Detection of pathogenic *Vibrio* spp. in foods: polymerase chain reaction-based screening strategy to rapidly detect pathogenic *Vibrio parahaemolyticus*, *Vibrio cholerae*, and *Vibrio vulnificus* in bivalve mollusks and preliminary results

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Abstract

The majority of human diseases attributed to seafood are caused by *Vibrio* spp., and the most commonly reported species are *Vibrio parahaemolyticus*, *Vibrio vulnificus,* and *Vibrio cholerae*. The conventional methods for the detection of *Vibrio* species involve the use of selective media, which are inexpensive and simple but time-consuming. The present work aimed to develop a rapid method based on the use of multiplex real-time polymerase chain reaction (PCR) to detect *V. parahaemolyticus*, *V. vulnificus,* and *V. cholerae* in bivalve mollusks. 30 aliquots of bivalve mollusks (*Mytilus galloprovincialis*) were experimentally inoculated with two levels of *V. parahaemolyticus*, *V. vulnificus,* and *V. cholerae*. ISO 21872-1:2017 was used in parallel for qualitative analysis. The limit of detection of 50% was 7.67 CFU/g for *V. cholerae*, 0.024 CFU/g for *V. vulnificus,* and 1.36 CFU/g for *V. parahaemolyticus*. For *V. vulnificus* and *V. cholerae,* the real-time PCR protocol was demonstrated to amplify the pathogens in samples seeded with the lowest and highest levels. The molecular method evaluated showed a concordance rate of 100% with the reference microbiological method. *V. parahaemolyticus* was never detected in samples contaminated with the lowest level, and it was detected in 14 samples (93.33%) seeded with the highest concentration. In conclusion, the developed multiplex real-time PCR proved to be reliable for *V. vulnificus* and *V. cholerae.* Results for *V. parahaemolyticus* are promising, but further analysis is needed. The proposed method could represent a quick monitoring tool and, if used, would allow the implementation of food safety.

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Introduction

In seawater, many different bacterial pathogens are frequently encountered, such as *Escherichia coli*, *Salmonella* spp., and *Proteus* spp.; however, the majority of human diseases attributed to the natural microbiota of aquatic environments and seafood are caused by *Vibrio* spp. (Baker-Austin *et al*., 2018).

Vibrio spp. are gram-negative and halophilic bacteria widely distributed in the aquatic environment worldwide. To the genus *Vibrio* belong more than 100 species, of which 13 have been associated with human infection, and among them, *Vibrio cholerae, Vibrio parahaemolyticus*, and *Vibrio vulnificus* are the most important (Castello *et al*., 2022). Outbreaks of *V. cholerae* are common both in developed and developing countries. *V. cholerae* is responsible for acute diarrheal disease, and it represents, globally, an important cause of morbidity and mortality in vulnerable populations with a lack of access to clean water and sanitation facilities. In particular, in 2021, cholera-associated deaths surged globally. The infection is caused by the ingestion of the microorganism through contaminated food, and the incubation period ranges from 2 hours to 5 days (Castello *et al*., 2022). *V. parahaemolyticus* is mainly associated with gastrointestinal infections, but it can also cause extraintestinal infections (Fleischmann *et al*., 2022). It is considered an emerging pathogen, and the infection in humans

mainly occurs due to the consumption of contaminated foods, in particular undercooked fish and shellfish (Castello *et al*., 2022; Ma *et al.*, 2023). The virulence of *V. parahaemolyticus* depends on the presence of two virulence genes: a thermostable direct hemolysin (TDH) and a thermostable related hemolysin, encoded by the *tdh* and *trh* genes, respectively. *V. parahaemolyticus* is often associated with human infections in the United States of America, Chile, and Japan, and to a lesser extent in the European Union (Castello *et al*., 2022). *V. vulnificus* can be responsible for seafood-related gastroenteritis but is mainly associated with wound infection, which may lead to septicemia and even death (Fleischmann *et al*., 2022).

In the latest years, the incidence of vibriosis has increased, probably due to sea warming caused by climate change worldwide (Baker-Austin *et al*., 2018). Indeed, besides nutrients and salt, temperature is the most important factor influencing *Vibrio* spp. occurrence in water (Fleischmann *et al*., 2022).

Moreover, *Vibrio* spp. is one of the main bacterial diseases affecting mollusk bivalves. Mollusks, being filter-feeding organisms, may concentrate unicellular algae, bacteria, and other contaminants diluted in the environment (La Tela *et al*., 2021). The incidence of foodborne outbreaks associated with their consumption is increasing (Marceddu *et al.*, 2017). Historically, *E. coli* has been used as an indicator bacterium to measure the potential presence of other pathogens. However, the use of *E. coli* has been recently questioned since it has been shown that it is poorly predictive of the presence of microorganisms adapted to the aquatic environment (*Vibrio* spp.) (Marceddu *et al*., 2017).

