Handbook of Seafood and Seafood Products Analysis

DETECTION OF THE PRINCIPAL FOODBORNE PATHOGENS IN SEAFOODS AND SEAFOOD-RELATED ENVIRONMENTS.

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ABSTRACT

The number of bacterial gastroenteritis associated to seafood products has been increased considerably during the last decades. Between the bacterial pathogen that can produce gastroenteritis associated to seafood products, three can be considered as a primary threat: the enteropathogenic *Vibrio*, *Listeria monocytogenes* and *Salmonella* spp. Microbiological quality control programs are being increasingly applied throughout the seafood production chain in order to minimize the risk of infection for the consumer. In this chapter we will revise the most relevant aspect of the detection strategies developed for those main bacterial pathogens.

Keywords: Food Safety, Public Health, Detection, Bacteria, Salmonella, Listeria, Vibrio, Outbreaks, molecular methods, PCR, culture.

INTRODUCTION

The importance of foodborne pathogens in Public Health is substantial. They cause more than 14 million illnesses, 60,000 hospitalisations and 1,800 deaths per year in the United States (1) with annual medical and productivity losses above 6,500 million dollars (2). In England and Wales, the figures are similar, and they cause 1.3 million illnesses, 20,759 hospitalisations and 480 deaths each year (3). The number of bacterial gastroenteritis associated to seafood products has been increased considerably during the last decades by the rapid globalisation of the food market, the increase of personal and food transportation and profound changes in the food consumption habits (1,4). Between the bacterial pathogen that can produce gastroenteritis associated to seafood products, three can be considered as a primary threat: the enteropathogenic *Vibrio*, *Listeria monocytogenes* and *Salmonella* spp.

Three *Vibrio* species, *V. parahaemolyticus*, *V. vulnificus* and *V. cholerae*, are well-documented human pathogens, specially associated to the consumption of raw or undercooked seafood products (5,6,7). *V. parahaemolyticus* is an important seafood-borne pathogen worldwide (8). It was first identified as a cause of food-borne illness in Japan in 1950 (9), and it has been reported to account for 20–30% of foodborne illnesses in Japan (10) and a common cause of seafood-borne gastroenteritis in Asian countries (11, 12). In contrast, infections are occasional in Europe, and only sporadic outbreaks have been reported in Spain and France (13). In The Unites States, *V. parahaemolyticus* is the leading cause of gastroenteritis associated with seafood consumption, and between 1973 and 1998 approximately 40 outbreaks were reported (14). Consumption of raw or undercooked seafood, particularly shellfish, contaminated with *V. parahaemolyticus* may produce a self-limiting gastroenteritis involving symptoms such as vomiting, nausea, diarrhoea with abdominal cramps, headache, and low-grade fever. *V. parahaemolyticus* is disseminated worldwide in estuarine, marine, and coastal water environments (15). Some environmental factors such as the water temperature, salinity, zooplankton blooms, tidal flushing,

and dissolved oxygen modulate its spatial and temporal distribution (16). The increase of the prevalence of *V. parahaemolyticus* in raw shellfish is also correlated to the warm seawaters. The *V. parahaemolyticus* loads in oysters is usually lower than 10^3 cfu g^{-1} (17), but it can increase notably when the shellfish is cultivated in warmer seawater (18).

There is *V. parahaemolyticus* pandemic clone, the O3:K6 clone, which is distributed worldwide since its emergence in 1996 in India (19), and it has been involvesd in seafood-related outbreaks in 1998 in Japan (20) and in the USA (14). In Chile, the O3:K6 clone has also been responsible for most of gastroenteritis cases since 2004 (21). However, other serotypes have also been involved in V. *parahaemolyticus* outbreaks: serotype O6:K18 in Alaska after the consumption of oysters (22), serotypes O4:K12 and O4:K on the US Atlantic coast (23) after the consumption of shellfish and seafood. In Spain, a massive *V. parahaemolyticus* outbreak (serotypes O4:K12 and O4:KUT) was reported linked to the people the consumption of shrimp during a food banquet on a cruise (24). Recently, a new emerhing serotype, O4:K8, has been reported in southern China linked to seafood diarrheal cases (25)

V. vulnificus produces one of the most severe foodborne infections, with a case-fatality rate greater than 50% (26). It can cause fatal septicaemia, wound infections and gastroenteritis especially in immuno-compromised individuals (27). It was first isolated by the Center for Disease Control (CDC) in 1964 (28). This organism is also disseminated worldwide in waters of different temperatures and salinities (29). Environmental conditions such as water temperature, salinity modulate the variation in its prevalence (30). Most of the *V. vulnificus* cases have been reported in Japan (31,32,33,34), in the USA (35,36,37,38,39,40), as well as in New Caledonia (41), Korea (42), and the Gulf of Mexico (43) during summer generally associated to the consumption of raw seafoods.

V. cholerae is the causative agent of the cholera outbreaks and epidemics. The World Health Organization reported 172,454 cases of cholera in 2015 including 1304 deaths in a total of 42

countries including six European countries (44). There is a direct relationship between the consumption of raw, undercooked, contaminated, or re-contaminated seafood and outbreaks produced by *V. cholerae* (5,45,46). Foodstuff can be contaminated by this pathogen through contaminated irrigation water or human origin-fertilizer (45,47). The O1 serogroup is the group predominantly isolated in cholera epidemics (46), and a new pathogenic serogroup, O139, has been also identified (48). *V. cholerae* O1 was reported as the responsible for an outbreak in Haiti in October 2010 (49, 50). Another V. cholerae O1 outbreak occurred in a wedding in the Dominican Republic in January 2011 linked to the consumption of shrimps contaminated via the ice on which they seafood was stored (51). Non-O1/O139 serogroups are sporadically involved in cholera-like diarrheal episodes, but infrequently in outbreaks (52,53, 54,55,56). Toxigenic *V. cholerae* O1 is rarely isolated and no isolations of serogroup O139 have been reported in western countries. In contrast, non-O1/O139 isolates are commonly found in estuarine water and shellfish (57). Various O1 strains have become endemic in many regions in the world, including Australia and the US Gulf Coast (58, 59).

