Annex to: EFSA's BIOHAZ Panel Scientific opinion "Public health aspects of *Vibrio* spp. related to the consumption of seafood in the EU". doi:10.2903/j.efsa.2024.8896

Annex D – Analytical methods for the detection and enumeration of potentially enteropathogenic *Vibrio* spp. in seafood

Table D.1: Overview of the analytical methods for the detection and enumeration of potentially enteropathogenic *Vibrio* spp. in seafood.

Standardised methods for detection						
Scope	**Field of application / sample type**	***Vibrio* species**	**Brief description of procedure**	**Genetic markers**	**Performance characteristics**	**Applicability / limitations**
ISO 21872-1:2017/Amd 1:2023. Microbiology of the food chain – Horizontal method for the determination of *Vibrio* spp. Part 1: Detection of potentially enteropathogenic *Vibrio parahaemolyticus*, *Vibrio cholerae* and *Vibrio vulnificus*. International Organization for Standardization, Geneva, Switzerland.						
Horizontal method for the detection of enteropathogenic *Vibrio* spp. (human illness in or via the intestinal tract)	Products for human consumption, animal feeding, environmental samples	*V. cholerae*	**Enrichment:** test portion up to 25 g or 25 mL, 1 medium, 2 consecutive enrichments and 2 temperatures **Colony isolation:** 2 selective media **ID:** biochemical tests **ID confirmation:** cPCR **Pathogenicity**: serology (O1/O139) or cPCR	**ID confirmation**: 16S-23S rRNA ITS **Pathogenicity:** *ctx* gene	**ILS[a]** Matrix: cooked prawns LOD50: 11.34 CFU/g Sensitivity: 92.5% Specificity: 84%	Widely used method for the detection of Vp, Vv, Vc. Well standardised protocols. Primer sets for *Vibrio* spp. ID confirmation and Vp virulence associated genes. Subculture of a small number of colonies could lead to miss potentially pathogenic strains.
		V. parahaemolyticus	**Enrichment:** test portion up to 25 g or 25 mL, 1 medium, 2 consecutive enrichments and 2 temperatures **Colony isolation:** 2 selective media **ID:** biochemical tests **ID confirmation:** cPCR and rtPCR **Pathogenicity**: cPCR and rtPCR	**ID confirmation**: *toxR* gene (cPCR and rtPCR) **Pathogenicity:** *tdh* and *trh* genes (cPCR), *tdh* gene (rtPCR)	**ILS[a]** Matrix: raw oysters LOD50: 0.43 CFU/g Sensitivity: 72.5-100% for Vp, 86% for *tdh*+ Vp by cPCR, 94% for *trh*+Vp by cPCR, 73% for *tdh*+ Vp by rtPCR Specificity: 111%[b] for Vp, 89% for *tdh*+ Vp by cPCR, 91% for *trh*+Vp by cPCR, 94% for *tdh*+ Vp by rtPCR Matrix: cooked prawns LOD50: unknown Sensitivity: 100% Specificity: 99%	
		V. vulnificus	**Enrichment:** test portion up to 25 g or 25 mL, 1 medium, 2 consecutive enrichments and 1 temperature **Colony isolation:** 2 selective media **ID:** biochemical tests **ID confirmation:** cPCR and rtPCR	**ID confirmation**: *vvhA* gene (cPCR and rtPCR)	**ILS[a]** Matrix: raw oysters LOD50: 12.31 CFU/g Sensitivity: 37.5% Specificity: 77.5% Matrix: cooked prawns	

