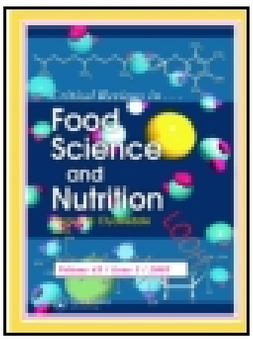

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Vibrio species involved in seafood-borne outbreaks (*Vibrio cholerae*, *V. parahaemolyticus* and *V. vulnificus*): review of microbiological *versus* recent molecular detection methods in seafood products

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Abstract

Seafood products are widely consumed all around the world and play a significant role on the economic market. Bacteria of the *Vibrio* genus can contaminate seafood and thus pose a risk to human health. Three main *Vibrio* species, *V. cholerae*, *V. parahaemolyticus* and *V. vulnificus*, are potentially pathogenic to humans. These species are responsible for a dramatic increase of seafood-borne infections, worldwide. Hence, early detection of total and pathogenic *Vibrio* is needed and should rely on quick and effective methods. This review aims to present the standard methods FDA-BAM, ISO/TS 21872-1:2007 and TS 21872-2:2007 and compare them to recent molecular biology methods including endpoint PCR, quantitative real-time PCR (qPCR) and PCR-derived methods with a focus on LAMP (loop-mediated isothermal amplification). The available methods presented here are dedicated to the detection and identification of the *Vibrio* species of interest in seafood.

Keywords

seafood, *Vibrio cholerae*, *Vibrio parahaemolyticus*, *Vibrio vulnificus*, FDA-BAM, ISO/TS 21872, PCR, qPCR, LAMP

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Introduction

The *Vibrio* genus (*Vibrionaceae* family, Gammaproteobacteria class) is now composed of 142 species (Sawabe, *et al.*, 2013), although the taxonomy is constantly changing with the progress of molecular techniques.

These Gram-negative halophilic bacteria, described as rod-shaped, are ubiquitous and indigenous in aquatic environments (estuarine, coastal waters and sediments) and many of them are associated with marine organisms such as seawater fish, molluscs and crustaceans. *Vibrio* can infect all animals including humans (Austin, 2010). Several species of the genus *Vibrio* have been identified and classified among the 15 pathogens causing 95% or more of all foodborne illnesses, hospitalizations, and deaths in the United States (Batz, *et al.*, 2012). Recently, the European Food Safety Authority (EFSA) has highlighted rising seawater temperatures and alerted of the growth of *Vibrio* spp. in oceans as a potential emerging issue. In fact foodborne *Vibrio* pathogens carrying several virulence factors have emerged, in response to climate changes and especially global ocean warming (Vezzulli, *et al.*, 2013) that is why EFSA recommended further investigations and vigilance (EFSA, 2016).

Among the 12 *Vibrio* species known as pathogenic to humans, the three most commonly reported are *Vibrio cholerae*, *Vibrio parahaemolyticus* and *Vibrio vulnificus* (Robert-Pillot, *et al.*, 2014). Contamination by seawater, raw or undercooked seafood, or work surfaces is responsible for most cases of vibriosis (Daniels, *et al.*, 2000) following the consumption of seafood products. Due to the increase in seafood consumption worldwide, there has been a global

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rise in seafood production from capture fisheries and aquaculture. Total fish production amounted to 158 million tons in 2012, including around 136 million tons for human consumption (FAO, 2014). Consequently, zoonotic infections dramatically increase, leading to economic consequences (Hoffmann, *et al.*, 2015). For instance, 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 (World Health Organization, 2016). Autochthonous cholera has been eradicated from most areas of the developed world, but imported contaminated food should be considered a potential source of infection for sporadic cases of domestically acquired cholera (Schurmann, *et al.*, 2002). Infection is often mild or asymptomatic. Nevertheless, for 5--10% of infected persons, severe cholera infection results in various symptoms such as vomiting, rapid heart rate and intense dehydration, even leading to death. Of more than 220 O-antigen serogroups identified as *V. cholerae*, only the O1 and O139 serogroups have been identified in cholera epidemics and pandemics (Reidl and Klose, 2002), (Islam, *et al.*, 2004), (Mahapatra, *et al.*, 2014). The main virulence factor associated with *V. cholerae* O1 and O139 is the cholera toxin (CTX) with the *ctx* genes (*ctx*A and *ctx*B) encoding the production of this protein. For example, *V. cholerae* O1 was reported in association with shellfish ingestion in 136 cases of infection during the period from 1965 to 1991 (Potasman, *et al.*, 2002). Later, seafood contaminated through seawater in fish tanks was involved in an outbreak of *V. cholerae* O1, serotype Inaba in Hong Kong during the summer of 1994 (Kam, *et al.*, 1995). All these investigations clearly show the link between the water contamination by *V. cholerae* O1 and its transmission to humans *via* seafood products which are therefore considered as vehicles of transmission. *V. cholerae* O1 was responsible for an outbreak in Haiti in October 2010 (Hill, *et*

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al., 2011), (Baron, *et al.*, 2013). Moreover, *V. cholerae* O1 was isolated in two samples composed of fish and crabs or bivalves; (Hill, *et al.*, 2011). Other *V. cholerae* O1 infections occurred in the Dominican Republic in January 2011 after a wedding due to the consumption of shrimp in particular (Jiménez, *et al.*, 2011). In this case, contamination was linked to the ice on which the shrimp was stored. Other non-toxigenic, non-O1 and non-O139 serogroups have been involved in sporadic gastroenteritis (Restrepo, *et al.*, 2006), (Albuquerque, *et al.*, 2013), (Lu, *et al.*, 2014).

V. parahaemolyticus is described as the most frequent cause of *Vibrio*-associated gastroenteritis in the USA (Daniels, *et al.*, 2000) and the symptoms are associated with the production of Thermostable Direct Hemolysin (TDH) or TDH-Related Hemolysin (TRH) proteins. Within the *V. parahaemolyticus* species, 13 O-serogroups and 71 K-serogroups have been reported (Chen, *et al.*, 2012) among them, serotype O3:K6 has most commonly been involved in outbreaks.

The O3:K6 pandemic clone, distributed worldwide since its emergence in 1996 in India (Velazquez-Roman, *et al.*, 2014), was involved in outbreaks in 1998 in Japan (Hara-Kudo, *et al.*, 2012) and in the USA (Daniels, *et al.*, 2000). In Chile, this O3:K6 clone was also responsible for 1500, 3600 and 900 cases in 2004, 2005 and the summer of 2006, respectively (Fuenzalida, *et al.*, 2007). Other serotypes have also been involved in *V. parahaemolyticus* outbreaks: serotype O6:K18 in Alaska after the consumption of oysters (McLaughlin, *et al.*, 2005), serotypes O4:K12 and O4:K (unknown) on the US Atlantic coast (Newton, *et al.*, 2014) after the consumption of shellfish and seafood. In Spain, 100 people were poisoned during a food banquet on a cruise boat due to the consumption of shrimp contaminated by *V. parahaemolyticus*

(Martinez-Urtaza, *et al.*, 2016). Of the isolated strains, seven were both *tdh*- and *trh*-positive and belonged to the O4:K12 and O4:KUT (O4:K UnTypeable) serotypes. Recently, serotype O4:K8 was isolated in southern China in foodborne diarrheal cases (Li, *et al.*, 2017).

Another pathogenic *Vibrio* is *V. vulnificus*, which can cause two different types of symptoms: firstly, primary septicemia following ingestion of raw shellfish or oysters potentially leading to fever, nausea and hypotension (Linkous and Oliver, 1999). Primary septicemia is distinguished from infections of existing wounds by contact with seawater or contaminated fish. *V. vulnificus* should be considered a highly pathogenic heterogeneous bacterial species which has been subdivided into three biotypes, each one involved in wound infections (Oliver, 2005). Biotype 1 causes sporadic cases of human vibriosis. Biotype 2 can infect both fish and humans (Sanjuán, *et al.*, 2011). Biotype 3 is geographically restricted to Israel and causes outbreaks of human vibriosis after handling fish (Jones and Oliver, 2009). *V. vulnificus* infections have mainly been described in raw shellfish and particularly oysters (Shapiro, *et al.*, 1998), (Horseman and Surani, 2011). In Florida, during the 1981--1992 period, 72 cases due to raw oysters contaminated by *V. vulnificus* occurred, half of which led to death (Hlady, *et al.*, 1993). *V. vulnificus* cases have mostly been reported in Japan (Inoue, *et al.*, 2008), (Nagao, *et al.*, 2009), (Matsumoto, *et al.*, 2010), (Matsuoka, *et al.*, 2013), in the USA (Daniels, 2011), (Horseman and Surani, 2011), as well as in New Caledonia (Cazorla, *et al.*, 2011), Korea (Jung, *et al.*, 2005), and the Gulf of Mexico (Shapiro, *et al.*, 1998) with fresh seafood and raw oysters as contamination vehicles. Furthermore, most cases have occurred in summer, when seawater temperatures increase (Motes, *et al.*, 1998), (Matsuoka, *et al.*, 2013). A survey carried out by Matsuoka, *et al.* (2013) on *V. vulnificus* infection showed that 67% of the studied cases occurred between June and September.