Conventional methods for the detection of *Vibrio* spp. involve the use of selective media, which are inexpensive and simple but time-consuming (take up to 5 days). The molecular methods included in ISO 21872-1 (ISO, 2017a) for detecting the different *Vibrio* species in a food sample reduced the detection time. However, using three simplex polymerase chain reactions (PCR) is still considered time-consuming and expensive. The development of rapid methods is important to conduct proper control of the contaminated products, identify outbreak sources quickly, and prevent the spread of illness (Wang and Salazar, 2016). Therefore, the aim of the present study was the development of a rapid method based on the use of multiplex real-time PCR (qPCR), to detect *V. parahaemolyticus*, including potentially enteropathogenic strains, *V. vulnificus* and *V. cholerae,* in mollusk bivalves.

Materials and Methods

Strains and inoculum preparation

Vibrio strains

V. parahaemolyticus ATCC 17802*, V. cholera* ATCC 9459*, V. vulnificus* ring trial LGC 2021 IB supplied by the collection of the Experimental Zooprophylactic Institute of Southern Italy were included in this study. Bacterial strains were cultured in blood agar (CM0055, OXOID, Basingstoke, UK) for 18 hours at 37°C and then activated in alkaline saline peptone water (ASPW) (Biolife, Monza, Italy) and incubated at 37°C for 18 hours. Subsequently, 10-fold serial dilutions of the culture were prepared in ASPW, and the cell concentration was measured by plating onto blood agar after incubation at 37°C for 24 hours.

Escherichia coli and Salmonella Typhimurium strains

E. coli and *S.* Typhimurium strains supplied by the collection of the Experimental Zooprophylactic Institute of Southern Italy were included in this study. Bacterial strains were cultured in blood agar for 18 hours at 37°C and then activated in buffered peptone water (CM1049, OXOID, Basingstoke, UK) and incubated at 37°C for 18 hours. Subsequently, 10-fold serial dilutions of the culture were prepared in peptone water, and the cell concentration was measured by plating onto plate count agar (CM0325, OXOID, Basingstoke, UK) after incubation at 37°C for 24 hours.

Inoculation of the food matrix

A total of 60 aliquots of bivalve mollusks (*Mytilus galloprovincialis*) were collected at different shellfish farms along the Campania coast in southern Italy. To check the absence of *Vibrio* spp., *E. coli* and *Salmonella*, the aliquots were first analyzed, respectively, by UNI EN ISO 21872-1:2017 (ISO, 2017a), UNI ISO TS 16649-1:2018 (ISO, 2018), and UNI EN ISO 6579-1:2017/Amd1:2020 (ISO, 2017b). To point out the optimal protocol for *Vibrio*'s detection, 30 aliquots were experimentally contaminated with *V. parahaemolyticus, V. vulnificus*, and *V. cholerae*. In particular, 25 g of flesh and intervalvular liquid of each aliquot were placed in a sterile stomacher bag and homogenized for 3 minutes at 230 rpm using a peristaltic homogenizer (BagMixer®400 P, Interscience, Saint Nom, France) after the addition of 195 mL (1:10, W/W) of sterilized ASPW (Biolife, Monza, Italy).

For the detection of *Vibrio* species, 15 aliquots were inoculated with 10 mL of dilutions with a bacterial load of 1.5x10 CFU/25 g of *V. parahaemolyticus* and *V. vulnificus* and 1.5×10^3 CFU/25 g of *V. cholerae* (low concertation), and 15 aliquots with a concentration of 1.5×10^6 CFU/25 g of *V. parahaemolyticus* and *V. vulnificus* and 1.5×10^5 CFU/25 g of *V. cholerae* (high concentrations) as indicated in Annex E of the ISO 21872-1:2017 (ISO, 2017a). 30 aliquots were experimentally contaminated with *E. coli* and *S.* Typhimurium (negative controls). In particular, 25 g of flesh and intervalvular liquid from each aliquot were placed in a sterile stomacher bag and homogenized for 3 minutes at 230 rpm using a peristaltic homogenizer (BagMixer®400 P, Interscience, Saint Nom, France) after the addition of 205 mL (1:10, W/W) of sterilized ASPW (Biolife, Monza, Italy). Subsequently, 15 aliquots were inoculated with 10 mL of *E. coli* and 10 mL of *S.* Typhimurium dilutions, both with a bacterial load of 1.5×10^3 CFU/g (low concertation) and 15 aliquots with a concentration of 1.5×10^5 CFU/g (high concentrations). After inoculation, all samples were shaken for 1 minute to optimize the distribution of the inoculum, and then they were incubated at 37°C for 18 hours. After the incubation, 1 mL of each incubated homogenate was subjected to the DNA extraction phase. Moreover, samples seeded with the Vibrio species were tested in parallel with ISO 21872-1:2017 (ISO, 2017a).