Listeria monocytogenes is an important foodborne pathogen, which usually (20-50% of the cases) produces a fatal infection. It has been isolated from a wide range of sources, and seafood and seafood-related environments have been reported as important niches for this bacterium (60). Cao et al. (61) reported the recurrent presence of this pathogen in shrimp samples and a frozen shrimp processing line environment, without a positive correlation between its presence and the accompanying environmental microbiota. Farber (62) reported a low incidence of *L. monocytogenes* in imported seafood products between 1996–1998 (below 1%), and a complete absence in Canadian seafood products. Van Coillie et al. (63) studied the prevalence of *L. monocytogenes* in different ready-to-eat (RTE) seafood products on the Belgian market. The occurrence of *L. monocytogenes* was 23.9%, and the contamination levels were low in most cases (84% below 100 cfu g^{-1}). The most prevalent serotype was 1/2a and serotypes 1/2b, 1/2c and 4b were also present. In a longitudinal study in seafoods between 2001 and 2005 in France, Midelet-Bourdin et al. (64) observed similar

findings (a prevalence of 28% with a low level of contamination). The presence of *L. monocytogenes* in tropical fish and shellfish in Mangalore, India was 17% and 12%, respectively (65). Similar results were obtained by Nakamura et al. (66,67) in RTE seafood products commercially available or in a cold-smoked fish processing plant in Osaka, Japan (13% and 7%, respectvely). Its incidence was mainly in the summer and autumn, and it was only isolated in cold-smoked fish samples and in low numbers (below 100 cfu g^{-1}). The serotype 1/2a was the most prevalent in both studies, and serotypes 1/2b, 3b, 4b and 3a were also present. In a survey conducted in Thailand, a total of 595 samples were collected from raw material, seafood products and related environments, and *L. monocytogenes* was found in 22 (3.7%) samples (68). In another study conducted in Spain, 250 refrigerated ready-to-eat seafood products were tested for the presence of *L. monocytogenes, and* 4.8% of smoked salmon samples were positive with low levels (<10 cfu/g) (69). The consumption of seafoods and outbreaks of listeriosis is well documented (60). For example, in a small human outbreak occurred in Ontario, Canada, the relationship between the presence of *L. monocytogenes* in seafood products (imitation crab meat) and the outbreak was clearly established (62). Although all the foodstuffs obtained from the refrigerator of the two patients contained *L. monocytogenes*, three of them were heavily contaminated: imitation crab meat, olives, and salad. Molecular typing of the isolates by randomly amplified polymorphic DNA (RAPD) and pulsed-field gel electrophoresis (PFGE) typing demonstrated that the imitation crab meat and the clinical strains were indistinguishable. In addition, challenge studies performed with a pool of *L. monocytogenes* strains showed that imitation crab meat, but not olives, supported growth of this pathogen.

Salmonella spp. is a major public health problem because of its large and varied animal reservoir, the existence of human and animal carrier states, and the lack of a concerted nationwide program to its control (70). Furthermore, *Salmonella* is the main cause of documented foodborne human illnesses in most developed countries (71,72,73). Of the outbreaks of foodborne illness recorded in the WHO report for 1993 to 1998, Salmonellae were most often reported as causative

agent (54.6 % of cases) (74). Food items with a greater hazard include raw meat and some products intended to be eaten raw, raw or undercooked products, such as seafood and seafood products (75). A recent meta-analysis study has identified a specific serovar distribution in seafood (76): while the serovar Hadar ia the most frequently found in Latin America, the serovar Typhimurium is the most prevalent in Europe, followed by Senftenberg, and the serovars Weltevreden and Newport are the most prevalent serotype in Asia and in North America, respectively. The presence of *Salmonella* spp. in tropical seafood products collected from different landing centres and open markets in Mangalore, India was studied by Kumar et al. (77). The overall incidence of *Salmonella* spp. was 17%, suggesting that the contamination of seafoods with *Salmonella* may be occurring during post-process handling and processing. A similar study was conducted in fish, shellfish, ice and water obtained from the market and fish-landing centre in Mangalore, India (78). Twenty percent of the samples were positive using conventional methods, but the number of positives increased up to 52% when PCR was used, indicating the prevalence of *Salmonella* in seafood may be much more than that reported by conventional isolation techniques. The most prevalent serotype was *S. enterica* serotype Weltevreden, and *S. enterica* serotype Worthington and *S. enterica* serotype Newport were also present.

DETECTION OF THE PRINCIPAL SEAFOOD-BORNE PATHOGENS

As a consequence of the potential hazards described above, microbiological quality control programs are being increasingly applied throughout the seafood production chain in order to minimize the risk of infection for the consumer. Classical microbiological methods to detect the presence of those microorganisms involve enrichment and isolation of presumptive colonies of bacteria on solid media, and final confirmation by biochemical and/or serological identification. It is laborious and time consuming, and usually more than 3-5 days are needed for definitive results. Although remaining the approach of choice in routine analytical laboratories, the adoption of alternative techniques such as molecular-based methods in microbial diagnostics has become an

alternative approach, as they possess inherent advantages such as shorter time to results, excellent detection limits, specificity, and potential for automation.

Detection of pathogenic Vibrio species in seafoods and seafood-related environments

V. parahaemolyticus

The most widely used methods for the detection of *V. parahaemolyticus* in foods are the International Organization for Standardization (ISO) standard 8914:1990 (79) and the most probable number (MPN) method described in the US Food and Drug Administration (FDA) Bacterial Analytical Manual (BAM) (80). In the International Standard ISO 8914:1990, food samples are incubated at 35 ºC for 7–8 h in parallel in two enrichment broths (salt polymyxin B broth and alkaline saline peptone water or saline glucose culture medium with sodium dodecyl sulfate), and then streaked on two selective media [thiosulfate–citrate–bile salts–sucrose agar (TCBS) and triphenyltetrazolium chloride soya tryptone agar (TSAT)]. After incubation for 18 h on TCBS or 20–24 h on TSAT, colonies being 2–3 mm, smooth, and green on TCBS or 2–3 mm, smooth, flat, and dark red on TSAT can be considered presumptive colonies of *V. parahaemolyticus*, and they must be confirmed by biochemical tests. Recently a new ISO standard (ISO/TS 21872-1:2007) has been published describing a horizontal method in food for detection of *V. parahaemolyticus* and *V. cholerae* (81). In the FDA BAM method, after the MPN analysis, the tubes must be plated on TCBS selective medium and several presumptive isolates must be confirmed by biochemical testing. In both cases, these methods are cumbersome and laborious, and definitive results can be only obtained after more than 4-5 days. To overcome those disadvantages, different PCR methods have been developed for detection of *V. parahaemolyticus* in seafood products and seafood-related environments (Table 1).

Some authors have reported PCR methods for the detection *V. parahaemolyticus* independently of the pathogenic capacity of the strains detected. For this purpose, different PCR targets and DNA protocols have used (82) Lee et al. (83) developed a PCR method based on a

specific fragment, *pR72H*, cloned and sequenced in that laboratory. To determine its selectivity, 124 *V. parahaemolyticus* and 50 non-*V. parahaemolyticus* isolates were assayed. The PCR assay was 100% selective. Finally, the applicability of the method was evaluated in oysters. Ten ml of oyster homogenate were inoculated with decreasing amounts of *V. parahaemolyticus*, and one millilitre of each homogenate was then mixed with 9 ml of TSB (Tryptose soy broth) containing 2.5% NaCl and incubated at 35 ºC. After enrichment, the DNA was extracted following three different protocols (by heating, by addition of 10% Triton X-100 and heating; and by enzymatic digestion with lysozyme and proteinase followed by boiling). The limit of detection after 3 hours-enrichment, using enzymatic digestion and boiling was as few as 9.3 cfu g^{-1}.