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Furthermore, several studies underlined the importance of hygiene conditions especially during the post-harvesting period (Paydar, *et al.*, 2013), (Malcolm, *et al.*, 2015). and the significance of hygiene practices in the seafood process from 'farm-to-fork' has been particularly highlighted in WHO/FAO Report No. 16 of 2011 (Skovgaard, 2012).

Considering the epidemiological significance of these foodborne pathogens, there is an essential need to detect the bacteria with relevant microbiological and molecular biological techniques. These techniques will be implemented in the routine monitoring of *Vibrio* species posing substantial public health risks. Health organizations recommend the use of standardized microbiological procedures such as the Food & Drug Administration's Bacteriological Analytical Manual (FDA-BAM, 2004) and ISO/TS 21872-1:2007 (International Organization for Standardization, 2007) and ISO/TS 21872-2:2007 (International Organization for Standardization, 2007). These standards are both time-consuming, requiring several days to confirm the presence of a *Vibrio* pathogen isolated from a seafood product. Over the last few years, several alternatives to microbiological methods have been developed to detect and quantify total and pathogenic *Vibrio*. These methods are based on the DNA amplification principle by polymerase chain reaction (PCR).

The purpose of this review is to describe, compare and discuss the overall standard reference methods as well as the molecular methods focusing on studies published since 2010. Recent progress in the detection of *V. cholerae*, *V. parahaemolyticus* and *V. vulnificus* in seafood matrices will be also considered, introducing PCR- and qPCR-derived methods such as the LAMP one but also emerging methods including biochemical and physico-chemical ones.

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Microbiological methods for *Vibrio* species enumeration and/or detection *versus* molecular PCR and qPCR methods for identification

The obvious link between vibriosis and seafood consumption raises a need for the development of reliable tools enabling the early detection of these pathogens in food products. Indeed, the 'Guidelines on the application of general principles of food hygiene to the control of pathogenic *Vibrio* species in seafood' (CAC/GL 73--2010) aimed to reduce the risk by adopting appropriate hygiene measures (Codex alimentarius, 2010). Furthermore, the European Commission Regulation (2005)2073/2005/EC of the 'Hygiene Package'(2005) emphasized compliance with 'Good Hygiene Practices' and suggested the development of reliable methods to assess *Vibrio* microbial risks. Recent studies have been published in order to accurately identify and quantify total and pathogenic *Vibrio* species (Garrido-Maestu, *et al.*, 2014), (Kim, *et al.*, 2014), (Kim and Lee, 2014), (Yi, *et al.*, 2014), (Di, *et al.*, 2015). This research topic is constantly being enhanced in order to develop faster and more sensitive methods that can quickly be implemented for both food manufacturing controls and epidemiological monitoring.

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Microbiological methods

In this section, the Food &Drug Administration – Bacteriological Analytical Manual (FDA-BAM) and the ISO/TS 21872-1:2007 and ISO/TS 21872-2:2007 standard methods are broken down. The FDA-BAM method was developed for the detection and enumeration of bacteria while the ISO/TS 21872-1:2007 and ISO/TS 21872-2:2007 methods were developed for detection alone. Both methods contribute to the identification of *Vibrio* species.

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FDA-BAM standard

Chapter 9, *Vibrio*, of the FDA-BAM method, revised in 2004, describes the species characteristics often associated with human illness related to seafood consumption. It describes the distribution, sources of contamination and isolation methods for *V. cholerae* in a first part (Fig. 1). Fig. 1 describes the sample preparation and the enrichment step in Alkaline Peptone Water (APW), followed by the isolation step. The final step of this method, consisting of biochemical tests, allows to be ensured that it is or not *V. cholera*. The entero-toxigenicity of the isolates is also investigated through a Y-1 mouse adrenal cell assay testing the production of Cholera Toxin (CTX). Nevertheless, most food and environmental isolates do not produce CTX.

Analog methods, for the detection of other *Vibrio* including *V. parahaemolyticus* and *V. vulnificus*, are described in the second part of Chapter 9 distinguishing between "products that have been processed" and "molluscan shellfish." For the enumeration of *V. parahaemolyticus* and *V. vulnificus*, among the three possible analytical schemes, the Most Probable Number (MPN) procedure, which is the most commonly used in laboratories is detailed in the Fig. 2. However, two other analytical schemes exist: the membrane filtration procedure using the Hydrophobic Grid Membrane Filter (HGMF) for *V. parahaemolyticus* enumeration, and DNA probe colony hybridization for *V. parahaemolyticus* and *V. vulnificus* enumeration.

The ISO/TS 21872-1:2007 and TS 21872-2:2007 international standard methods

As in the FDA-BAM standard, the ISO standard methods are based on three main microbiological steps: enrichment, isolation and confirmation. The International Organization for Standardization described two horizontal methods for the detection of potentially entero-pathogenic *Vibrio* species in food: the ISO/TS 21872-1:2007 and ISO/TS 21872-2:2007 methods

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(Fig. 3). The first is dedicated to *V. cholerae* and *V. parahaemolyticus*, while the second is focused on other *Vibrio* spp. including *V. vulnificus*. They are almost similar, except for the incubation temperature during the enrichment period. For both methods, the two successive enrichment steps are in fact done in APW with similar incubation times but with different temperatures according to the food matrix state (fresh or frozen) and to the targeted bacterial species The isolation and identification steps consist of the plating of the enrichment broths on selective TCBS agar and on a second selective medium for *Vibrio*. After 24 hours of incubation at 37°C blue-green colonies on TCBS are specific to *V. parahaemolyticus* or *V. vulnificus* (sucrose-negative), while yellow colonies are characteristic of *V. cholerae* (sucrose-positive). In order to confirm the isolated strains, biochemical tests are then carried out in five characteristic colonies. However, results using TCBS agar are often difficult to interpret (Hara-Kudo, *et al.*, 2001).and this medium cannot always accurately distinguish between *V. parahaemolyticus* and *V. alginolyticus* (Yamazaki, *et al.*, 2011). Hence, the use of a second selective medium is required. Furthermore, the ISO standard method recommends that the *Vibrio* species diagnosis should be confirmed in a reference laboratory and a further study is promoted for *V. cholerae*, *V. alginolyticus* and *V. parahaemolyticus*. For instance, some authors highlighted the fact that the ISO standard method is time-consuming and failed to properly detect any *V. parahaemolyticus* or *V. cholera* in living bivalve molluscs and they therefore suggested reconsidering this method (Rosec, *et al.*, 2012). Indeed, the ISO standard has recently been revised (ISO 21872-1:2017) by the working group (ISO/TC 34/SC 9).

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Use of other selective media for specific Vibrio isolation

To counter the relative inefficiency of TCBS medium, commercial chromogenic agar media such as CHROMagar Vibrio and Chrom ID Vibrio have been implemented and compared to TCBS agar by several authors. Efforts to develop effective selective agar media underline the identification complexity of potential human pathogenic *Vibrio* species. In fact, the CHROMagar Vibrio medium exhibited better results than TCBS agar in distinguishing between pathogenic *Vibrio* spp. and other species (Messelhäusser, *et al.*, 2010). Comparative studies have confirmed the higher discriminating power of the CHROMagar Vibrio medium compared with TCBS agar (Di Pinto, *et al.*, 2011), (Nigro and Steward, 2015). Nigro and Steward especially underlined the complementarity of both media reducing the number of false-positives from 1.9- to 2.7-fold. This complementarity is consistent with what is prescribed by the ISO standard method. Garrido, *et al.* (2012) demonstrated better productivity with ChromID Vibrio agar for three tested *V. parahaemolyticus* strains, compared to CHROMagar Vibrio. Other media such as Cellobiose-Colistin (CC) agar can be used for the isolation of *V. parahaemolyticus*, but this has been mainly used for *V. vulnificus* isolation to supplement TCBS agar (Bhattacharyya and Hou, 2013). Colistin Polymyxin β-Cellobiose (CPC) agar was also used for the isolation of *V. vulnificus* by Garrido-Maestu, *et al.* (2014). Indeed, the recommendations of Chapter 9, *Vibrio*, of the FDA-BAM (2004) for the analysis of *V. vulnificus* described the isolation step using TCBS and CC or mCPC. The study by Cruz, *et al.* (2013), concerning the detection of *V. vulnificus* in oysters, compared the standard method of Chapter 9, *Vibrio*, of the FDA-BAM FDA-BAM (2004) with commercial methods using CHROMagar supplemented by TCBS and CC media. A triple-plating method, based on the sequential use of CHROMagar Vibrio, CPC+ (modified CPC agar), and