DNA extraction

DNA was extracted using the Chelex-100-resin method (Bio-Rad, Hercules, CA, USA), whereby one colony of each strain was suspended in 300 µL of 6% Chelex 100 by vortexing and incubated for 20 minutes at 56°C and again for 8 minutes at 100°C. The suspension was immediately chilled on ice for 1 minute and centrifuged for 5 minutes at 10,000×g at 4°C (Peruzy *et al.*, 2020). 5 µL of DNA extracted was used as the template for the qPCR.

Real-time polymerase chain reaction

For the detection of *Vibrio* spp., the *Vibrio* multiplex screening assay kit (Generon, San Prospero, Italy) was used. The kit allowed the individual identification of the virulence genes: *tdh* and TDH-related hemolysin (*trh*) genes in *V. parahaemolyticus*, *V. vulnificus*

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hemolysin (*vvh*A) gene in *V. vulnificus,* and the presumptive cytotoxin (*rtx*A) in *V. cholerae.* The primer sequences of *V. parahaemolyticus* and *V. vulnificus* used in the present work are reported in UNI EN ISO 21872-1:2017 (ISO, 2017a), while those of *V. cholerae* are reported in the study by Chow *et al*. (2001). The kit contained the PATHfinder OLIGO Mix*, the* GENERase ULTRA Plus Mastermix*,* the diluent, and a positive and negative control. Moreover, the kit included a non-competitive internal control (IAC) to determine the inhibition in the biological samples and to identify false negative results. The reaction was run at 95°C for 3 minutes, followed by 45 cycles at 95°C for 10 seconds and 1 cycle at 60°C for 45 seconds. Amplification reactions were performed with the Bio-Rad CFX96 platform using a 96-well PCR multiplate from Bio-Rad (Hercules, CA, USA). While awaiting the reverse transcription-PCR results, the enrichment broths were stored at 4°C.

Bacterial isolation and identification through sma-trix-assisted laser desorption ionization-time of flight mass spectrometry

PCR-positive samples were seeded in CHROMagarVibrio and thiosulphate-citrate-bilesaltsucrose agar and incubated at 37°C for 24 hours. Presumptive *Vibrio* spp. colonies were analyzed through matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) (Bruker Daltonics, Bremen, Germany). In brief, isolates were smeared in duplicate onto a 96-spot plate (Bruker Daltonics, Bremen, Germany) and allowed to dry at room temperature. Subsequently, they were covered with 1µL matrix solution containing 10 mg/mL α- cyano-4-hydroxycinnamic acid in acetonitrile, deionized water, and trifluoracetic acid (50:47.5:2.5, [vol/vol/vol]). Bruker's bacterial test standard (Bruker Daltonics, Bremen, Germany) was used as a mass calibration and reference standard before each series of MALDI measurements. Samples were processed in the Microflex™ LT/SH smart MALDI-TOF mass spectrometer (Bruker Daltonics, Bremen, Germany) equipped with a nitrogen laser operating in linear positive ion detection mode using MALDI Biotyper Automation Control 2.0 (Bruker Daltonics, Bremen, Germany). Identifications were obtained by comparing the mass spectra to the Bruker MSP database using the Bruker Compass software (Bruker Daltonics) at default settings. Identification score criteria were classified according to the manufacturer's identification criteria; scores between 2.0 and 3.0 indicated highly probable species identification, between 1.7 and 1.99 for certain genera and probable species identification, whereas values lower than 1.7 indicated a non-reliable identification. The analysis was repeated when the spots resulted in 'no peaks found'.

Verification of microbiological measurement

To calculate the limit of detection of 50% (LOD_{50}), the analysis of one sample seeded with the highest load, one sample contaminated with the lowest load, and one negative sample was repeated five, twenty, and five times, respectively. LOD_{50} was calculated by using the limit of detection program for qualitative microbiology methods (ISO 5725-1) (ISO, 2023). To evaluate the inter-individual variation, analyses were repeated by different operators. In particular, according to ISO 21872-1:2017 (ISO, 2017a), two operators repeated the analysis on five randomly selected positive samples and five randomly selected negative samples. The different operators analyzed the same ten selected samples.