Other gen marker used for *V. parahaemolyticus*-specific detection is the thermolabile haemolysin (*tlh*) gene. Wang and Levin (84) observed a linear relationship between the fluorescent intensity of the *tlh* PCR products in the agarose gel and the bacterial populations. Kaufman et al. (85) devised an alternative strategy for detection of *V. parahaemolyticus* in oyster. They used mantle fluids as food matrix instead of homogenized oyster tissues, since they observed that the levels of natural contamination of *V. parahaemolyticus* were similar in mantle fluids and oyster tissues. They developed a *tlh*-specific real-time PCR, which was 100% selective as determined using 37 *V. parahaemolyticus, 27* other Vibrio and 37 non-*Vibrio* isolates. A strong linear correlation between the PCR results and the concentration of cells inoculated into mantle fluids was observed, and the mantle fluid exhibited less PCR inhibition than the homogenized oyster tissue.

Kim et al. (86) reported a PCR method based on the toxin transcriptional activator (*toxR)* gene. After testing 373 *V. parahaemolyticus* isolates and 290 isolates of other bacterial species, they concluded that the method was 100% selective. Similarly, Takahashi et al. (87) developed a *toxR*-based real-time PCR method. It was fully selective as tested 25 *V. parahaemolyticus* and 30 non- *V. parahaemolyticus* isolates. They also evaluated tits applicability in shellfish. Twenty-five grams of short-neck clams were homogenised with PBS (phosphate buffered saline), artificially contaminated

with decreasing amounts of *V. parahaemolyticus*., and the DNA was extracted with the MagExtractor-Genome Kit (Toyobo). The real-time PCR detected as few as 100 cfu g^{-1}.

Venkateswaran et al. (88) reported a PCR method based on the B subunit of DNA gyrase (*gyrB*) gene. The selectivity of the method was evaluated using 117 strains of *V. parahaemolyticus* isolated from various environments, food, and clinical sources, and 150 isolates of other species. Twenty five-grams samples of shrimp were homogenized in 225 ml of APW (alkaline peptone water) and artificially contaminated with decreasing amounts of *V. parahaemolyticus* and *V. alginolyticus*, and incubated at 37 °C. The homogenates were centrifuged and resuspended in 1 ml of sterile PBS. Ten microliters were used for PCR without extraction of DNA. The analytical sensitivity was as few as 1.5 *V. parahaemolyticus* cfu g^{-1} of homogenate. Similarly, Cai et al. (89) designed a *gyrB*-based real-time PCR. The selectivity was confirmed using 27 *V. parahaemolyticus* and 10 non- *V. parahaemolyticus* isolates. One gram oyster meat homogenate were artificially contaminated and 1 ml aliquot was used for the DNA extraction using the Wizard genomic DNA purification (Promega). The limit of detection of the method was 100 cfu ml^{-1} of oyster homogenates. When 300 seafood samples collected from local supermarkets in eastern China were tested, 32% of the samples were positive using the method. However, only 26% of the samples were positive using the conventional culture method. Interestingly, all culture-positive were also real-time PCR-positive, indicating that the real-time PCR method was more sensitive that the conventional culture method.

PCR methods have been also developed for the only specific detection of pathogenic strains of *V. parahaemolyticus*. Tada et al. (90) developed a PCR method based on the thermostable haemolysin (*tdh*) gene and *tdh*-related haemolysin (*trh*) gene. The selectivity was demonstrated using 263 *V. parahaemolyticus*, and 133 isolates of other species. Karunasagar et al. (91) reported a PCR method for the detection of Kanagawa positive strains in seafoods. The primers targeted the *tdh* gene. It was fully selective as tested in 4 Kanagawa positive *V. parahaemolyticus*, 20 Kanagawa negative *V. parahaemolyticus*, and 31 other *Vibrio* isolates. For the detection in seafoods, 50 g of

samples were homogenised with 450 ml APW. One millilitre of homogenate was centrifuged at 100 ×*g*, and the supernatant was again centrifuged, resupended and lysed by heating. The analytical sensitivity was less than 10 cells of *V. parahaemolyticus* after 8 h enrichment. A real-time PCR method was also developed using the same molecular marker, *tdh* (92). The sensitivity was demonstrated using 42 *tdh*+ *V. parahaemolyticus* isolates, 12 *tdh*- *V. parahaemolyticus* isolates, and 103 non-target isolates. For detection of the pathogenic strains in oyster samples, a 50-ml aliquot of 1:1 oyster homogenate was added to 200 ml of APW and enriched overnight at 35 ºC. After the enrichment, 1 ml was boiled and 2.5 µl of the supernatant was used for PCR. The real-time PCR detected as few as 1 cfu per reaction. Finally, 131 natural oyster samples collected from Alabama, USA were analyzed by both conventional microbiological methods and real-time PCR. Forty-two percent of negative samples for the microbiological method were positive for the real-time PCR indicating a significantly higher detection rate ($p < 0.05$) and only a 20 % of the samples positive for the microbiological method were negative for the real-time PCR method.

Hara-Kudo and co-workers (93) optimised a PCR method using different DNA extraction procedures for the detection of the pathogenic *V. parahaemolyticus* in seafoods. The primers targeted the *tdh* gene, which PCR selectivity had been tested previously (90). Three different DNA extraction methods were evaluated: a silica membrane method using the NucleoSpin Tissue Kit (Macherey-Nagel), a glass fibber method using the High Pure PCR Template Precipitation Kit (Roche), or a magnetic separation method using the MagExtractor-Genome Kit (Toyobo). The use of the silica membrane and the glass fibber methods increased notably the analytical sensitivity.

Taking in consideration the importance for Public Health of this pathogen, distinguishing between potentially pathogenic and non-pathogenic *V. parahaemolyticus* isolates is of critical importance. Bej and collaborators (94) reported a multiplex PCR method for the detection of total and haemolysin-producing *V. parahaemolyticus* in shellfish. The method targeted the *tlh* gene for the detection of all *V. parahaemolyticus* strains and the *tdh* and *trh* genes for the specific detection of the pathogenic strains. The selectivity of the method was evaluated using 111 *V.*

parahaemolyticus isolates from different origins and 19 non-*V. parahaemolyticus* isolates. The *tlh* primers were 100% selective. Fifty four percent of the *V. parahaemolyticus* isolates showed positive PCR amplification for the *tdh* primers, and 39% showed amplification of the *trh* primers. Interestingly, 3 isolates showed no *tdh*- and *trh*-PCR amplification but were Kanagawa positive, and three other isolates were *tdh*-PCR positive, but produced a negative Kanagawa reaction. Finally, 10 g of oyster homogenate were artificially contaminated with decreasing amounts of *V. vulnificus* strains with different *tl*/*tdh*/*trh* profiles, diluted in 350 ml of APW and incubated at 35 ºC for 6 hours. DNA was extracted following a previously described method (95). The limit of detection for all the three PCR primers was 100 cells for the *tdh*-primers, and 10 cells for *tlh*- and *trh*-primers. Using the same set of primers, Luan and co-workers (96) used a rapid MPN-PCR method for quantification of this pathogen in seafood samples purchased at local retail markets in Qingdao, China. Seventy-three percent of the samples were *V. parahaemolyticus* (*tlh*-) positive with values higher than 719 MPN g^{-1}, and 41.5% of samples were positive for *tdh* gene-possessing cells, indicating the presence of pathogenic strains.

