automatic or manual for use with 1ml and 10ml open-ended pipette tips
- Pipette, single channel, variable volume, 2 – 20µl, 20 – 200µl
- Sterile plastic spreader rods
- Sterile scissors
- Sterile glassware
- Sterile bijoux and universal bottles

[1] ISO 21872:2017 replaces ISO/TS 21872-1:2007 and ISO/TS 21872-2:2007, which have been technically revised. It also incorporates ISO/TS 21872-1:2007/Cor.1:2008.

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- Sterile loops, 1µl and 10µl
- Fine tip water proof marker (black)
- Centrifuge tubes 0.5ml, 1.5ml and 2ml
- Heating block 95±2˚C
- Ultraviolet transilluminator
- Vortex
- Conventional or real-time PCR machine
- Conventional PCR Thermocycler
- Micro-centrifuge capable of running at 10 000g

6. Media and reagents [2] [3]

- Ethanol
- Alkaline salt peptone water (ASPW); formula per litre - de-ionised water 1±0.01 litre, alkaline saline peptone water (CM1117) 40±0.5g, pH 8.6±0.2.
- Thiosulphate citrate bile sucrose agar (TCBS); - formula per litre - de-ionised water 1±0.01 litre, Cholera medium TCBS (CM0333) agar 88±0.5g, pH 8.6±0.2.
- Saline Nutrient agar (SNA); formula per litre - de-ionised water 1±0.01 litre, Meat extract 5±0.1g, Peptone 3±0.1g, Sodium chloride 10±0.1g, agar-agar 8g to 18g (depending on agar- agar strength), pH 7.2±0.2.
- Oxidase reagent (Oxidase); formula per 100ml - de-ionised water 100±0.2 ml, N, N, N', N'-Tetramethyl-p-phenylenediamine dihydrochloride 1±0.1g.
- L-Lysine decarboxylation medium (LDC); formula per litre - deionised water 1±0.01 litre, L-Lysine monohydrochloride 5±0.1g**,** Yeast extract 3±0.1g, Glucose 1±0.1g, Bromocresol purple 0.015±0.05g, Sodium chloride 10±0.1g, pH 6.8±0.2.
- Arginine dihydrolase saline medium (ADH); formula per litre - de-ionised water 1±0.01 litre, Arginine monohydrochloride 5±0.1g**,** Yeast extract 3±0.1g, Glucose 1±0.1g, Bromocresol purple 0.015±0.05g, Sodium chloride 10±0.1g, pH 6.8±0.2.
- ß-galactosidase detection (ONPG reagent); formula per 20ml – Buffer solution 5±0.1ml, ONPG solution 15±0.1ml. (Buffer solution; formula per 50 ml - de-ionised water 45±0.1ml, Sodium dihydrogen-orthophosphate (NaH_2PO_4) 6.9±0.1g, adjust to pH 7.0±0.2 using Sodium hydroxide (0.1mol/L solution), Make up to 50ml using de-ionised water. ONPG solution; formula per 15ml - deionised water 15±0.1ml, 2-orthonitrophenyl-ß-D-galactosipyranoside 0.08±0.01g)
- Tryptophan saline medium (Indole); formula per litre - de-ionised water 1±0.01 litre, Enzymatic digest of casein 10±0.1g, DL-trytophan 1±0.1g, Sodium chloride 10±0.1g, pH 7.0±0.2.
- Kovacs reagent (Kovacs); formula per 75 ml – Dimethylamino-4 benzaldehyde 5±0.1g, Hydrochloric acid, (p=1.18 g/ml to 1.19 g/ml) 25±0.1ml, Methyl-2 butan-2-ol 75±0.1ml.
- Saline peptone water (SPW); formula per litre - de-ionised water 1±0.01 litre, Peptone 10±0.5g, Sodium chloride (0g, 60±0.1g or 100±0.1g), pH 7.5±0.2.
- Sodium chloride solution (0.85% NaCl solution); formula per litre - de-ionised water 1±0.01 litre, Sodium chloride 8.5±0.5g, pH 7.5±0.2.
- Tris acetate EDTA buffer (TAE); formula per litre - de-ionised water 1±0.01 litre, 50X TAE buffer 20±0.1ml.
- 2% agarose gel; formula per 100ml - 1X TAE buffer (or equivalent buffer) 100±0.2ml, Agarose 2±0.1g.
- Molecular reagents for PCR; (See 8.9.2)

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[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

7. Microbiological reference strains for control purposes

The EURL recommends the use of the positive and negative controls throughout the procedure. The strains and criteria included in Table 1 are recommended for use as a minimum.