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TCBS agar, was specifically developed by Williams, *et al.* (2013) to detect *V. vulnificus*. This method and especially the first plating on CHROMagar Vibrio, which appeared to be the most selective medium, reduced the number of false-positives. The triple-plating method was compared to the single use of CHROMagar Vibrio for artificially seeded oyster samples and 100% and 43.5% of the isolates were respectively identified as *V. vulnificus*. The triple-plating method's limit for *V. vulnificus* was biotype 3 in addition to sucrose-positive strains which could not be detected. VVX agar (*V. vulnificus* X-Gal agar), formulated by Griffitt and Grimes (2013), was used for the isolation of *V. vulnificus* from oyster tissue. The use of X-Gal, as a chromogenic lactose analog, made it possible to distinguish between non-lactose-fermenting *V. vulnificus* and other bacteria. Froelich, *et al.* (2014) compared the efficency of specific media (CPC+, CHROMagar Vibrio, VVX) and the triple-plating method in isolating *V. vulnificus* from oysters. The results showed that VVX was the most selective media (19% failing to identify *V. vulnificus* isolates) followed by CHROMagar Vibrio (26%), the triple-plating method (32%) and the CPC+ medium (56%). All these experiments clearly showed that the use of strictly conventional culture methods is not relevant enough to accurately retrieve *Vibrio* species. Indeed, differentiation between the *Aeromonas* and *Vibrio* genera is still ambiguous, since *Aeromonas* strains can grow on the same media as *Vibrio* and they share genetic similarities (Teh, *et al.*, 2010). Furthermore, species differentiation within the *Vibrio* genus remains difficult since *V. parahaemolyticus* and *V. alginolyticus* are often mistaken for one another, as are *V. cholerae* and *V. mimicus* (Neogi, *et al.*, 2010), (Teh, *et al.*, 2010). Biochemical misidentification can also occur. For example, the API 20E method fails to distinguish between *V. parahaemolyticus* and *V. vulnificus* strains (Yi,

et al., 2014). Therefore, a molecular step is usually required to confirm identification of the *Vibrio* species (Di Pinto, *et al.*, 2011), (Nigro and Steward, 2015).

Molecular methods for identification

Characterization of bacteria and detection of virulence factors by endpoint Polymerase Chain Reaction (PCR) and real-time PCR (qPCR)

Over the past 20 years, the development of molecular-based techniques has improved the identification of *Vibrio* species. Indeed, the method in Chapter 9 of the FDA-BAM (2004) (Kaysner and DePaola Jr, 2004) recommends using a PCR method as a final step for the identification procedure. PCR also contributes to accurately characterizing the pathogenicity of the isolates whereas the interpretation of biochemical tests is sometimes questionable and requires sending the isolates to a reference laboratory for confirmation. The targeted genes are the *ctx* gene encoding the Cholera Toxin (Koch, *et al.*, 1995); the *tlh*, *tdh* and *trh* hemolysin genes simultaneously detected in multiplex PCR (Bej, *et al.*, 1999); and the cytolysin gene *vvha* (Hill, *et al.*, 1991) for the detection of *V. cholerae*, *V. parahaemolyticus* and *V. vulnificus* (Kaysner and DePaola Jr, 2004). Furthermore, the method in Chapter 28 of the FDA-BAM (2004) entitled "Detection of Enterotoxigenic *Vibrio cholerae* in Foods by the Polymerase Chain Reaction" (Koch, *et al.*, 1995) clearly promotes the use of PCR. Unlike the recommendations of both FDA-BAM methods, the ISO/TS 21872-1:2007 (International Organization for Standardization, 2007) and ISO/TS 21872-2:2007 (International Organization for Standardization, 2007) international standard methods do not mention the PCR technique to confirm *Vibrio* species identification. However, the ISO standard has evolved since June 2017 (International Organization for Standardization, 2017) and now provides an alternative, in

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addition to biochemical tests, to use PCR or qPCR to confirm *Vibrio* species. Indeed to cope with fastidious culture methods, more molecular DNA-based tests have been developed to improve the rapidity, sensitivity and accuracy of detection methods with applications specifically for species identification or pathogenic marker detection.

PCR (Table 1) as well as qPCR (Table 2) methods dedicated to seafood are described in this review. Each molecular method is approached species by species starting with *V. cholerae, V. parahaemolyticus* and then *V. vulnificus*, with or without the simultaneous detection of virulence factors. Methods enabling several species to be identified simultaneously are then presented. The term 'multiplex' refers to the detection of both a species and its virulence factors in the same experiment, or to the detection of several *Vibrio* species.

A multiplex PCR assay was developed to detect both total and toxigenic *V. cholerae* in fish and fishery products (Jeyasekaran, *et al.*, 2011) with previously designed primers. The *rpoA* gene was targeted for genus identification, whereas the *ctxA* and *RtxA* genes were considered as toxin-producing genes. Two strains, isolated from fish and shrimp respectively, were positive for the three targeted genes, whereas the strain isolated from seawater was only positive for *rpoA*. Regarding the detection limit, a 12-hour enrichment period in APW was necessary to detect the three bacteria with the three sets of primers, in an artificially seeded shrimp sample. A minimum of eight hours of enrichment were necessary to detect the lowest dilutions of *V. cholera* inoculated in 25 g of previously sterilized shrimp. In another study, primers were developed to target the *tdh* and *trh* genes encoding hemolysins and the *groEL* gene which is a phylogenetic marker. These primers were tested to detect *V. parahaemolyticus* strains in artificially

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contaminated shellfish and seawater (Hossain, *et al.*, 2013). DNA was extracted after five hours of incubation at 37°C and the detection limit was 4.10^4 CFU/g. Furthermore, No, *et al.* (2011) used the Histone-like Nucleoid Structure (H-NS) gene to identify *V. parahaemolyticus* among other *Vibrio* spp. strains in spiked oyster samples. The *hns* gene's detection limit was $1.8.10^5$ CFU/g. A multiplex PCR method was secondly developed to distinguish between virulent and non-virulent *V. parahaemolyticus* strains targeting the *tdh* and *trh* genes in addition to the *hns* gene. Indeed, Yu, *et al.* (2010) used a comparative genomic approach to design the *irgB* gene marker encoding IrgB, an iron-regulated virulence regulatory protein for *V. parahaemolyticus*. The specificity of the PCR assay was assessed with 293 *V. parahaemolyticus* strains from clinical, environmental and seafood samples, as well as other *Vibrio* species and other genera. The *irgB* fragment's detection limit on agarose gel was 0.17 pg of purified genomic DNA. Primers specific to the *irgB* gene were used simultaneously with primers previously published targeting the *tdh* (Nordstrom, *et al.*, 2007) and *trh* (Bej, *et al.*, 1999) genes to detect total and virulent *V. parahaemolyticus* strains. However, this method has not been applied to naturally or even artificially contaminated matrices. Other studies offer more comprehensive methods. In fact, Espineira, *et al.* (2010) suggested a sequential method based on several multiplex PCR assays applicable to seafood such as shrimp, crabs, salmon, scallops, oysters and clams. The three-step method started with a PCR analysis to distinguish between five *Vibrio* species: *V. cholerae* by targeting the *ctxA* gene, *V. parahaemolyticus* by targeting the *tlh* gene, and *V. vulnificus* (as well as *Vibrio alginolyticus* and *Vibrio mimicus*) by targeting the *dnaJ* gene. The second step was used to differentiate between viable and dead bacteria. Thirdly, other multiplex PCR assays were performed to determine the O1 or O139 serogroup of *V. cholerae* by *wbeO*

gene detection. For *V. cholerae* O1, an additional multiplex PCR assay targeting the *tcpA* gene was able to distinguish between *V. cholerae* O1 El Tor and O1 Classical biotype. Regarding *V. parahaemolyticus*, the virulence factors *tdh* and *trh* were also investigated. This hierarchical methodology was assessed with 40 artificially contaminated fish samples, spiked with 1 to 10^5 CFU of *Vibrio* spp. The detection limit was 1 CFU/mL. Sixty-three seafood samples were then analyzed and two mussel samples were positive for *V. parahaemolyticus*. Only one was positive for the *trh* gene (Espineira, *et al.*, 2010).