Nordstrom et al. (97) developed a multiplex real-time PCR method for detection of the total and pathogenic strains of this organism in oysters using the same targets; *tlh, tdh* and *trh* genes, but this method included an internal amplification control (IAC). The IAC is a non-target nucleic acid sequence present in every reaction, which is amplified simultaneously with the target sequence (98). In PCR diagnostics, IACs are essential to identify false negative results (99) as in a reaction with an IAC, a control signal will always be produced when there is no target sequence present. The selectivity was evaluated using 117 *V. parahaemolyticus* isolates with different *tlh*/*tdh*/*trh* profiles and 36 isolates of other species of the genus *Vibrio*. A perfect correlation was shown between the results obtained for the *V. parahaemolyticus* isolates and the *tlh*/*tdh*/*trh* profiles, however 75% of the *V. hollisae* strains gave a low positive signal for *tdh*. Twenty-seven natural oyster samples were collected at Alaska, and one gram of homogenate was added to 10 ml of APW and incubated overnight at 35 °C. After the enrichment, 1 ml aliquots were boiled and 2 µl of supernatant was

used for PCR. Forty four percent, 44% and 52% of the oyster samples were positive for *tlh*, *tdh*, and *trh*, respectively. However, only 33%, 19% and 26% were positive for *tlh*, *tdh*, and *trh* using conventional culture methods. Davis and collaborators (100) used a similar strategy to evaluated the *V. parahaemolyticus* strains isolated from mussels and associated with a foodborne outbreak happening in 2002, in Florida, USA. The selectivity of the assay was confirmed using 20 *V. parahaemolyticus* isolates. The mussels were the only food sample with positive results. More than 21 % of the mussels samples were positive for *tlh* indicating the presence of the *V. parahaemolyticus* in the samples, and almost 17% of the samples were positive for *tdh*, indicating the presence of pathogenic variants in those samples.

The emergence of the O3:K6 serotype and its widespread distribution have fostered the development of detection methods to detect such pathogenic variants. Myers et al. (101) developed a PCR method for the specific detection of this serotype. The PCR target was the open reading frame 8 of phage f237 (*orf8*). They tested 37 *V. parahaemolyticus* O3:K6 serotype, 123 *V. parahaemolyticus* non-O3:K6 serotype, 114 isolates from other species, and they observed that the method was 100% selective. The method could detect down to 10^4 cells per 100 ml of water samples after the DNA purification using the FastDNA SPIN kit (Bio 101). Rizvi et al. (102) designed *orf8* primers coupled with *tlh* primers for the simultaneous detection of total *V. parahaemolyticus* and pandemic O3:K6 serovar using a multiplex real-time PCR. The selectivity of the assay was evaluated using 37 *V. parahaemolyticus* O3:K6, 26 *V. parahaemolyticus,* 7 non-*parahaemolyticus Vibrio* and 9 non-*Vibrio* isolates. All the *V. parahaemolyticus* and all the *V. parahaemolyticus* O3:K6 isolates were positive for the *tlh*- and *orf8*-PCRs, respectively, and none of the non-target isolates was positive. One gram oyster tissue homogenates and Gulf water were artificially contaminated with *V. parahaemolyticus* O3:K6, and incubated at 37 ºC. After the enrichment, DNA extraction was performed using the Instagene matrix (Bio-Rad). The limit of detection of the real-time PCR method was 1 cfu of pandemic *V. parahaemolyticus* O3:K6 serovar per ml of Gulf water or 1 g of oyster tissue homogenate after 8 hours enrichment. Ward and Bej,

(103) developed a multiplex real-time PCR assay for the simultaneous detection of *V. parahaemolyticus* using the *tlh* gene, pathogenic strains using the *tdh* and *trh* genes, and the pandemic O3:K6 serotype using the *orf8*. Detection of 1 cfu per gram of oyster tissue homogenate was possible after overnight enrichment. Finally the method was applied to 33 natural samples from the Gulf of Mexico, Alabama (USA). Fifty-two percent of the samples were positive for *tlh* indicating the presence of *V. parahaemolyticus* in these samples, and 12% were positive for *tdh* indicating the samples contained pathogenic *V. parahaemolyticus* strains.

Luan et al. (104) compared the performance of four PCR assays for the detection of *V. parahaemolyticus*. The PCR assays targeted the *toxR* (105), *tlh*, *tdh* and *trh* (94), *gyrB* (88) and the *V. parahaemolyticus* metalloprotease (*vpm*) gene. Eighty-six *V. parahaemolyticus* and 16 non- *V. parahaemolyticus* isolates were tested with the four set of primers. All the four PCR assays were 100% selective. However the analytical sensitivity varied: the *vpm*-PCR assay detected as few as 4 pg of genomic, whereas the *toxR*-PCR, *tlh*-PCR and *gyrB*-PCR detected a minimum of 375, 100 and 800 pg, respectively.

V. vulnificus

The current guidelines recommended by the ISSC indicates that less than 30 cfu g^{-1} in post-harvest-treated oysters is the threshold to consider a food item as safe for consumption (60). The detection protocol approved by the FDA BAM method is based on the MPN enrichment series in APW coupled with isolation in selective medium and biochemical or molecular confirmation of *V. vulnificus* and on the direct isolation on minimally selective media followed by identification of *V. vulnificus* by colony blot DNA-DNA hybridization (80). Recently the ISO/TS 21872-1:2007 standard has been published describing a horizontal method in food for detection of other potentially enteropathogenic *Vibrio* species than *parahaemolyticus* and *V. cholerae* (106), which is based in similar principles. In Table 2 are summarized the currently available selective media for *V. vulnificus*.