					LOD50: 81.2 CFU/g Sensitivity: 61.25-71.5% Specificity: 60%	
FDA-BAM (2004). Bacteriological Analytical Manual Chapter 9: Vibrio. Food & Drug Administration, Bacteriological Analytical Manual, Washington DC[c]						
Detection of human pathogenic Vibrionaceae (*V. cholerae*, *V. parahaemolyticus* and *V. vulnificus*) in seafood	Food and water	*V. cholerae (V. mimicus)*	**Enrichment:** test portion 25 g, 1 medium, 1 or 2 consecutive enrichments and 2 temperatures **Colony isolation:** 2 selective media **ID:** biochemical tests, serological typing **Pathogenicity**: cell assay, cPCR	**Pathogenicity:** *ctx* gene	NR	Presumptive identification of Vc (no molecular characterisation or confirmation). Biotyping and other tests recommended or optional.
	Seafood (molluscan shellfish, processed food)	*V. parahaemolyticus*	**Direct plating:** test portion 0.1 g of homogenate, 1 selective medium, incubation at 1 temperature **ID:** colony hybridization (AP-probe) or multiplex cPCR **Pathogenicity**: colony hybridization (AP-probe and DIG-probe) or multiplex cPCR	**ID:** *tlh* gene (AP-probe, cPCR) **Pathogenicity**: *tdh* gene (AP-probe, cPCR), *trh* gene (DIG-probe, cPCR)	NR	The use and specificity in given conditions of the *tlh* gene for Vp identification has been questioned (Yáñez et al., 2015)
	Seafood (molluscan shellfish, processed food)	*V. vulnificus*	**Direct plating:** test portion 0.1 g of homogenate, 1 selective medium, incubation at 1 temperature **ID:** colony hybridization (DIG-probe) and cPCR	**ID:** *vvhA* gene (DIG-probe, cPCR)	NR	
Standardised methods for enumeration						
ISO/TS 21872-2:2020. Microbiology of the food chain – Horizontal method for the determination of *Vibrio* spp. Part 2: Enumeration of total and potentially enteropathogenic *Vibrio parahaemolyticus* in seafood using nucleic acid hybridization. International Organization for Standardization, Geneva, Switzerland.						
Method for the direct enumeration of total and potentially enteropathogenic *V. parahaemolyticus* in seafood	Seafood	*V. parahaemolyticus*	**Direct plating:** test portion 0.1 g of homogenate, 1 non-selective medium, incubation at 1 temperature, transfer of colonies to nylon membrane **ID:** colony hybridization (DIG-probes) for total Vp and for *tdh*+ and/or *trh*+ Vp	**ID**: DIG *toxR* gene (DIG-probe) **Pathogenicity**: *tdh1, tdh2, trh1, trh2* genes (DIG-probe)	**Single-laboratory validation** Matrix: bivalve shellfish, finfish, cephalopods, crustaceans. Selectivity: (a) oligoprobes inclusivity: *toxR* 99.4%, *tdh* 96.7%, *trh* 97.8%; (b) oligoprobes exclusivity: 100% for all targets Sensitivity: LOD 10 CFU/g, LOQ 100 CFU/g (defined in accordance with ISO 7218) Performance parameters: accuracy and linearity, repeatability.	Accurate and specific but time consuming and technically demanding method; 0.1 g of sample analysed only.
FDM-BAM (2004) Bacteriological Analytical Manual Chapter 9: Vibrio. Food & Drug Administration, Bacteriological Analytical Manual, Washington DC[c]						
Detection of human pathogenic Vibrionaceae (*Vibrio cholerae*,	Seafood (molluscan shellfish, processed food)	*V. parahaemolyticus*	**MPN format enrichment:** test portion 1 g and following dilutions, 1 medium, incubation at 1 temperature **Colony isolation:** 1 selective medium **ID:** biochemical tests or colony hybridization (AP-probe) or multiplex cPCR	**ID:** *tlh* gene (AP-probe, cPCR) **Pathogenicity**: *tdh* gene (AP-probe, cPCR), *trh* gene (cPCR)	NR	Time consuming (MPN: 4-5 days, HGMF: 3-4 days). Identification is only presumptive if no