Several qPCR methods have been developed with the aim of targeting either a single or multiple species using a multiplex approach. For example, Garrido-Maestu, *et al.* (2015) developed a multiplex qPCR assay for the detection of both the *lolB* and *ctxA* genes of *V. cholerae*. The detection limit was evaluated with spiked boiled mussels at 2 CFU/25 g, after an 18+/- 2-hour enrichment step in APW at 37°C. Moreover, other non-spiked seafood samples such as fish, crustaceans, cephalopods and bivalves, as well as water samples, were analyzed by both this method and the ISO 21872-1 method. For all of the 152 tested seafood samples, the results indicated that eight out of 25 bivalve samples were positive for the *lolB* gene. Still concerning *V. cholerae*, a TaqMan qPCR method was developed for spiked *pangasius* fillets for the specific detection of O139 *V. cholerae*, targeting the *ctx* operon, before testing commercial fish samples (Chapela, *et al.*, 2010). This molecular method was compared to the FDA-BAM culture method in order to correlate them. Of the commercial fish samples analyzed, 14 were positive with the FDA-BAM method, but qPCR detection of the cholera toxin was negative.

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The advantage of the methodology developed by Garrido, *et al.* (2012) is the ability to detect pathogenic *V. parahaemolyticus* strains containing two forms of the *trh* gene (*trh1* and *trh2*) in a multiplex qPCR assay also targeting the *tdh* gene with another probe. The detection limit was evaluated with boiled frozen mussels for both methods (qPCR and the ISO/TS 21872-1 standard) and was determined as 6 CFU/25 g for *tdh*, 11 CFU/25 g for *trh1* and 8 CFU/25 g for *trh2*. The determined values for the sensitivity, specificity and efficiency of the qPCR method were 94%, 100% and 94% respectively. Moreover, in order to detect total and pathogenic *V. parahaemolyticus*, Rizvi and Bej (2010) developed a SYBR-Green multiplex qPCR assay targeting the *tlh*, *tdh* and *trh* genes. A five-hour enrichment period at 37°C prior to DNA extraction was required to improve detection sensitivity in artificially seeded and natural oysters. Furthermore, Robert-Pillot, *et al.* (2010) developed a TaqMan qPCR method on MPN enrichment cultures for the detection and enumeration of total and pathogenic *V. parahaemolyticus* in frozen shrimps (R72H, *tdh* and *trh* target sequences). These TaqMan qPCR results were correlated with those obtained by plate count assay.

Regarding the distinction between pathogenic and non-pathogenic *V. vulnificus* strains, a qPCR method using TaqMan technology was developed to target the *pilF* gene (Baker-Austin, *et al.*, 2012). It was linked with the qPCR method targeting the *vcgC* gene (Baker-Austin, *et al.*, 2010) that is known to detect pathogenic *V. vulnificus* biotype 1 strains. This assay was tested on DNA extracted from bio-accumulated Pacific oysters after six hours of exposure to 10^9 cells, and detected almost 7600 genome copies of *pilF* per reaction. Canigral, *et al.* (2010) analyzed natural samples collected in the Mediterranean coastal area, such as seawater, raw sewage and seafood,

by TaqMan qPCR on the *vvhA* gene, after a 20-hour enrichment period in APW at 37°C. *V. vulnificus* prevalence was 32% in seawater, 13% in wastewater and 10% in seafood samples.

Compared to the methods described above, the following are able to detect several *Vibrio* species in a single run. Garrido-Maestu, *et al.* (2014) developed a multiplex qPCR assay to detect *V. cholerae*, *V. parahaemolyticus* and *V. vulnificus* targeting the *ompW*, *tlh* and *vvha* genes, respectively. Furthermore, 58 natural non-contaminated samples were analyzed using the ISO/TS 21872-1/2:2007 method and this qPCR assay. Regarding fish matrices, bivalves and algae samples, the number of positive results was higher with the qPCR method than with the ISO method. Kim and Lee (2014) established a multiplex TaqMan qPCR assay to detect total bacteria, *V. parahaemolyticus* and *V. vulnificus* (as well as *Vibrio anguillarum*) targeting 16SrDNA, the *tlh* gene and the *vvhA* gene respectively. In natural samples tested by qPCR, seven out of 10 seawater samples were positive for *V. vulnificus* and two out of 10 for *V. parahaemolyticus*; three out of 20 fish fillet samples were positive for *V. vulnificus*. Results obtained by plating on TCBS were well correlated with the qPCR results only for three seawater samples and for no fish samples. On the contrary, three fish samples giving positive results on TCBS did not show any signal by qPCR. Furthermore, Kim, *et al.* (2012) suggested a SYBR Green-based multiplex qPCR method with primers targeting the *zot*, *vmrA* and *vuuA* genes, specific to *V. cholerae*, *V. parahaemolyticus* and *V. vulnificus* respectively. Oysters, crab meat and raw fish, spiked with 10^0 to 10^5 CFU/g, were incubated for eight hours at 37°C in APW. The minimum detection level was 10^0 CFU per reaction for all species, corresponding to the inoculum before enrichment. More comprehensive methods have also been developed to combine the detection of total *Vibrio* with that of their virulence factors. Indeed, specific primers

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and probes were designed targeting the *tlh*, *tdh* and *trh* genes of *V. parahaemolyticus* and the *vvp* gene of *V. vulnificus* (Taminiau, *et al.*, 2014). Raw shrimps, cooked shrimps and raw mussels were spiked with six bacterial concentrations ranging from 0 to 10^3 CFU/25 g, followed by a 24-hour enrichment period. Regarding seafood seeding by *V. parahaemolyticus* ATCC 43996, the detection limit was much lower for the qPCR method than for the ISO 21872-1 standard method. However, the detection of *V. vulnificus* ATCC 27562 varied in relation to the nature of the seafood and was also dependent on the methods used. Robert-Pillot, *et al.* (2014) developed a TaqMan multiplex qPCR assay containing an Internal Amplification Control (IAC) and targeting the *V. parahaemolyticus* R72H fragment, the *tdh* and *trh* genes, the *V. cholerae ctxA* gene as well as the intergenic spacer region (ISR) and *V. vulnificus* hemolysin (*hly*). The method was applied to 167 fresh and frozen seafood samples retailed in France (crustaceans, fish and shellfish) after a six-hour enrichment period at 37°C (frozen product) or 41.5°C (fresh product) in APW. Of the samples, *V. parahaemolyticus* was the most common species with an occurrence of 31.1%, followed by *V. vulnificus* (12.6%) and *V. cholerae* (0.6%). Furthermore, *V. parahaemolyticus* and *V. vulnificus* were simultaneously present in 9.6% of samples. Virulence genes (*tdh* and *trh*) were present in 25% of the *V. parahaemolyticus*-positive samples. The only *V. cholerae* strain detected was a non-toxigenic strain. Messelhäusser, *et al.* (2010) developed a TaqMan qPCR assay targeting the *vvha*, *toxR* and *sodB* genes for the distinction of the *V. vulnificus*, *V. parahaemolyticus* and *V. cholerae* species respectively. Specific primers for *tdh*, *trh1* and *trh2* on the one hand and *ctx* and *toxR* on the other hand were designed to test for the *V. parahaemolyticus* and *V. cholerae* virulence genes respectively. Of a total of 338 seafood

samples analyzed, including prawns, shellfish, and raw sea fish, a single *V. parahaemolyticus* strain was positive for *trh2* and 10 *V. cholerae* strains were positive for *toxR*.

PCR- and qPCR-derived methods

Many PCR- and qPCR-derived methods have been developed for the detection of *Vibrio*. Some examples dedicated to seafood are introduced here. A Dual Priming Oligonucleotide (DPO) multiplex PCR assay, different from classical PCR methods especially in terms of primer design, was developed to detect *V. cholerae*, *V. parahaemolyticus* and *V. vulnificus* in a single run targeting the *mdh*, *toxR* and *vvhA* genes (Xu, *et al.*, 2017). The DPO system was tested with 810 seafood samples after an enrichment period and in relation to endpoint PCR, this new method had greatly improved specificity. A total of 22 non-O1/non-O139 *V. cholerae*, 34 *V. parahaemolyticus* and four *V. vulnificus* were thus detected. Regarding PCR-derived methods, electrochemiluminescence PCR (ECL-PCR) was developed to detect *V. parahaemolyticus*. Based on the amplification of the *gyrB* gene, in a mixture of *V. alginolyticus* and *V. fluvialis*, the detection limit was 1.6 pg of purified *V. parahaemolyticus* genomic DNA per µg of non-specific DNA from other species (Wei, *et al.*, 2010). The authors used a four-hour enrichment period in sodium chloride violet purple broth containing the seafood samples. Fykse, *et al.* (2012) compared the TaqMan qPCR method with the Nucleic Acid Sequence Based Amplification (NASBA) method to detect *V. cholerae* in ballast water. Probes and primers were designed to target the *groEL* marker and the *tcpA* toxin gene. Toxigenic *V. cholerae* was detected within seven hours by qPCR, including a four-hour enrichment step, and the detection limit was 1 CFU/100 mL, whereas the NASBA RNA-based technique for *V. cholerae* detection in spiked ballast water was effective after six hours of enrichment in APW. Kang, *et al.* (2011) focused

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their research on the *tdh* gene and developed a two-step ultra-rapid qPCR assay named UR Real-time PCR. In this method, annealing/extension, at a temperature of 66°C, was carried out in one step. The main benefit of this study was the rapidity of *tdh* gene detection, performed within 10 minutes, including melting point analysis.