Table 1: Microbiological reference strains used for control purposes

Media type		Control strain	WDCM [a]	Characteristic reaction
Alkaline salt peptone water (ASPW)		*Vibrio parahaemolyticus*	00185 [b]	Turbid
		Vibrio vulnificus	00187 [b]	
Thiosulphate citrate bile sucrose agar (TCBS)		*Vibrio parahaemolyticus*	00185 [c]	Green colony, (sucrose negative)
		Vibrio furnissii	00186 [c]	Yellow colony, (Sucrose positive)
		Escherichia coli [b d]	00012, 00013 or 00090	Total inhibition
Saline nutrient agar (SNA)		*Vibrio parahaemolyticus*	00185	Good growth
		Vibrio vulnificus	00187	
L-lysine decarboxylase saline (LDC) [e]		*Vibrio parahaemolyticus*	00185	Turbid with purple (positive)
		Vibrio vulnificus	00187	
		Vibrio furnissii	00186	Turbid with yellow (negative)
Arginine dihydrolase saline (ADH) [e]		*Vibrio furnissii*	00186	Turbid with purple (positive)
		Vibrio parahaemolyticus	00185	Turbid with yellow (negative)
		Vibrio vulnificus	00187	
Detection of ß-galactosidase (ONPG) [e]		*Vibrio vulnificus*	00187	Yellow colour change (positive)
		Vibrio parahaemolyticus	00185	No colour (absence) (negative)
Detection of Indole [e]		*Vibrio parahaemolyticus*	00185	Red ring appears (positive)
		Vibrio vulnificus	00187	
		Vibrio furnissii	00186	Yellow-brown ring appears (negative)
Growth in saline peptone water with	0% NaCl [e]	*Vibrio mimicus*	11435 [f]	Turbid growth (positive)
		Vibrio parahaemolyticus	00185	No growth (negative)
		Vibrio vulnificus	00187	
	6% NaCl [e]	*Vibrio parahaemolyticus*	00185	Turbid growth (positive)
		Vibrio vulnificus	00187	
		Vibrio mimicus	11435 [f]	No growth (negative)
	10% NaCl [e]	*Vibrio alginolyticus*	12160 [f]	Turbid growth (positive)
		Vibrio parahaemolyticus	00185	No growth (negative)
		Vibrio vulnificus	00187	

[a] Follow the link (http://www.phe-culturecollections.org.uk/products/bacteria/WDCMStrains.aspx) to obtain NCTC number 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] Some national restrictions and directions may require the use of a different *E. coli* serovar. Make reference to national requirements relating to the choice of *E. coli* serovars.
[e] Strains provided for guidance; the strain selected must produce the required characteristic.
[f] NCTC reference strain number.

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8. Procedure

8.1 Sample transport and receipt [4] [5]

Samples should be cooled immediately after collection to between 7˚C and 10˚C and received in an intact food grade plastic bag and properly packed in a cool box with ice packs. 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.

8.2 Sample storage

Upon receipt in the laboratory the temperature of the samples should be recorded. Samples should preferably be examined immediately – If storage in the laboratory is necessary then samples should be stored at 3±2˚C for no more than 24 hours until examination [6].

8.3 Sample selection

Choose shellfish that are alive according to the following points:

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

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

8.4 Sample preparation

Mud and sediment adhering to the shell should be removed prior to opening by rinsing/scrubbing under cold, running tap water of potable quality. Shellfish should not be re-immersed in water as this may cause them to open. Open all selected shellfish as described below with a flame sterilised shucking knife or equivalent and empty meat and liquor into a sterile container [7]. If sterilised by heating allow the knife to cool before using. When opening shellfish ensure that the hand holding the shellfish is protected with a heavy-duty safety glove to prevent cuts.

8.4.1 The oyster cracker

Place a single animal in a weighing boat underneath the shucking device and rest the shucking leaver 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.

8.4.2 Shucking with a knife

Using a sterilised shucking knife, open all selected shellfish as described below:

8.4.2.1 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.