As for *V. parahaemolyticus*, a battery of PCR-based methods have been devised to overcome the disadvantages of the microbiological culture methods (Table 1). Hill et al. (107) reported a PCR method based on the cytolysin gene (*vvhA*). The selectivity of the primers was evaluated by testing 5 *V. vulnificus*, 12 non-*vulnificus Vibrio* and 10 non-*Vibrio* strains. The PCR method was fully selective. Using the *vvhA* gene as PCR target, Brauns et al. (105) confirmed the selectivity of the PCR assay testing 1 *V. vulnificus*, 5 non-*vulnificus Vibrio* and 9 non-Vibrio isolates. Campbell and Wright (108) developed a real-time PCR method based on the same gene. The selectivity of the assay was evaluated with 28 *V. Vulnificus* and 22 non-*V. vulnificus* isolates, showing to be 100%. Detection of *V. vulnificus* in pure cultures was possible down to 10^2 cfu ml^{-1}. The applicability of this method for detection of *V. vulnificus* in oysters was evaluated using natural and artificially contaminated oysters. Thirty grams of oyster meat was 1:10 diluted in ASW and homogenized for 90 s. Ten millilitres of oyster homogenates were artificially contaminated with decreasing amounts of *V. vulnificus*. DNA was extracted using the QIAamp DNA minikit and concentrated with precipitation with ethanol. The results obtained by real-time PCR correlated well with plate counts based on colony blot hybridization enumeration. Similarly, another real-time PCR method using SYBR Green was developed targeting the *vvhA* gene (109). The method was fully selective as 80 *V. vulnificus* isolates produced PCR signals and 47 isolates from other species did not produce any PCR amplification. One gram-aliquots of oyster tissue homogenate were 10-fold serially diluted in sterile GWP-16 and artificially contaminated with *V. vulnificus* and incubated for 5 h at 37 °C. After the enrichment, 5 ml-aliquots were used for DNA extraction using the Instagene matrix (Bio-Rad). The real-time PCR method detected as few as 1cfu of *V. vulnificus* in 1 g of oyster homogenate. Using the same SYBR Green real-time PCR assay, Wang and Levin (110) optimised a DNA extraction protocol for clam samples. One gram homogenates were artificially contaminated with decreasing amounts of *V. vulnificus*. The aliquots were centrifuged at 1000 ×*g* for 5 min, and the supernatants were washed twice and lysed with TZ lysis. The DNA was purified using Micropure EZ minicolumns. The real-time PCR detected as few as 100 cfu g^{-1} of clam tissue and 1

cfu g^{-1} after an enrichment step for 5 h at 37 ºC. Panicker and Bej (111) compared 3 previously reported sets of primers targeting the *vvhA* gene (108,109,112). A TaqMan probe was developed for the first two sets of primers, and the probe previously described was used for the former (108). The selectivity was evaluated using 81 *V. vulnificus*, and 37 isolates from other species. The first two PCR systems were 100% selective, however the former was not fully selective as detected more than 32 % of non-*V. vulnificus* isolates. Both PCR systems were used for detection of *V. vulnificus* in naturally and artificially contaminated oysters. For artificially contaminated oysters, 1 g- aliquots homogenized samples were added to 50 ml of GWP-18 and the solution was artificially contaminated with decreasing amounts of *V. vulnificus*, and incubated at 37 °C for 5 h. One millilitre aliquots were used for the DNA extraction using the Instagene matrix (Bio-Rad). The PCR methods detected as few as 1 cfu g^{-1}.

Other PCR targets have been used for the detection of *V. vulnificus*. Kumar et al. (113) developed a PCR method based on the *gyrB* gene. The PCR assay was 100% selective as tested with 45 *V. vulnificus* and 49 other *Vibrio* isolates. The analytical sensitivity was evaluated using *V. vulnificus* pure cultures and artificially contaminated oyster meat. For artificially contaminated samples, one gram of fresh homogenates was spiked with decreasing amounts of *V. vulnificus*, and lysed by heating. The PCR method detected as low as 3 *V. vulnificus* cfu ml^{-1} of pure cultures, and 300 cfu g^{-1} in artificially contaminated oyster homogenate without enrichment or 30 cfu g^{-1} after 18 h- enrichment in APW. The method was also evaluated in 79 natural oyster samples collected from four different estuaries along the Mangalore coast, India. The homogenates were incubated 0, 6 and 18 hours. The best results were obtained after 18 h enrichment, where *V. vulnificus* was detected in 75% of natural oyster samples, while the conventional microbiological method (isolation on mCPC agar plates after 18 h enrichment) only detected *V. vulnificus* in 45.5% of samples.

Vickery et al. (114) reported a real-time PCR method for the classification of *V.vulnificus* based on 16S rRNA genotype (type A or B). A re-evaluation of the 67 U.S. isolates demonstrated that 45.5% of the isolates originally identified as 16S rRNA type A were actually type AB, and 76%

of clinical isolates tested were type B, 9% type A, and 15% type AB, and in contrast, 91% of non-clinical isolates were found to be of either type A or type AB, and only 9% type B. Other additional 18 strains were also examined, and all of the isolates were classified as type A, all the Biotype 3 strains isolated from an outbreak in Israel were type AB. Using a similar approach, Gordon et al. (115) distinguished *V. vulnificus* strains form environmental and clinical sources. In addition, no amplification was observed with any of the non- *V. vulnificus* isolates tested. Tissues from single oysters collected, in USA were 1:10 diluted in APW, artificially contaminated with *V. vulnificus* and incubated at 37 °C for 4 and 24 h. After enrichment, the homogenates were ten-fold diluted. Two ml were boiled and 2 µl were used for PCR. The limits of detection were 10^3 and 10^2 cfu per reaction for type A and type B, respectively. Using this method, the authors described that the type A/B ratio of Florida clinical isolates was 19:17. The ratio in oysters harvested from restricted sites in Florida with poor water quality was 5:8, but it was 10:1 in oysters from permitted sites with good water quality. A substantial percentage of isolates from oysters (19.4%) were type AB.

V. cholerae

The FDA BAM method for detection of *V. cholerae* in foods relies on the overnight enrichment in APW of 25 g of food samples at 42 °C, the isolation on selective medium and final confirmation for biochemical and molecular tests (80). Similarly the ISO Committee has developed a reference method for this pathogen, the ISO/TS 21872-1:2007 (81).

Another analytical approach is the screening of the samples for toxigenic *V. cholerae* with PCR assays targeting a portion of the *ctx* operon without or after enrichment (Table 1). Koch et al. (116) developed a PCR method, which targeted the cholera toxin operon, *ctxAB*. The selectivity was tested using 3 *V. cholerae* and 10 non-*V. cholerae* isolates, showing to be 100%. Analytical sensitivity was tested in artificially contaminated crab or oysters with *V. cholerae* before homogenization in APW. Ten percent APW homogenates were prepared and 1-ml aliquots were taken immediately and again after the 37 °C incubation, boiled and 2-5 µl of supernatants were used

for PCR. Crabmeat homogenates inoculated with as few as 4×10^4 *V. cholerae* cfu g^{-1} without further enrichment (equivalent to 10 cells in the reaction) and oysters homogenates artificially contaminated with as few as 10 *V. cholerae* cfu g^{-1} after 8 hour enrichment produced positive amplification. De Paola and Wang (117) evaluated the effects of dilutions, incubation times, and incubation temperatures on detection of *V. cholerae* by a *ctxA*-based PCR method. PCR detection of *V. cholerae* was significantly improved using oyster homogenates diluted 1:100 in APW and incubated at 42 °C for 18-21 h.