Regarding the numerous detection methods available for both *V. parahaemolyticus* and *V. vulnificus*, some authors such as Jones, *et al.* (2012) decided to evaluate and compare molecular techniques including different kinds of qPCR and LAMP (loop-mediated isothermal amplification) assays (Table 3). The BAX® System qPCR assay (DuPont Qualicon, Wilmington, DE), the FDA multiplex qPCR (Nordstrom, *et al.*, 2007) and a specific LAMP assay (Nemoto, *et al.*, 2011) were all implemented in different studies for the detection of total *V. parahaemolyticus* in oysters and fish intestines. Pathogenic *tdh*+ and *trh*+ *V. parahaemolyticus* were detected by the FDA multiplex qPCR. A LAMP assay was also used for *tdh*+ *V. parahaemolyticus* (Nemoto, *et al.*, 2009). Srisuk, *et al.* (2010) developed a LAMP reaction targeting the *ompW* gene to detect total *V. cholerae* in contaminated seafood. This method's sensitivity was 8 CFU/reaction for pure culture and 20 CFU/reaction for spiked shrimp samples without enrichment. In comparison, the 'classical' PCR protocol exhibited detection limits of 20 CFU/reaction and 100 CFU/reaction in pure culture and in seeded seafood respectively. Moreover, Di, *et al.* (2015) developed a LAMP method targeting the *V. parahaemolyticus tlh/ldh* gene. The detection limit was 2 CFU/g of spiked oysters after three hours of enrichment in APW at 35°C. *V. parahaemolyticus* was detected in 11 of the 70 analyzed natural seafood samples (cuttlefish, sleevefish, jellyfish, etc.). Yi, *et al.* (2014) developed a real-time LAMP assay using the ESE-Quant tube scanner to detect the *tlh/ldh* gene of *V. parahaemolyticus*. The LAMP outer primers designed were also used in a

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qPCR reaction using a SYBR Green I kit to compare the sensitivity of both methods. A comparison was also established with the API 20E identification method. These methods were evaluated with *V. parahaemolyticus* strains isolated from different seafood products (shrimp, fish, oysters, crabs, lobster, cuttlefish, abalone) worldwide. All the tested strains were effectively detected by the LAMP method, while 98% were correctly detected by qPCR using the outer primers of the LAMP assay. Lastly, 90% of the strains were positive with the API 20E method. With this method, six of 91 *V. parahaemolyticus* strains were identified as *V. vulnificus*. The ESE-Quant tube scanner was also used by Zeng, *et al.* (2014) to create a nanoparticle-based immunomagnetic separation (IMS) combined with a LAMP assay to detect *V. parahaemolyticus* in spiked oysters. Furthermore, a qPCR method, based on TaqMan technology, was performed by Nordstrom, *et al.* (2007) using primers and probe designed for targeting the *tlh* gene, and was implemented alone or in combination with IMS (IMS-rPCR). For a 1.9 CFU/g inoculation of the *V. parahaemolyticus* ATCC 17802 strain in oysters, the IMS-LAMP and IMS-rPCR methods required a six-hour enrichment period to give a positive result, while LAMP and rPCR without IMS required a 10-hour enrichment period. Pathogenic *V. parahaemolyticus* were also targeted by a LAMP assay in the study by Malcolm, *et al.* (2015) in which seafood samples were analyzed after an 18-hour enrichment period on APW at 35°C. Samples were then analyzed by a multiplex PCR assay targeting the *toxR*, *tdh* and *trh* genes and a LAMP assay for the *tdh* and *trh* genes. Of a total of 232 samples (bloody clams, surf clams and shrimps from Malaysian wet markets and hypermarkets), 229 were positive for *V. parahaemolyticus*, of which 77 were *tdh* positive and 16 were *trh* positive. In relation to the PCR method, the LAMP method was more sensitive overall for detecting low numbers of pathogenic *V. parahaemolyticus* strains. Lastly,

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more comprehensive LAMP methods were designed to detect both total and pathogenic *V. parahaemolyticus*. This bacterial species was detected in naturally contaminated seafood after the incubation of 25 g samples in APW for 16--18 hours at 37°C (Yamazaki, *et al.*, 2011). The *tlh* gene was targeted at the same time as the *tdh, trh1 and trh2* genes. LAMP reactions for the *tdh, trh1* and *trh2* genes were all negative, but the results revealed that 43 out of 171 samples were positive for the *tlh* gene. Detection of total and pathogenic *V. parahaemolyticus* was also performed by LAMP assays with specific primers targeting the *rpoD* and *tdh* genes respectively (Jones, *et al.*, 2012) following the protocol described by Nemoto, *et al.* (2009) and Nemoto, *et al.* (2011). Nemoto, *et al.* (2011) developed a LAMP assay targeting the *toxR* and *rpoD* genes to detect *V. parahaemolyticus* and a most probable number (MPN) method combined with the rpoD-LAMP assay (MPN-rpoD-LAMP assay).This alternative method was compared to the conventional MPN method with the use of chromogenic agar (MPN-CV method). The results showed that the rpoD-LAMP assay was more sensitive than the toxR-LAMP assay. Short-necked clams were spiked with *V. parahaemolyticus* at different concentrations and then mixed in APW for analysis with the MPN method. After an 18-hour enrichment period at 37° C, DNA was extracted and analyzed by the MPN-rpoD-LAMP assay. While enriched samples were inoculated onto CHROMagar Vibrio agar, suspected isolates were tested with biochemical tests. The MPN-rpoD-LAMP assay exhibited better sensitivity and rapidity than the MPN-CV method.

LAMP assays have also been applied to other *Vibrio* species. More specifically, this method was evaluated in combination with amplicon detection by chromatographic lateral flow dipstick (LFD), for the detection of *V. vulnificus* in spiked oyster samples. It appeared to be sensitive with the detection of $1.2.10^4$ CFU/g (Surasilp, *et al.*, 2011). Six primers were designed to specifically

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hybridize the *rpoS* gene encoding the RNA polymerase subunit sigma factor S (Kim, *et al.*, 2008). A FITC-labeled probe for LFD assay was designed and was able to hybridize biotin-labeled LAMP amplicons, thus improving specificity for the detection of the *rpoS* gene from *V. vulnificus*. Furthermore, LAMP and PCR assays were compared, showing that the LAMP-LFD assay was 100 times more sensitive than PCR in pure culture strains and spiked oyster samples. Han and Ge (2010) quantified *V. vulnificus* on the basis of *vvha* gene recognition. Two LAMP assays, using either the SYTO 9 fluorescent DNA-intercalating dye or a turbidimeter to quantify the magnesium pyrophosphate by-product, were evaluated and compared to two PCR reactions, using two sets of primers: LAMP outer primers and the primers described by the FDA-BAM. In pure culture, PCR was 100-fold less sensitive than real-time LAMP, which could detect 1 to 10 CFU per reaction. In spiked oysters, the detection limit for the *V. vulnificus* ATCC 27562 strain was $6.4.10^4$ CFU/g by RT-LAMP and $6.4.10^7$ CFU/g by PCR, without any enrichment. Regarding the rapidity of the techniques, the fluorescent-based RT-LAMP was faster (20 minutes) than the turbidity-based assay (30 minutes) but was less reliable for low-contaminated food samples.