Vibrio parahaemolyticus and *Vibrio vulnificus*) in seafood			**Pathogenicity:** colony hybridisation (AP-probe) or multiplex cPCR			molecular confirmation is applied. Not suitable for routine analysis. The use and specificity of the *tlh* gene in given conditions for Vp identification has been questioned (Yáñez et al., 2015)
			HGMF: test portion 0.5 g, culture on filter on 1 selective medium, incubation at 1 temperature **ID:** presumptive enumeration based on colony colour		NR	
			Direct plating: test portion 0.1 g of homogenate, 1 non-selective medium, incubation at 1 temperature), **ID:** colony hybridisation (AP-probe) or multiplex cPCR **Pathogenicity:** colony hybridisation (AP-probe) or multiplex cPCR	**ID:** *tlh* gene (AP-probe, cPCR) **Pathogenicity**: *tdh* gene (AP-probe, cPCR), *trh* gene (cPCR)	NR	
		V. vulnificus	**MPN format enrichment:** test portion 1 g and following dilutions, 1 medium, incubation at 1 temperature **Colony isolation:** 2 selective media **ID:** biochemical tests or colony hybridisation (DIG-probe) or cPCR	**ID:** *vvhA* gene (DIG-probe, cPCR)	NR	

Other methods (national standards)

Scope	Field of application / Sample type	*Vibrio* sp.	Brief description of procedure	Genetic markers	Performance characteristics	Applicability / Limitations
NMKL Method N°156, 2nd ed. 1997 (Scandinavian countries) Pathogenic *Vibrio* species. Detection and enumeration in foods						
Detection and enumeration of *V. parahaemolyticus*, *V. cholerae*, *V. vulnificus* and *V. alginolyticus* in food	Microbiological control of foods, especially shellfish, crayfish and fish. Microbial investigations in connection with disease outbreaks	*V. parahaemolyticus, V. cholerae, V. vulnificus, V. alginolyticus*	**Detection**: enrichment (2 media, incubation at 1 temperature), colony isolation, colony identification (type based on colour, size, aspect), ID confirmation (biochemical tests, O1/O139 antisera Vc) **Enumeration**: direct plating (1 selective medium, incubation at 1 temperature)		Not validated in a collaborative study	Presumptive ID of colony (colony type description, colour, size, aspect) Enumeration based on colony type (aspect)
MFLP-37 (2019). Detection, isolation, and enumeration of *Vibrio parahaemolyticus* and/or *Vibrio vulnificus* in Seafood. Laboratory Procedures for the Microbiological Analysis of Foods. Volume 3. Government of Canada.						
Detection, isolation and enumeration of *V. parahaemolyticus* and/or *V. vulnific*us in seafood	Seafood (fish, shellfish, crustaceans)	*V. parahaemolyticus*	**Enumeration**: MPN format enrichment (test portion 1g and following dilutions, 1 medium, incubation at 1 temperature) **Colony isolation**: 1 selective medium **ID**: biochemical tests, multiplex cPCR (described in method MLFP-23, March 2006[d]), multiplex rt PCR (described in method MLFP-102, July 2017[e]) **Pathogenicity**: multiplex cPCR or multiplex rt PCR (as in the identification)	**ID**: R72H fragment, *tlh* gene **Pathogenicity**: *tdh* and *trh* genes (multiplex cPCR or multiplex rtPCR)	NR	