Blackstone and collaborators (118) developed a real-time PCR method for detection of toxigenic *V. cholearae* in seafood and seafood-related environments. The system targeted the cholera toxin (*ctxA*) gene, found in toxigenic *V. cholerae* strains. The real-time PCR assay was 100% selective as tested with 32 toxigenic *V. cholerae* and 59 non- *V. cholerae* isolates as well as DNA from different environments and eukaryotic organisms. The limit of detection of the method was less than 1 cfu per reaction in oyster. Finally, 6 shellfish and 10 related environmental samples collected in Mobile Bay, USA were evaluated. Twenty-five grams of oyster homogenate were added to 2475 ml of APW and incubated overnight at 42 °C. A 1-mL aliquot of enrichment was boiled and 2–2.5 µl of the boiled aliquot was used for PCR. For environmental samples, 25 g of sediment and ballast water were added to 225 ml of APW and incubated overnight at 42 °C. None of the seafood and environmental samples showed a positive signal for toxigenic *V. cholerae.*

Detection of Listeria monocytogenes in seafoods and seafood-related environments

ISO has developed reference methods for detection and enumeration of *L. monocytogenes*: ISO 11290-1 and 11290-2, respectively (119,120,121,122). In the ISO 11290-1, 25 g of food sample are homogenized in a primary enrichment medium (Half Fraser broth) and incubated at 30 ºC for 24 h. Subsequently, primary culture is plated on Agar Listeria according to Ottaviani and Agosti (ALOA) and in other selective medium (e.g. Oxford or PALCAM media) and incubated at 37 ºC for 24 hours, and in parallel 0.1 ml primary enrichment aliquot is also transferred into a tube

with 10 ml of the secondary enrichment medium, and incubated at 35 or 37 ºC for 48 h. Afterwards, the secondary enrichment is also streaked on ALOA and other selective medium (e.g. Oxford or PALCAM media), and incubated at 37 ºC for 24 hours. Finally, the typical *L. monocytogenes* colonies (green-blue colonies surrounded by an opaque halo in ALOA plates) are confirmed by biochemical tests. In the protocol for detection of *Listeria monocytogenes* recommended by the FDA (123), 25 g of seafoods are homogenized in 225 ml of buffered *Listeria* enrichment broth base containing sodium pyruvate without selective agents (BLEB), and incubated at 30 °C for 4 h, and then the selective agents are added and incubated for 44 h more at 30 °C. At 24 and 48 h, BLEB culture are plated onto one selective isolation medium such as Oxford agar, PALCAM agar, modified Oxford agar (MOX), and Lithium chloride-phenylethanol-moxalactam (LPM) agar fortified with esculin and Fe^{3+}, and incubated at 35 °C for 24-48 h for Oxford, PALCAM or MOX plates or at 30 °C for 24-48 h for fortified LPM plates. In addition primary cultures must be plated onto one *L. monocytogenes*-*L. ivanovii* differential selective agar (e.g. BCM, ALOA, RapidL'mono, or CHROMagar Listeria) after 48 h of enrichment (optionally at 24 h, too). Finally the typical *L. monocytogenes* colonies are confirmed by biochemical tests.

In the ISO 11290-2, ten-fold dilutions of the seafood product homogenate are prepared and plated on ALOA, and incubated at 37 ºC for 24 hours for the enumeration of *L. monocytogenes*. After the enrichment, the typical *L. monocytogenes* colonies are confirmed by biochemical tests. However, in the FDA protocol for enumeration of *L. monocytogenes,* only the positive food samples for presence of *L. monocytogenes* are tested by colony count on *L. monocytogenes* differential selective agar in conjunction with MPN enumeration using selective enrichment in BLEB with subsequent plating on ALOA or BCM differential selective agar.

A study compared the reference ISO methods (ISO 11290-1 and 11290-2) with an in-house method in 543 seafood product samples collected from 21 different companies between 2001 and 2005 in France (64). For the in-house method, 25 g of seafood product was homogenized with 225 ml of Listeria repair broth (LRB) (124,125), and left at room temperature up to 60 min. To

enumerate *L. monocytogenes*, homogenates were spread over Listeria selective agar (LA) plates (126) and incubated at 37 ºC for 48 h. To detect *L. monocytogenes*, 0.90 ml of selective supplement LRB (Oxoid, UK) was added to the homogenate, and incubated at 30 ºC for 24 h, and subsequently streaked on ALOA and *L. monocytogenes* blood agar (LMBA) plates (126) and incubated 37 ºC for 48 h. For the second enrichment step, 0.1 ml of the 24-h culture was transferred to a tube with 10 ml of the Fraser broth, and the mixture was incubated at 37 ºC for 48 h. This second enrichment culture was streaked on ALOA and on LMBA plates and incubated at 37 ºC for 48 h. For each plate with suspect *L. monocytogenes* colonies, several colonies were spread on LA plates and incubated at 37 ºC for 48 h, and subsequently re-spread on Trypticase Soy Agar supplemented with yeast extract (TSAYE). Isolated colonies were taken into microcentrifuge tube containing 100 µl of sterile distilled water, and lysed by heating at 95 ºC for 25 min, then centrifuged and 3 µl of the supernatant was used for confirmation by PCR. Four sets of primers were used; one for the identification of *Listeria* spp. targeting the16S rRNA gene (127), and three specific for the identification of *L. monocytogenes* targeting the *hly* (128, 129), and *iap* (127) genes. Twenty eight percent of the samples were positive by at least one of the methods and 16% were positive by both methods. The sensitivity of the methods was higher than 78%, being slightly higher in the case of the in-house method than 79.5%, and the efficiency of isolation was different depending on the nature of the seafood product. The international standard methods confirmed as positive more samples in smoked salmon and herb-flavoured slices of smoked salmon, but the in-house method in carpaccio-like salmon, herb-flavoured slices of raw salmon, and smoked trout.

Agersborg et al. (130) were the first to develop a specific PCR method for the detection of *L. monocytogenes* in seafood products. They artificially contaminated 5 g of fish cakes, fish pudding, peeled frozen shrimps, salted herring and marinated and sliced coalfish in oil with 500, 10, 5 and 1 *L. monocytogenes* cells. The seafood samples were homogenised in 20 ml of Tryptone Soy Broth or universal pre-enrichment broth (UPB) and incubated for 24 h. Afterwards, 0.5 ml aliquots were inoculated to 5 ml of UPB and incubated for other 24 h, and subsequently 1.5 ml aliquots were

centrifuged for 10 min at 16,000 ×g, and submitted to bacterial DNA extraction. Three different protocols were used by the DNA isolation: the bacterial pellets were resupended (i) in 500 µl of double distilled (dd-)water and treated by heating; (ii) in 750 µl of dd-water and treated with lysozyme and proteinase K; (iii) in 400 µl of dd-water and 400 µl of 2% Triton X-100 was added. In all the cases, the DNA solutions were centrifuged, and 10 µl of the supernatants were used by the PCR. The PCR systems targeted different regions of the *hly* (131, 132) and *iap* genes (133). Lysis by Triton X-100 was the most reliable DNA extraction procedure. After 48 h of incubation, samples inoculated with one to five *L. monocytogenes* cells were clearly positive for the three different set of primers.