8.4.2.2 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, inoculation and incubation of primary enrichment broth

Weigh 25±1g of shellfish flesh and intravalvular fluid into at least three stomacher bags, to avoid small pieces of

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[4] The recovery of certain *Vibrio* spp. may be improved by the use of different incubation temperatures based upon the target species or state of the food matrix. The recovery of *V. parahaemolyticus* and *V. cholerae* in fresh products is enhanced by enrichment at 41.5°C whereas for *V. vulnificus,* and for *V. parahaemolyticus* and *V. cholerae* in deep frozen (<−18°C), dried or salted products, recovery is enhanced by enrichment at 37 °C.

[5] The sample transport criteria has been extracted from ISO 6887-3, BAM method and guidance from the EURL.

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

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

shell from puncturing the bags. Remove excess air from the bag and operate the stomacher for 3 minutes at normal speed. Add 75±1ml from a bottle of 225±5ml sterile ASPW. Stomach at 'normal' speed for a further 3 minutes. Transfer the stomached homogenate into a sterile beaker and add the remaining ASPW (150ml). Label as the 10^{-1} (primary enrichment).

Note: Repeat step 8.5 to obtain an identical primary suspension if required based on the target *Vibrio* spp. to be identified (See Table 2).

Table 2: Primary and secondary enrichment incubation temperatures for *Vibrio* spp. [8]

Incubation temperature	*V. parahaemolyticus*	*V. vulnificus*	Un-inoculated
37 ± 1 °C	X	✓	✓
41.5 ± 1 °C	✓	X	✓

For the positive controls inoculate individual bottles containing 10±0.2ml of ASPW with *V. parahaemolyticus* WDCM 00185 and/or *V. vulnificus* WDCM 00187 using a 1µl loop. For the negative controls an un-inoculated bottle containing 10±0.2ml of ASPW is included. Incubate the primary enrichment broths and controls at 37±1°C and/or 41.5±1°C for 6±1 hours dependent on the *Vibrio* spp. of interest (see Table 2).

8.6 Subculture of primary and secondary enrichment broths [9]

8.6.1 Subculture of primary enrichment

Following incubation of the ASPW primary enrichment broth/s and controls, from just below the surface and without mixing, subculture using a 1µl loop onto the surface of one TCBS plate and one plate of a second isolation medium [10] streaking with the objective to obtain well-isolated colonies. Transfer 1±0.1ml of primary enrichment broth to 10±0.2ml of ASPW (secondary enrichment).

Incubate the inoculated ASPW (secondary enrichment) for 18±1 hours at the same temperature the primary enrichment broth was incubated. For TCBS plates incubate at 37±1˚C for 24±3 hours. Incubate the second isolation plates in accordance with the manufacturer's instructions.

8.6.2 Subculture of secondary enrichment

Following incubation of the ASPW secondary enrichment broth/s and controls, from just below the surface and without mixing, subculture using a 1µl loop onto the surface of one TCBS plate and one plate of the second isolation 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 plates in accordance with the manufacturer's instructions.

8.7 Isolation and identification [11] [12]

Examine all inoculated TCBS and the second isolation plates (sub-cultured from both primary (8.6.1) and secondary enrichments (8.6.2)) for the presence of typical characteristic colonies of *V. parahaemolyticus* and *V. vulnificus:*

TCBS: *V. parahaemolyticus* - Smooth, green (negative sucrose) and 2 - 3 mm in diameter
V. vulnificus - Smooth, green (negative sucrose) and 2 - 3 mm in diameter

Second isolation plates: Follow the manufacturer's instructions for colony characterises of *V. parahaemolyticus* and *V. vulnificus*.

If no typical *V. parahaemolyticus* and/or *V. vulnificus* colonies are present on isolation plates sub-cultured from primary and/or secondary enrichment, then report the result as '*V. parahaemolyticus* and/or *V. vulnificus* NOT detected in 25g.'

If typical *V. parahaemolyticus* and/or *V. vulnificus* colonies are present on isolation plates sub-cultured from primary and/or secondary enrichment, confirmation must be carried out as described in 8.8.

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[8] The optimum temperature for *V. cholerae* to grow is 41.5±1˚C
[9] PCR may be used to screen for the presence of target bacterium in primary and secondary enrichment broths following incubation. This may enable downstream targeting of identification.
[10] The second isolation medium selection is the testing laboratories choice.
[11] PCR based confirmation can reduce the subjective interpretation of biochemical identification tests and accelerate the identification process.
[12] *V. cholerae* on TCBS plates are smooth, yellow (positive sucrose) colonies and are 1 - 2 mm in diameter.