Concluding remarks and future prospects in the detection of *Vibrio cholerae*, *Vibrio parahaemolyticus* and *Vibrio vulnificus* in seafood

The presence of potentially pathogenic *Vibrio* for humans is the subject of numerous studies worldwide (Raghunath, *et al.*, 2008), (Huehn, *et al.*, 2014), (Suffredini, *et al.*, 2014); (Caburlotto, *et al.*, 2016). Indeed, the *Vibrio* risk linked to seafood consumption increases with global ocean warming (Baker-Austin, *et al.*, 2017), the spread of pathogenic *Vibrio* species through the

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international exchanges with the transmission of bacteria *via* ballast water (Fykse, *et al.*, 2012) and the seafood growing demand (Johnson and Schantz, 2017). Furthermore, the antibiotic resistance of pathogenic species (Baker-Austin, 2015), which can easily and quickly adapt through the Horizontal Gene Transfer (HGT) of virulence genes (Metzger and Blokesch, 2016), must be considered. HGT can occur between closely related species (Gonzalez-Escalona, *et al.*, 2006). For example, a STX element isolated from *Vibrio fluvialis* appears as a conjugative transposon derived from *Vibrio cholerae* and responsible for multi-antibiotic resistance (Ahmed, *et al.*, 2005). Due to the importance of the HGT phenomenon into the *Vibrio* genus and the genetic complexity of *Vibrio* species including *V. parahaemolyticus* with the rapid turnover of pathogenic variants (Baker-Austin, *et al.*, 2017), it is relevant to detect all the *Vibrio* including non-toxigenic environmental strains.

To date, the reference methods used to detect *Vibrio* rely mainly on microbiological techniques, *via* enrichment steps and isolation on culture media. Nevertheless, according to the emergence of hazards associated with the seafood / *Vibrio* couple, studies on molecular methods have been increasingly developed in recent years. Microbiological methods have obviously some limitations compared to molecular methods. Indeed, several weeks are needed to get a result. Culture media are more or less specific and the reading of biochemical tests during the confirmation step can induce misidentifications due to the close genetic and phenotypic relations between certain *Vibrio* species. The sequential use of several media (like TCBS, CHROMagar Vibrio and CC) is more discriminant between species (Cruz, *et al.*, 2013). However, it is important to have non-destructive isolation and purification steps in order to store the strains for future analysis. Nevertheless, these microbiological techniques do not provide information about

the pathogenicity. Thus, a confirmation step by nucleic acid amplification methods, such as PCR or qPCR, is needed. PCR is therefore more specific, more sensitive and faster than the microbiological method. The species are more accurately identified and its pathogenicity can be determined if a virulence gene is targeted. The qPCR method allows a quantification with the determination of optimal detection limits and a shortened enrichment step. Finally, the LAMP method is faster than the conventional PCR thanks to the fact that LAMP works under isothermal conditions. It also offers better sensitivity compared to the PCR (Yu, *et al.*, 2013) and it is more suited for an *in situ* screening. However, the VBNC state is not taken into account by microbiological methods whereas basic qPCR allows the detection of both live and dead cells. This detection of DNA from dead cells can lead to an overestimation of Vibrio or a false-positive result. Zhu, *et al.* (2012) developed a qPCR with a PMA (propidium monoazide) treatment to detect *tdh*-positive and viable strains of *V. parahaemolyticus*. Similarly, Zhong, *et al.* (2016) developed a PMA-LAMP to distinguish between viable and dead *V. parahaemolyticus* in seafood.

It is not generally so easy to make intra-relations and comparisons between the molecular methods mentioned because of the following differences: the targeted gene(s), the seafood matrix origin, the DNA extraction procedure, the enrichment time and medium and the equipment used. Thus, the detection limits vary widely from one method to another.

As detailed in this review, PCR and qPCR methods targeting mostly virulence genes encoding toxins, have been widely developed in recent years. Prospects for the future are the development of miniaturized detection models for *in situ* diagnosis. This kind of model might be suitable for

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industry in order to release seafood batches more quickly, to be consistent with HACCP procedure for example. The methods currently developed provide time saving with a reduced enrichment period, a sensitivity increase and a lower cost. Moreover, an additional argument is the ease of use. These methods are at the border of several skill domains such as microbiology, biochemistry and biophysics. From that point, *Vibrio* detection is based on the use of biosensors (Cecchini, *et al.*, 2016), in particular regarding seafood (Pengsuk, *et al.*, 2013), (Jadeja, *et al.*, 2015); (Khemthongcharoen, *et al.*, 2015), (Sha, *et al.*, 2016); (Liu, *et al.*, 2017); (Park and Choi, 2017). These tests mainly rely on immunological assays and use specific devices like chips and nanoparticles. In addition to the development of biosensors, the next-generation sequencing (NGS) technologies have rapidly progressed in recent years. In the near future, NGS could be applied to *Vibrio* and could be used for epidemiological investigations, to get more information about the distribution and the spread of pathogenic clones worldwide, as it is already the case for *V. parahaemolyticus* (Kimura, 2017). Thus, traceability studies could be achieved. NGS could be useful to get the full map of virulence markers for each strain detected as *Vibrio* genomes are particularly dynamic. Moreover this technique will allow the reduction of identification and serotyping issues by the use of whole genome sequencing (Perez Chaparro, *et al.*, 2011) as well as identifying new clones (Gonzalez-Escalona, *et al.*, 2016). NGS becomes useful to confirm foodborne outbreaks and identify the vehicle, as it was the case for *Salmonella* strains suspected responsible of food poisoning event (Du, *et al.*, 2017). Nevertheless, NGS could be a good promising tool to detect and better identify *Vibrio* but cannot be used for *in situ* diagnosis because it remains expensive and needs specific laboratory skills for a routinely use.

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Table 1: Genes targeted by PCR methods for the detection of total and pathogenic *V. cholerae*, *V. parahaemolyticus* and *V. vulnificus* in seafood

PCR	Species	Genes	Gene products	References
Detection of total *Vibrio*	*V. cholerae*	*rpoA*	genus-specific RNA polymerase subunit A gene	Jeyasekaran *et al.*, 2011
	V. parahaemolyticus	*groEL*	heat shock protein (phylogenetic marker)	Hossain *et al.*, 2013
		hns	histone-like nucleoid structure gene	No *et al.*, 2011
		tlh	thermolabile hemolysin gene	Espineira *et al.*, 2010 FDA-BAM (Bej *et al.*, 1999)
	V. vulnificus	*dnaJ*	heat shock protein	Espineira *et al.*, 2010
Detection of pathogenic *Vibrio*	*V. cholerae*	*ctx*	cholera toxin	FDA-BAM (Koch *et al.*, 1995)
		ctxA	cholera toxin subunit A	Jeyasekaran *et al.*, 2011 Espineira *et al.*, 2010
		rtxA	repeat in toxin subunit A	Jeyasekaran *et al.*, 2011
		wbeO	O-antigen biosynthesis	Espineira *et al.*, 2010
		tcpA	toxin-coregulated pili	
	V. parahaemolyticus	*tdh & trh*	thermostable direct hemolysin & TDH-related haemolysin	Hossain *et al.*, 2013 No *et al.*, 2011 Yu *et al.*, 2010 Espineira *et al.*, 2010 Taminiau *et al.*, 2014 FDA-BAM (Bej *et al.*, 1999)
		irgB	iron-regulated virulence regulatory protein IrgB	Yu *et al.*, 2010
	V. vulnificus	*vvhA*	cytolysin	FDA-BAM (Hill *et al.*, 1991)

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Table 2: Genes targeted by qPCR methods for the detection of total and pathogenic *V. cholerae*, *V. parahaemolyticus* and *V. vulnificus* in seafood

qPCR	**Species**	**Genes or detection markers**	**Gene products**	**References**
Detection of total *Vibrio*	*V. cholerae*	*sodB*	superoxide dismutase	Messelhausser *et al.*, 2010
		Intergenic Spacer Region (ISR)	-	Robert-Pillot *et al.*, 2014
		ompW	outer membrane protein	Garrido-Maestu *et al.*, 2014
		lolB	outer membrane lipoprotein	Garrido-Maestu *et al.*, 2015
	V. parahaemolyticus	*tlh*	thermolabile hemolysin gene	Garrido-Maestu *et al.*, 2014 Rizvi & Bej, 2010 Kim & Lee, 2014 Taminiau *et al.*, 2014
		R72H DNA sequence	-	Robert-Pillot *et al.*, 2010 Robert-Pillot *et al.*, 2014
	V. vulnificus	*vvp*	metalloprotease	Taminiau *et al.*, 2014
Detection of pathogenic *Vibrio*	*V. cholerae*	*ctx*	cholera toxin	Chapela *et al.*, 2010 Messelhausser *et al.*, 2010
		ctxA	cholera toxin subunit A	Robert-Pillot *et al.*, 2014 Garrido-Maestu *et al.*, 2015
		zot	zonular occludens toxin	Kim *et al.*, 2012
		toxR	global regulatory gene	Messelhausser *et al.*, 2010