Isonhood et al., (134) developed an upstream processing method to facilitate the detection by PCR of *L. monocytogenes* in RTE (ready to eat) seafood salads. Eleven grams of the salads were diluted in 99 ml of sterile saline, and artificially contaminated with decreasing amounts of *L. monocytogenes*. After homogenising, 80 ml of the filtrate was removed for a two-steps centrifugation, consisting of one centrifugation step (119 ×g for 15 min at 5 ºC) to remove large food particulates and a second centrifugation step (11,950 ×g for 10 min at 5 ºC) to concentrate the bacterial cells in the supernatant that was recovered after the first centrifugation. DNA extraction was done on the 1-g bacterial pellets using DNAzol (Invitrogen). The DNA was serially diluted and subjected to dilution series PCR amplification using a set of primers targeting the 16S rDNA gene (135) and confirmed by chemiluminescent Southern blot hybridization. The mean recovery after the two-step method was 49.0 %, and consistent PCR detection of *L. monocytogenes* was possible down to 103 cfu g^{-1}.

Destro et al. (136) combined RAPD and PFGE analysis to trace *L. monocytogenes* contamination in a shrimp processing plant in Brazil, over a 5-month period (May to September 1993). Two random primers were used for the RAPD analysis, generating more than 10 different RAPD profiles, a lower number than reported previously. PFGE was performed using *Sma*I and *Apa*I restriction endonucleases, obtaining more than 12 restriction endonuclease digestion profiles

(REDP), a number similar to previous studies. The combined profile generated when the two RAPD primers and the two PFGE enzymes were used, increased the discriminatory ability to detect differences among isolates of *L. monocytogenes* within serogroups. The combination of these two typing methods allowed tracking the origin of the isolates; *i.e*, natural isolates from inside the processing plant, and isolates introduced from outside the plant and restricted to the receiving area.

Detection of Salmonella spp. in seafoods and seafood-related environments

The International reference method for detection of *Salmonella* is the ISO 6579 (137, 138). In this standard, 25 g of food sample are homogenized with buffered peptone water (BPW), and incubated at 37 °C for 18 h. Subsequently, a 0.1 ml pre-enrichment aliquot is transferred into 10 ml Rappaport-Vassiliadis medium with soya (RVS broth) and incubated for 24 h at 41.5 ºC and in parallel another 1 ml aliquot is transferred into 10 ml Muller-Kauffmann tetrathionate novobiocin (MKTTn) broth and incubated for 24 h are incubated at 37 °C. After the 24 h-incubation, a loop of the RVS and MKTTn broths are streaked onto xylose lysine desoxycholate (XLD) agar and other selective medium, and incubate the plates at 37 ºC for 24 h. Afterwards, typical Salmonella colonies (pink colonies with or without black centres in XLD agar) are confirmed by biochemical (TSI agar test, urea agar test, L-Lysine decarboxylation medium test, detection of β –galactosidase, Voges-Proskauer reaction, indole reaction), and serological tests. In the FDA protocol for detection of *Salmonella* (139) small differences can be noted. Twenty-five g of food sample is homogenized in 225 ml sterile lactose broth. After 1 hour at room temperature, 2.25 ml steamed Tergitol Anionic 7 or Triton X-100 are used, and the seafood homogenate is incubated for 24 h at 35 °C. Subsequently, a 0.1 ml pre-enrichment aliquot is transferred into 10 ml Rappaport-Vassiliadis (RV) medium and incubated for 24 h at 42 ºC and in parallel another 1 ml aliquot is transferred into 10 ml tetrathionate (TT) broth and incubated for 24 h at 35 °C. Afterwards, the RV and TT enrichments are streaked on bismute sulfite (BS) agar, XLD agar, and Hektoen enteric (HE) agar, and the plates are incubated for 24 h at 35 ºC. Finally, typical *Salmonella* colonies (brown, grey, or black colonies; sometimes with a metallic sheen in BS agar, pink colonies with or without black centres in XLD

agar; and blue-green to blue colonies with or without black centres in HE agar) are confirmed by biochemical or alternative tests.

As for pathogenic *Vibrio* and *L. monocytogenes* rapid alternatives based on molecular methods have been also devised. The research group led by Bej at the University of Alabama developed a multiplex PCR method for the simultaneous detection of *Escherichia coli*, *Salmonella enterica* serotype Typhimurium, *Vibrio vulnificus*, *V. cholerae*, and *V. parahaemolyticus* (140). The PCR primers targeted the *E. coli uidA*, *S. typhimurium invA*, *V. vulnificus cth*, *V. cholerae ctx*, and *V. parahaemolyticus tl* genes. The multiplex PCR was totally selective as each specific primer only detected the corresponding target. One gram of sterilized shellstocks from oysters obtained from local seafood restaurants were artificially contaminated with decreasing loads of these organisms. The sample was diluted in 30 ml of APW and incubated at 35 °C for 6 h. After the enrichment, the oyster homogenates were centrifuged and the DNA was extracted using the Chelex 100 resin (Biorad). To achieve maximum sensitivity, a 5-µl aliquot of the initial multiplex PCR-amplified products was subjected to a re-amplification by a second PCR. The minimum level of detection of each target in a single multiplex PCR was 100 cfu g^{-1}. However, the detection limit was improved to 10 cells cfu g^{-1} using the second PCR round. The same research group improved the detection of *Salmonella enterica* serotype Typhimurium, *Vibrio vulnificus*, *Vibrio cholerae* and *Vibrio parahaemolyticus* using a multiplex PCR followed by DNA–DNA sandwich hybridization (141). The target genes were the *Salmonella hns* and *spvB*, *V. vulnificus vvh*, *V. cholerae ctx*, and *V. parahaemolyticus tlh* genes. Oyster samples were processed according to standard methods and 1 g of oyster homogenates were diluted in 5 ml of APW and artificially contaminated with ten-fold dilutions of those 4 bacterial pathogens. The homogenates were enriched for 3 h at 37 ºC. The bacterial DNA extraction was performed as described above. The multiplex PCR allowed the detection of all four bacterial pathogens, and it was further confirmed by the non-radioactive and colorimetric CovaLinkk NH microtiter plate hybridization assay. The analytical sensitivity was down to 10^2 cells g^{-1} of oyster tissue homogenate

Vantarakis et al. (142) devised a multiplex PCR method for the simultaneous detection of *Salmonella* spp. and *Shigella* spp. in mussels. The multiplex PCR primers targeted specific nucleotide sequences of the *Salmonella invA* (215 bp) (143) and *Shigella virA* (275 bp) (144) genes. The PCR method was 100% selective as evaluated with six different Enterobacteriaceae genera. For the mussels analysis, 25 g of mussel meat was diluted in 90 ml of buffered peptone water (BPW). Decreasing amounts of *Salmonella* spp. and *Shigella* spp. were added to 1 ml of mussel homogenates and submitted to DNA extraction. Guanidine isothiocyanate was added to 1 ml-homogenates and incubated at 65 ºC for 90 min, diluted and boiled for 5 minutes. The samples were cooled to room temperature, then sodium acetate was added to the samples, and centrifuged at 14 000 ×*g* for 10 min. The supernatants were transferred to new tubes and extracted twice with an equal volume of chloroform. Finally the DNA was precipitated with 95% ethanol and the DNA was resuspended in sterile distilled water. The PCR method detected less than 10 *Salmonella* cells ml^{-1} of homogenate. However the authors introduced a pre-enrichment step to increase the analytical sensitivity as well as to guarantee the only detection of viable cells. After a 22-hour pre-enrichment in BPW, 10 to 100 cells of *Salmonella* spp. and *Shigella* per millilitre of homogenate were detected by the multiplex PCR