8.8 *Vibrio* confirmation [13]

Confirmation of presumptive *V. parahaemolyticus* and *V. vulnificus* from isolation plates can be achieved using biochemical and/or molecular PCR approaches as described in 8.9.1 – 8.9.2. Test at least one well isolated colony of each *Vibrio* spp. type. 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

On to the surface of an SNA plate [14] subculture one well-isolated colony from both the TCBS and second isolation plate showing typical characteristics of *V. parahaemolyticus* and *V. vulnificus,* using a 1µl loop to obtain well-isolated colonies. Incubate at 37±1˚C for 24±3 hours. Retain all isolation plates in the dark at room temperature until confirmation is complete [15].

After incubation check each SNA plate for purity. If pure (that is, all colonies have the same morphology and colour), then continue with identification (8.9). If the purity plate shows a mixed culture, re-streak onto more SNA plates to isolate the organisms until pure colonies have been obtained.

8.9 Presumptive identification [16]

Perform an oxidase test [17] on each SNA plate giving pure cultures in 8.8.1 using a 1µl loop [18], inoculate a filter paper moistened with oxidase reagent. A positive reaction will lead to a blue-purple colour and a negative reaction will show no colour change.

Continue identification by means of biochemical screening tests (8.9.1), Conventical PCR (8.9.2.2) or real-time PCR (8.9.2.3) on all SNA plates that gave an oxidase positive reaction. Reject any SNA plates that showed an oxidase negative reaction.

8.9.1 Biochemical screening testing [19]

Inoculate all oxidase positive colonies, using a 1µl loop and without recharging the loop, into 3.5±1.5ml of LDC, 3.5±1.5ml of ADH, 0.25±0.01ml of 0.85% NaCl solution [20], 5±0.1ml of Indole solution, 5±0.1ml of SPW containing 0%, 6% and 10% NaCl and finally streaking onto an SNA plate with the objective to obtain well-isolated colonies. Overlay the surface of inoculated LDC and ADH with 1±0.1ml of sterile mineral oil to help differentiate between weak positives from negatives. Add 1 drop of toluene to inoculated 0.85% NaCl solution and mix well before incubating at 37±1˚C for 5 minutes. Once incubated add 0.25±0.01ml of ONPG reagent and mix.

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

After incubation, add 1±0.1ml of kovac reagent to each tube containing Indole solution. A positive indole reaction is indicated by the formation of a red ring, whereas a negative reaction is indicated by a yellow - brown ring. Bacterial growth (turbidity) and violet colour change in LDC (decarboxylation of lysine) and ADH (dihydrolation of arginine) denotes a positive reaction, while a yellow colour change indicates a negative reaction. A positive ONPG reaction (β-galactosidase activity) is indicated by a yellow colouration. No colour change indicates a negative result. Bacterial growth (turbidity) in the SPW containing NaCl should be recorded as a positive reaction while absence of turbidity is a negative reaction [21]. Determine the identification of the *Vibrio* spp. from the biochemical screening test results using Table 3.

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[13] For samples that are considered of importance five colonies should be examined for the presence of pathogenic markers
[14] Saline nutrient agar or slants of saline nutrient agar or suitable medium of laboratories own choice.
[15] It is recommended to mark the location of colonies showing typical characteristics of *V. parahaemolyticus* and *V. vulnificus* on the base of the TCBS as colony colouration may change over time.
[16] In addition, ISO 21872 gives guidance on Gram stain and motility test isolated SNA colonies. Retain all colonies that are Gram negative and Motile positive.
[17] Commercially available Oxidase test kits can be used following the manufacturer's instructions.
[18] 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.
[19] Commercially available biochemical and/or molecular kits that have been shown to produce reliable results may be used as an alternative and should be used in accordance to the manufactures instructions.
[20] Commercially available ONPG disks can be used following the manufacturer's instructions.
[21] Following inoculation of SPW, it is recommended to subculture onto an SNA plate to ensure 'no growth' in 10% SPW is not due to dead cultures.

Table 3: Expected results of biochemical screening tests

Test		*V. parahaemolyticus*	*V. vulnificus*	*V. mimicus*	*V. alginolyticus*	*V. cholerae*
Oxidase		+	+	+	+	+
LDC		+	+	+	+	+
ADH		-	-	-	-	-
ONPG hydrolysis (ß-galactosidase detection)		-	+	+	-	+
Indole production		+	+	+	+	+
Growth in saline peptone water with	0% NaCl	-	-	+	+	-
	6% NaCl	+	+	-	-	+
	10% NaCl	-	-	-	-	+

8.9.2 PCR confirmation

For each oxidase positive SNA plate, prepare a DNA extraction as described in 8.9.2.1. The prepared DNA extraction can be used for both conventional PCR (8.9.2.2) and real-time PCR (8.9.2.3).