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	V. parahaemolyticus	*tdh*	thermostable direct hemolysin	Messelhausser *et al.*, 2010 Garrido *et al.*, 2012 Rizvi & Bej, 2010 Robert-Pillot *et al.*, 2010 Robert-Pillot *et al.*, 2014 Zhu et al., 2012
		trh	TDH-related haemolysin	Garrido *et al.*, 2012 Rizvi & Bej, 2010 Robert-Pillot *et al.*, 2010 Robert-Pillot *et al.*, 2014
		trh1	TDH-related haemolysin 1	Messelhausser *et al.*, 2010
		trh2	TDH-related haemolysin 2	
		vmrA	multidrug efflux pump	Kim *et al.*, 2012
		toxR	global regulatory gene	Messelhausser *et al.*, 2010
	V. vulnificus	*vvhA*	cytolysin	Messelhausser *et al.*, 2010 Garrido-Maestu *et al.*, 2014 Canigral *et al.*, 2010 Kim & Lee, 2014
		vuuA	vulnibactin outer membrane receptor	Kim *et al.*, 2012
		pilF	pilus-type IV assembly protein	Baker-Austin *et al.*, 2012
		vcgC	virulence-correlated gene	Baker-Austin *et al.*, 2010 Baker-Austin *et al.*, 2012
		hly	hemolysin	Robert-Pillot *et al.*, 2014

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Table 3: Genes targeted by LAMP methods for the detection of total and pathogenic *V. cholerae*, *V. parahaemolyticus* and *V. vulnificus* in seafood

LAMP	Species	Genes	Gene products	References
Detection of total *Vibrio*	*V. cholerae*	*ompW*	outer membrane protein	*Srisuk et al.*, 2010 Niessen et al., 2013
	V. parahaemolyticus	*tlh*	thermolabile hemolysin gene	Wang *et al.*, 2011 Yamazaki *et al.*, 2011 Wang *et al.*, 2013 Zeng *et al.*, 2014 Yi *et al.*, 2014 Di *et al.*, 2015 Zhong et al., 2016 Li *et al.*, 2017
		rpoD	RNA polymerase sigma factor	Nemoto *et al.*, 2011 Niessen et al., 2013
	V. vulnificus	*rpoS*	RNA polymerase subunit sigma factor S gene	Surasilp *et al.*, 2011 Niessen et al., 2013
Detection of pathogenic *Vibrio*	*V. parahaemolyticus*	*tdh*	thermostable direct hemolysin	Yamazaki *et al.*, 2011 Malcolm *et al.*, 2015 Li *et al.*, 2017 Niessen et al., 2013
		trh	TDH-related haemolysin	Malcolm *et al.*, 2015 Li *et al.*, 2017 Niessen et al., 2013
		trh1	TDH-related haemolysin 1	Yamazaki *et al.*, 2011; Niessen et al., 2013
		trh2	TDH-related haemolysin 2	
		toxR	global regulatory gene	Chen & Ge, 2010 Nemoto *et al.*, 2011 Niessen et *al.*, 2013
	V. vulnificus	*vvhA*	cytolysin	Han & Ge, 2010; Niessen et *al.*, 2013

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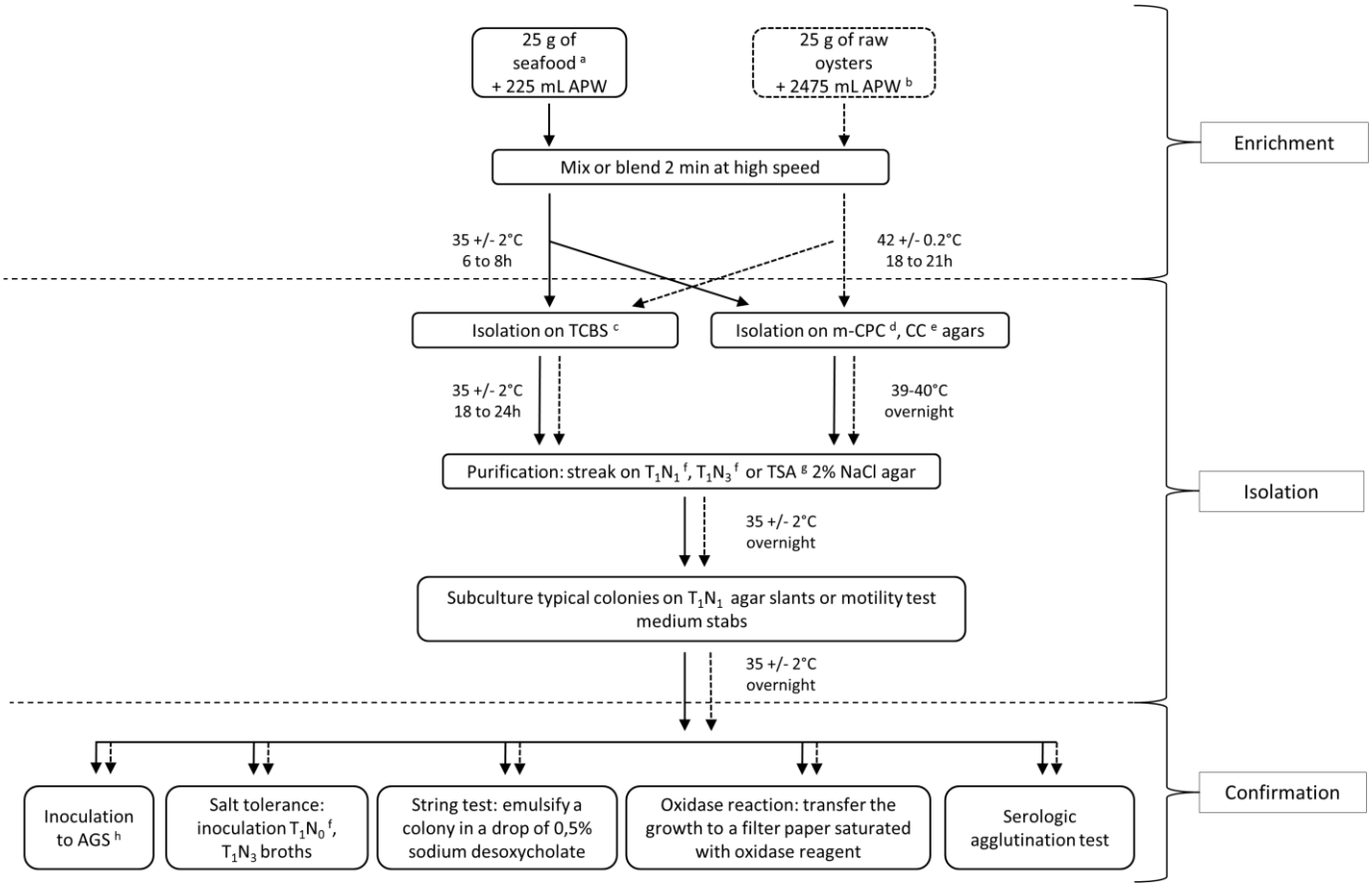

Figure 1: Detection of *Vibrio cholerae* by FDA-BAM (Chapter 9, Bacteriological Analytical Manual) Only microbiological identification is represented, excluding serological typing, the pathogenicity determination, and the genotypic detection of toxins. The tests described correspond to the Screening and Confirmation section (Chapter 9, part B: *V. cholerae*). The biochemical tests include the ability to grow in 1% tryptone without added NaCl, Gram staining, and API20E.

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[a] except raw oysters; dotted lines: conditions for raw oysters; [b] APW: Alkaline Peptone Water; [c] TCBS: Thiosulfate Citrate Bile Salts Sucrose; [d] m-CPC: modified-Cellobiose Polymyxin Colistin; [e] CC: Cellobiose Colistin; [f] T_1N_0, T_1N_1, T_1N_3: Tryptone Salt Agar with 0%, 1% or 3% of sodium chloride; [g] TSA: Trypticase (or Tryptic) Soy Agar; [h] AGS: Arginine Glucose Slants.