Wang and Yeh (145) developed a novel PCR method for the detection of *Salmonella enteriditis*, and evaluated its performance in different food samples, including seafoods. The PCR system targeted the *Salmonella IE* gene. All of the 24 *S. enteritidis* strains generated positive PCR signals. Ninety six non-*enteritidis Salmonella* and forty non-*Salmonella* isolates including strains of the family Enterobacteriaceae such as *E. coli*, *Shigella* and *Citrobacter*, did not produce any amplification signal, therefore, the PCR assay was 100% selective. The detection limit of the PCR assay was 10^2 cfu ml^{-1} of cell extracts prepared by heat lysis. For the analysis of seafood samples, the authors followed the FDA procedure, and 10 µl of the final enrichment was lysed by heating, and used for the PCR detection. None of the 15 samples were detected by either completed BAM method or by PCR.

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Table 1. *PCR-based method for the detection of pathogenic Vibrio species in seafood products.*

Organism	Method	Target sequence	Food Matrix	Reference
V. parahaemolyticus				
	PCR	pR72H	oyster	83
	PCR	*tdh*	oyster	91
	PCR	*gyrB*	shrimp	88
	PCR	*toxR*	- - -	86
	PCR	*tdh*	oyster	93
	PCR	*orf8*	- - -	101
	PCR	*vmp*	- - -	104
	PCR	*tlh*	fish	146
	PCR	*hns*	- - -	147
	PCR	*groEL*	- - -	148
	PCR	*irgB*	- - -	149
	Multiplex PCR	*tdh trh*	fish	146
	Multiplex PCR	*tdh trh*	- - -	147
	Multiplex PCR	*tdh, trh*	- - -	148
	Multiplex PCR	*tdh, trh*	- - -	150
	Multiplex PCR	*tdh, trh*	- - -	149
	multiplex PCR	*tdh, trh*	- - -	90
	multiplex PCR	*tlh, th, trh*	oyster	94
	multiplex PCR	*tlh, tdh,trh*	seafoods	96
	real-time PCR	*tdh*	oyster	92
	real-time PCR	*tdh*	- - -	151
	real-time PCR	*tdh*	- - -	152
	real-time PCR	*tdh*	- - -	153
	real-time PCR	*tdh*	- - -	154
	real-time PCR	*tdh*	- - -	155
	real-time PCR	*tdh*	- - -	156
	real-time PCR	*tlh*	- - -	110
	real-time PCR	*tlh*	oyster	85
	real-time PCR	*tlh*	- - -	157
	real-time PCR	*tlh*	shellfish	153
	real-time PCR	*tlh*	fish	158
	real-time PCR	*tlh*	- - -	150
	real-time PCR	*trh*	- - -	152
	real-time PCR	*trh*	shellfish	153
	real-time PCR	*trh*	- - -	154
	real-time PCR	*trh*	- - -	155
	real-time PCR	R72H DNA sequence	oyster	154
	real-time PCR	R72H DNA sequence	oyster	155
	real-time PCR	*vmrA*	clams	159

	real-time PCR	*toxR*	clams	87
	real-time PCR	*gyrB*	osyter	89
	multiplex real-time PCR	*trh1, thr2, toxR*	oyster	151
	multiplex real-time PCR	*tlh, tdh, trh*	mussels	100
	multiplex real-time PCR	*tlh,orf8*	oyster	102
	multiplex real-time PCR	*tlh,tdh, trh, orf8*	oyster	103
	multiplex real-time PCR	*tlh, tdh, trh*	oyster	97
V. vulnificus				
	PCR	*vvhA*	- - -	107
	PCR	*vvhA*	- - -	105
	PCR	*gyrB*	oyster	113
	PCR	*dnaJ*	fish	146
	Nested PCR	*23S rDNA*	fish	3
	Multiplex PCR	*vvhA*	oysters, shrimp	160
	Multiplex PCR	*vvhA*	oysters	140
	Multiplex PCR	*vvhA*	oyster	141
	RT PCR	*vvhA*	octopus	162
	Real-time PCR	*vvhA*	oyster	108
	Real-time PCR	*vvhA*	stools	112
	Real-time PCR	*vvhA*	oyster	109
	Real-time PCR	*vvhA*	clam	110
	Real-time PCR	*vvhA*	- - -	157
	Real-time PCR	*vvhA*	- - -	152
	Real-time PCR	*vvhA*	- - -	162
	Real-time PCR	*vvhA*	- - -	158
	Real-time PCR	*vuuA*	- - -	159
	Real-time PCR	16S rDNA	- - -	114
	Real-time PCR	16S rDNA	oyster	115
	Real-time PCR	*vvp*	- - -	150
	Real-time PCR	*pilF*	- - -	163
	Real-time PCR	*vcgC*	- - -	163
	Real-time PCR	*vcgC*	- - -	164
	Real-time PCR	*hly*	- - -	155
V. cholerae				
	PCR	*ctxAB*	oyster, crab	116
	PCR	*ctxA*	Oyster	117
	PCR	*ctxA*	Fish	165
	PCR	*ctxA*	Fish	146
	PCR	*rtxA*	Fish	165
	PCR	*wbeO*	Fish	146
	PCR	*tcpA*	Fish	146
	PCR	*ctx*	Oyster	118
	real-time PCR	*ctx*	- - -	156
	real-time PCR	*ctx*	- - -	151
	real-time PCR	*ctxA*	- - -	155
	real-time PCR	*ctxA*	- - -	157
	real-time PCR	*toxR*	- - -	151
	real-time PCR	*sodB*	- - -	151
	real-time PCR	ISR	- - -	155

real-time PCR	*lolB*	- - -	167
real-time PCR	*ampW*	- - -	157
real-time PCR	*zot*	- - -	159

Table 2: *Selective culture media for isolation and identification of V. vulnificus. Adapted form Harwood et al., 2004 (44)*

Medium	Abbreviation	Incubation temperature (ºC)	Carbon source	Colony colour	Reference
Thiosulphate citrate bile salt agar	TCBS	37	Sucrose	Green	168
Vibrio vulnificus agar	VV		Salicin	Grey, dark centre	169
SDS polymyxin sucrose agar	SPS		Sucrose	Blue with halo	170
Cellobiose polymyxin B colistin agar	COC	40	Cellobiose	Yellow	171
Modified cellobiose polymyxin B colistin agar	mCPC	40	Cellobiose	Yellow	172
Vibrio vulnificus enumeration agar	VVE	37	Cellobiose, lactose, X-Gal	Blue green	173
Cellobiose colistin agar	CC	40	Cellobiose	Yellow	174
Vibrio vulnificus médium	VVM	37	Cellobiose	Yellow	175
Vibrio vulnificus medium+colistin	VVMc	37	Cellobiose	Yellow	176