8.9.2.1 DNA extraction [22]

Prepare a bacterial suspension for each SNA plate retained in section 8.9. Select one well-isolated colony from the SNA plate, using a 1µl loop, inoculate 500±10µl of 0.85% NaCl [23] in a 1.5ml micro-centrifuge tube. Heat the inoculated tube in a heating block at 95±2˚C for 5±1 minutes, followed by centrifuging at 10 000g for 1±0.5 minutes. Retain the DNA extraction [24] (supernatant) for PCR testing.

For each batch of samples tested, run in parallel a positive and negative extraction control. Prepare a positive control for each *Vibrio* spp. to be identified, using a 1µl loop from a microbiological reference culture given in Table 5 and 6, based on the PCR assay to be performed. For the negative control use 500±10µl of 0.85% NaCl or nuclease free water.

8.9.2.2 Conventional Polymerase chain reaction (PCR)

Prepare a master mix, in a 1.5ml micro-centrifuge tube, as shown in Table 4, adjusting the volumes based on the number of reactions required (number of SNA plates prepared in 8.9.2.1). Select the PCR primers from Table 5 based on the *Vibrio* spp. to be identified.

Table 4: Composition of conventional PCR mastermix

Reagent	Volume per reaction (µl)
Reaction buffer (5x) [a]	10
$MgCl_2$ (25 mM)	5
dNTPs (20 mM)	0.625
Forward primer (nM) (100 µM)	0.5
Reverse primer (nM) (100 µM)	0.5
Water	30.625
Taq polymerase	0.25
Total volume	47.5
[a] Dependent upon the initial concentration of the reaction buffer	

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[22] Commercially available DNA extraction kits can be used in accordance to the manufactures instructions.
[23] Nuclease free water can be used instead of 0.85% NaCl.
[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.

Table 5: Conventional PCR target regions and control material

Target region	Primer sequence		Reference [a]	Control strains	WCDM [b]	Product size (bp)
VptoxR [c]	Forward	GTC TTC TGA CGC AAT CGT TG	Kim *et al.*, 1999	*Vibrio parahaemolyticus*	00185	368
	Reverse	ATA CGA GTG GTT GCT GTC ATG				
Vptdh [d]	Forward	GTA AAG GTC TCT GAC TTT TGG AC	Bej *et al.*, 1999	*Vibrio parahaemolyticus*	10884 [e]	269
	Reverse	TGG AAT AGA ACC TTC ATC TTC ACC				
Vptrh [d]	Forward	TTG GCT TCG ATA TTT TCA GTA TCT		*Vibrio parahaemolyticus*	00037	500
	Reverse	CAT AAC AAA CAT ATG CCC ATT TCC G				
VVH	Reverse	CGC CAC CCA CTT TCG GGC C	Hill *et al.*, 1991	*Vibrio vulnificus*	00139	519
	Forward	GTC TTC TGA CGC AAT CGT TG				

[a] Reference given were included in the ISO 21872 interlaboratory study. Alternative primers can be laboratories free choice.
[b] World Data Centre for Microorganisms (WDCM) strain catalogue available at http://refs.wdcm.org
[c] Denotes species identification.
[d] Indicates pathogenic strain of *Vibrio parahaemolyticus*.
[e] NCTC reference strain number.

Prepare an individual 0.5ml micro-centrifuge tube for each extracted DNA sample by adding 5µl of extracted DNA with 45µl of master mix. Load all micro-centrifuge tubes onto a PCR thermocycler block and set the cycling parameters as given in Table 6 based on the *Vibrio* spp. primer set selected.

Table 6: Conventional PCR cycling parameters

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

Prepare a 2% agarose gel [25] and allow to cool before adding a few drops of ethidium bromide [26] [27]and gently mix. Place a gel comb to the mould before pouring the gel. Allow to solidify before removing the comb. Load the gel, using a pipette, with 10µl of 100 bp DNA ladder. In the consecutive wells add 20µl of PCR (positive and negative) controls, followed by each previously prepared sample PCR product, using an appropriate loading dye as necessary. Run the gel at 130 V for 25-30 minutes [28]. Following electrophoresis visualize the gel using an ultraviolet transilluminator. The expected PCR products size for the primers used for each *Vibrio* spp. type are given in Table 5 [29].