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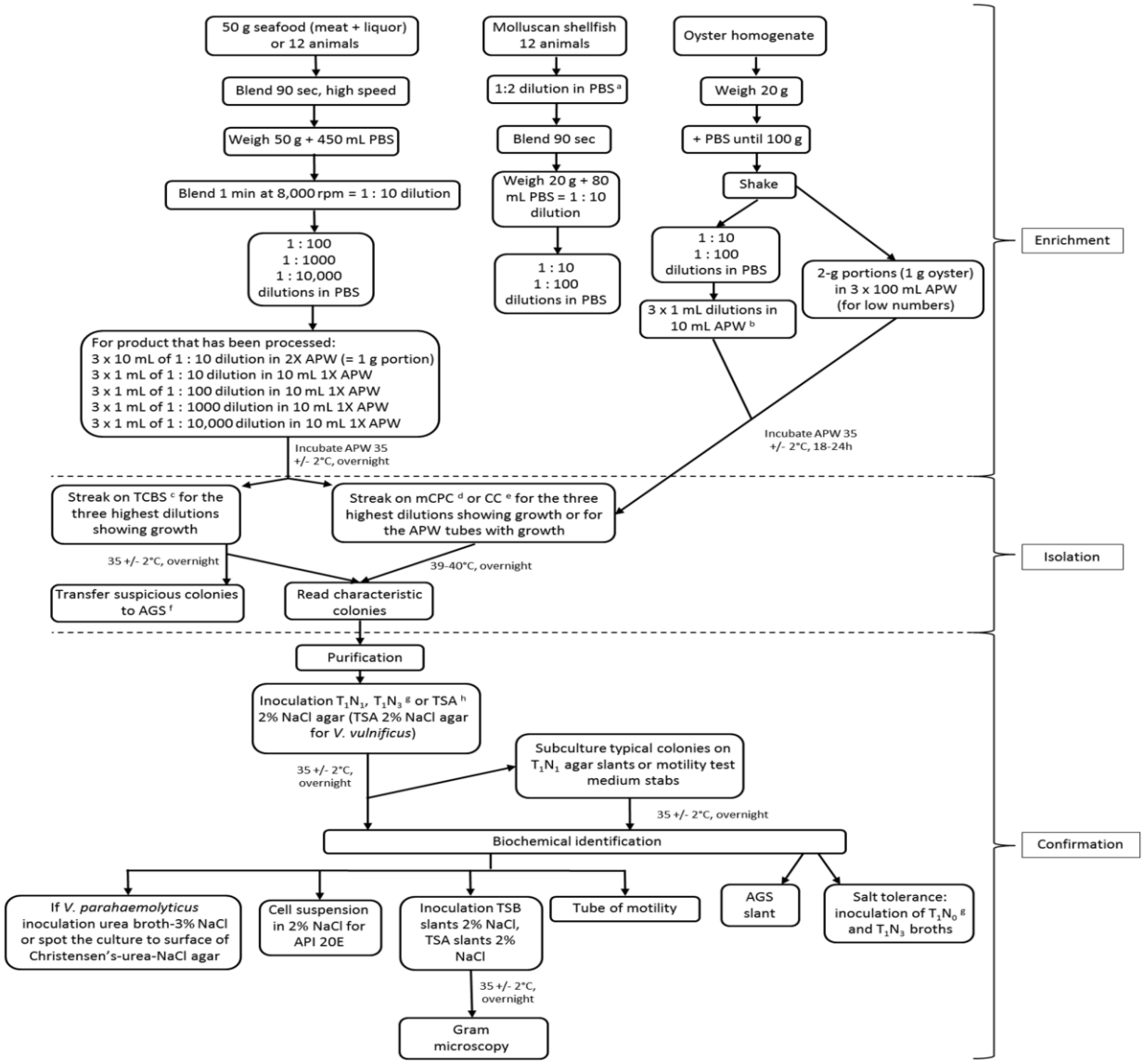

Figure 2: Detection of *Vibrio parahaemolyticus* and *Vibrio vulnificus* by FDA-BAM (Chapter 9, Bacteriological Analytical Manual)

The scheme describes the MPN (Most Probable Number) procedure for the enumeration of *V. parahaemolyticus* and *V. vulnificus* isolates. Biochemical identification includes the tests described in the Screening and Confirmation section (Chapter 9, part B: Other Vibrios).

[a] PBS: Phosphate Buffered Saline; [b] APW: Alkaline Peptone Water; [c] TCBS: Thiosulfate Citrate Bile Salts Sucrose;[d] m-CPC: modified-Cellobiose Polymyxin Colistin; [e] CC: Cellobiose Colistin; [f] AGS: Arginine Glucose Slants;[g] T_1N_0, T_1N_1, T_1N_3: Tryptone Salt Agar with 0%, 1% or 3% of sodium chloride; [h] TSA: Trypticase (or Tryptic) Soy /Agar.

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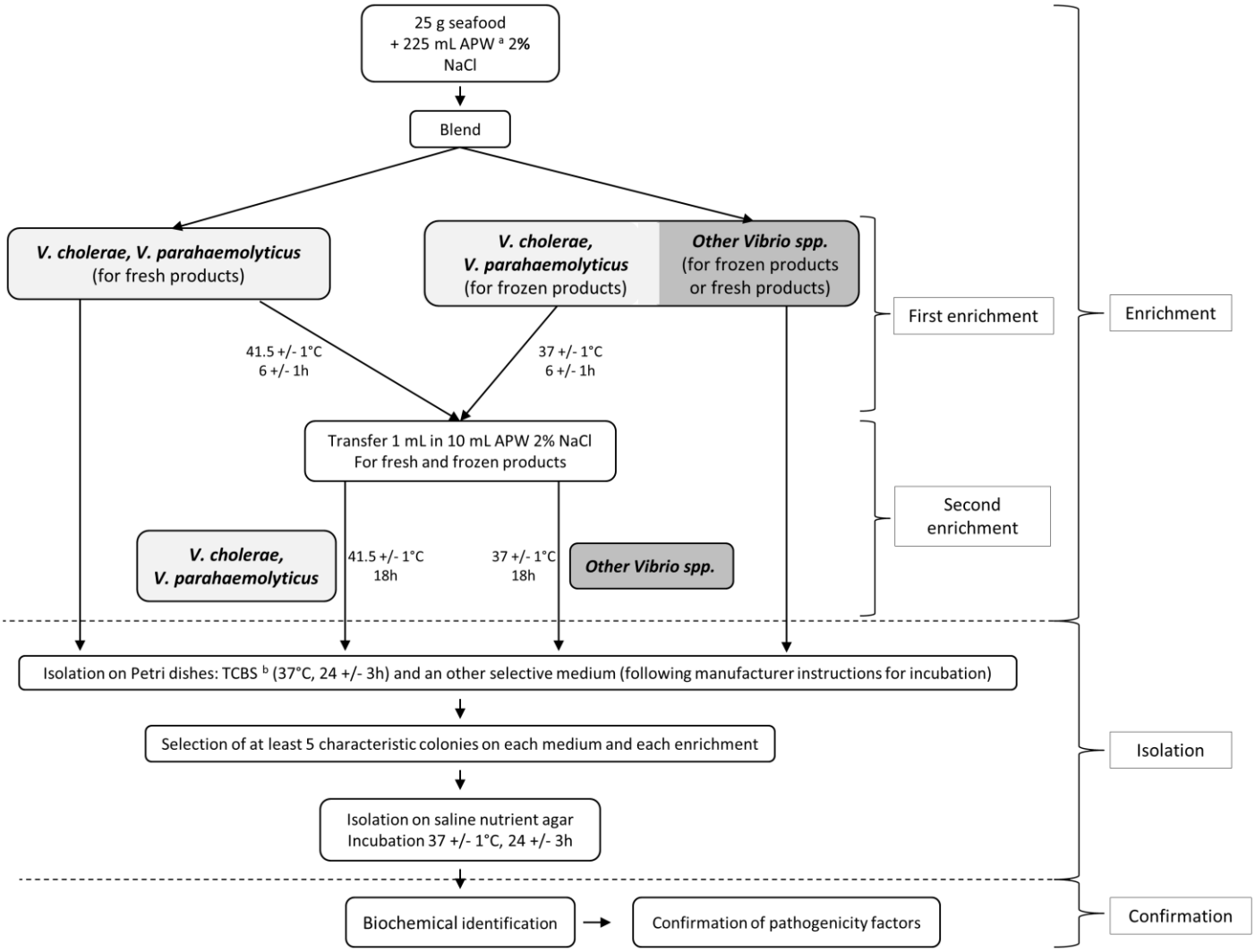

Figure 3: Horizontal method for the detection of potentially enteropathogenic *Vibrio* spp by International standards ISO/TS 21872-1:2007 (*Vibrio parahaemolyticus* and *Vibrio cholerae*) and ISO/TS 21872-2:2007 (species other than *Vibrio parahaemolyticus* and *Vibrio cholerae*)

Vibrio parahaemolyticus and *Vibrio cholerae*, in light gray

Species other than *Vibrio parahaemolyticus* and *Vibrio cholerae*, in dark gray

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Biochemical identification includes: Gram staining, oxidase, motility, API20E diagnostic strip and a halotolerance test with increased salt concentrations (0, 2, 6, 8, 10% NaCl) with incubation at 37°C for 24 +/- 3 hours.

[a] APW: Alkaline Peptone Water; [b] TCBS: Thiosulfate Citrate Bile Salts Sucrose.

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