		V. vulnificus	**Enumeration**: MPN format enrichment (test portion 1g and following dilutions, 1 medium, incubation at 1 temperature) **Colony isolation**: 1 selective medium **ID**: biochemical tests		Presumptive identification of Vv (no molecular characterisation or confirmation)
GB 4789.7-2013. National Food Safety Standard – Microbiological Examination of Food Hygiene – Examination of *Vibrio parahaemolyticus*. National Standard of the People's Republic of China					
Method for the inspection of *V. parahaemolyticus* in food	Fish, cephalopods, shellfish, crustaceans	*V. parahaemolyticus*	**Detection**: enrichment (test portion 25 g, 1 medium, incubation at 1 temperature), colony isolation (1 selective medium), colony identification (biochemical tests, serological typing) **Enumeration**: MPN format enrichment (test portion of 2.5 g and continuous dilutions, 1 medium, incubation at 1 temperature), colony isolation (1 selective medium), colony identification (biochemical tests, serological typing)		Presumptive identification of Vp (no molecular characterisation or confirmation)
GB 4789.44-2020. National Food Safety Standard – Microbiological Examination of Food Hygiene – Examination of *Vibrio vulnificus*. National Standard of the People's Republic of China					
Method for the examination of *V. vulnificus* in aquatic products	Fish, shrimp, crab, shellfish, cephalopods	*V. vulnificus*	**Detection**: enrichment (1 selective medium, incubation at 1 temperature), PCR testing and colony isolation (2 selective media), colony identification (biochemical tests and cPCR identification (optional) and typing/pathogenicity (optional)	**ID**: vvhA gene (cPCR) **Typing/pathogenicity**: 16S rRNA A/B, *SerE* gene, *Bt2* gene, *vcgC/E* genes cPCR)	NR

Abbreviations: AP-probe, alkaline phosphatase labelled probe; cPCR, conventional PCR; DIG-probe, digoxigenin labelled probe; HGMF, hydrophobic grid membrane filtration; ID, identification; ILS, inter-laboratory study; ITS, internal transcribed spacer; LOD, limit of detection; LOQ, limit of quantification; MPN, most probable number; NR, not reported; PCR, polymerase chain reaction; rtPCR, real-time PCR; Va, *Vibrio alginolyticus*; Vc, *V. cholerae*; Vm, *V. mimicus*; Vp, *V. parahaemolyticus*; Vv, *V. vulnificus.*

[a]An international inter-laboratory study, ILS (Hartnell et al., 2019) using the method specified in the ISO 21872:2017 was carried out for detection of *V. parahaemolyticus*, *V. vulnificus* and *V. cholerae* in raw and cooked seafood (Hartnell et al., 2019). Real-time PCR for identification of *V. cholerae* and detection of *trh* genes of *V. parahaemolyticus* was tested in this international ILS, but the data were not reliable and were therefore excluded from the final ILS dataset.

[b]The specificity calculated for *V. parahaemolyticus* (111%) was due to positive signals/identification obtained by cPCR in control samples.

[c]https://www.fda.gov/food/laboratory-methods-food/bam-chapter-9-vibrio, accessed on the 14 May 2024.

[d]MFLP-23, March 2006: Specific detection of *Vibrio parahaemolyticus* strains using a multiplex polymerase chain reaction (PCR) based on R72H taxonomic marker and the haemolysin genes TDH and TRH.

[e]MFLP-102, July 2017: Identification of *Vibrio parahaemolyticus* colonies by real-time polymerase chain reaction.

References

Hartnell, R. E., Stockley, L., Keay, W., Rosec, J. P., Hervio-Heath, D., Van den Berg, H., Leoni, F., Ottaviani, D., Henigman, U., Denayer, S., Serbruyns, B., Georgsson, F., Krumova-Valcheva, G., Gyurova, E., Blanco, C., Copin, S., Strauch, E., Wieczorek, K., Lopatek, M., … Baker-Austin, C. (2019). A pan-European ring trial to validate an international standard for detection of *Vibrio cholerae*, *Vibrio parahaemolyticus* and *Vibrio vulnificus* in seafoods. *International Journal of Food Microbiology*, *288*, 58–65. https://doi.org/10.1016/j.ijfoodmicro.2018.02.008

Yáñez, R., Bastías, R., Higuera, G., Salgado, O., Katharios, P., Romero, J., Espejo, R., & García, K. (2015). Amplification of *tlh* gene in other Vibrionaceae specie by specie-specific multiplex PCR of *Vibrio parahaemolyticus*. *Electronic Journal of Biotechnology*, *18*(6), 459-463. https://doi.org/10.1016/j.ejbt.2015.09.007