8.9.2.3 Real-time PCR

Prepare a master mix, in a 1.5ml micro-centrifuge tube, as shown in Table 6, adjusting the volumes based on the number of reactions required (number of SNA plates prepared in 8.9.2.1). Select the PCR primer

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[25] To prepare a 2% agarose gel add 2 g of agarose with 100ml of 1X TAE buffer (or equivalent).
[26] Alternative dye enabling visualization of PCR products can be used.
[27] Ethidium bromide is highly toxic. When handled appropriate Personal Protective Equipment (PPE) should be warn.
[28] Alternative volumes of PCR product and DNA ladder may be modified according the individual laboratory procedures and manufacturers' recommendations.
[29] PCR products should be confirmed by an appropriate method, following ISO 22174.

and hydrolysis probe from Table based on the *Vibrio* spp. to be identified.

Table 7: Composition of real-time PCR mastermix

Reagent	Volume per reaction (µl)
2 x TaqMan universal mastermix (1x) [a]	12,5
Forward primer (100 nM) [a]	0,45
Reverse primer (100 nM) [a]	0,45
Probe (500 nM) [a]	1
Water	5,6
Total volume	20
[a] Final concentration	

Prepare a 96 well plate, by adding 20µl of mastermix to individual wells followed by 5µl of extracted sample DNA [30]. For every batch of samples in a cycler run, prepare PCR controls by adding 5µl of undiluted sample DNA; 5µl of negative extraction control; 5µl of control DNA for each target assay and 5µl of nuclease free water into individual wells.

Table 8: Real-time PCR target regions and control material

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

[a] Probes labelled 5' 6-carboxyfluorescein (FAM), 3' 6-carboxytetramethylrhodamine (TAMRA).
[b] Reference given were included in the ISO 21872 interlaboratory study. Alternative primers can be laboratories free choice.
[c] World Data Centre for Microorganisms (WDCM) strain catalogue available at http://refs.wdcm.org
[d] Denotes species identification.
[e] Indicates pathogenic strain of *Vibrio parahaemolyticus*.
[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 at least 45 cycles of real-time PCR. Analyse the amplification plots using the approach recommended by the manufacturer of the real-time PCR machine. The threshold should ideally be set so that it crosses the area where the amplification plots (logarithmic view) are parallel (the exponential phase) [31].

Table 9: 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 s	45
	Annealing – extension	60 °C for 1 min	

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[30] Alternative volumes of PCR reagents may be modified according the individual laboratory procedures or from published protocols.
[31] 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.

Check the positive and negative control. Negative controls (nuclease-free water and negative extraction controls) should always be negative; if a positive result occur in a negative control then any samples giving positive results should be retested.

9. Results and reporting

Depending on the interpretation of the result, if at least one colony from the sample tested indicate that potentially pathogenic *Vibrio* spp. (*V. parahaemolyticus* and *V. vulnificus*) is detected in 25g, report as "*Vibrio spp.* detected in 25g" and specify the name of the *Vibrio* spp. and any pathogenicity characteristics if tested.

If no colonies from the sample tested conforms to the expected criteria of *V. parahaemolyticus and V. vulnificus* report the sample as " *V. parahaemolyticus and V. vulnificus* not detected in 25g"

10. Uncertainty of test results

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

11. References

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12 Appendices

12.1 Appendix 1: sub-sample sizes of shellfish required for Vibrio spp. analysis

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

Name [32]	Latin name [33]	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

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[32] The list given in Appendix 1 includes bivalve molluscs, echinoderms, tunicates and marine gastropods.

[33] Sample sizes given in this table have been provided by NRLs. The weight of shellfish flesh and liquor should be at least 50g (for very small species such as the *Donax* spp. a minimum amount of 25g is permitted). For species not given in the table, sufficient shellfish should be opened to achieve this minimum weight of flesh and liquor, with the provision that a minimum of ten animals should be used for very large species. In general, the more shellfish that are included in the initial homogenate, the less the final result will be influenced by the inherent animal-to-animal variation.

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Head office
Centre for Environment,
Fisheries & Aquaculture Science
Pakefield Road, Lowestoft,
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Web **www.cefas.defra.gov.uk**

Centre for Environment,
Fisheries & Aquaculture Science
Weymouth Laboratory,
Barrack Road, The Nothe, Weymouth,
Dorset DT4 8UB

Tel **+44 (0) 1305 206600**
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