Results

When *V. vulnificus* and *V. cholerae* were experimentally inoculated in bivalve mollusk samples to verify the application of the molecular method, the pathogens were always detected, both when the lowest ($1.5x10^3$ CFU/g) and the highest ($1.5×10^5$ CFU/g) concentrations were used (Table 1), and they were never detected in

Table 1. Comparison between real-time polymerase chain reaction and normalized microbiological isolation methods ISO 21872-1:2017 in the detection of *Vibrio cholerae, Vibrio parahaemolyticus,* and *Vibrio vulnificus* in mollusks experimentally contaminated with two bacterial levels.

Samples	*Vibrio* multiplex screening assay						ISO 21872-1:2017					
	Vibrio cholerae		*Vibrio parahaemolyticus*		*Vibrio vulnificus*		*Vibrio cholerae*		*Vibrio parahaemolyticus*		*Vibrio vulnificus*	
	LL	HL	LL	HL	LL	HL	LL	HL	LL	HL	LL	HL
1	+	+	N.D.	+	+	+	+	+	+	+	+	+
2	+	+	N.D.	+	+	+	+	+	+	+	+	+
3	+	+	N.D.	+	+	+	+	+	+	+	+	+
4	+	+	N.D.	+	+	+	+	+	+	+	+	+
5	+	+	N.D.	+	+	+	+	+	+	+	+	+
6	+	+	N.D.	N.D.	+	+	+	+	+	+	+	+
7	+	+	N.D.	+	+	+	+	+	+	+	+	+
8	+	+	N.D.	+	+	+	+	+	+	+	+	+
9	+	+	N.D.	+	+	+	+	+	+	+	+	+
10	+	+	N.D.	+	+	+	+	+	+	+	+	+
11	+	+	N.D.	+	+	+	+	+	+	+	+	+
12	+	+	N.D.	+	+	+	+	+	+	+	+	+
13	+	+	N.D.	+	+	+	+	+	+	+	+	+
14	+	+	N.D.	+	+	+	+	+	+	+	+	+
15	+	+	N.D.	+	+	+	+	+	+	+	+	+

LL, low level ($1.5×10^3$ CFU/g); HL, high level ($1.5×10^5$ CFU/g); N.D., not available.

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negative samples. *V. parahaemolyticus* was never detected in negative samples and at the lowest level, and it was detected in 14 samples (93.33%) seeded with the highest concentration (Table 1).

The cycle threshold ranged from 25.57 to 38.46 for *V. vulnificus,* from 20.25 to 27.43 for *V. cholerae,* and from 27.55 to 31.56 for *V. parahaemolyticus.*

The molecular method evaluated showed a concordance rate of 100% between the different operators and 100% with the reference traditional microbiological method (ISO 21872-1:2017) (ISO, 2017a). In particular, the two operators involved in the experiment correctly detected *Vibrio* species in positive samples and not in negative ones.

The LOD_{50} was 7.67 CFU/g for *V. cholerae* (reference range: 6.26-20.56 ufc/25 gr), 0.024 CFU/g for *V. vulnificus* (reference range: 8.46-17.91 ufc/25 gr), and 1.36 CFU/g for *V. parahaemolyticus* (reference range: 0.32-0.57 ufc/25 gr). Moreover, *Salmonella* and *E. coli* that were experimentally inoculated were never detected.

Discussion

Within *Vibrio* spp., *V. cholerae*, *V. vulnificus*, and *V. parahaemolyticus* are considered the most clinically relevant human pathogens (Ma *et al*., 2023). Human infection is generally associated with shellfish consumption, which is generally consumed live/raw or lightly cooked (Savini *et al*., 2021). Indeed, heating is an effective measure to reduce pathogen infection, and therefore, the greater risk of human infection is related to the consumption of raw or undercooked foods. In the latest year, the consumption of raw fish products has become more popular among consumers, and therefore the number of *Vibrio* infections is expected to increase (Ma *et al*., 2023).

In 2021, three strong-evidence foodborne outbreaks caused by *V. parahaemolyticus* and one caused by *V. cholerae* (nontoxigenic) were reported to the European Food Safety Authority (EFSA). In particular, the foodborne outbreak caused by *V. cholerae* was the first one since EFSA began collecting data on foodborne outbreaks (European Food Safety Authority and European Center for Disease Prevention and Control, 2022).

Vibrio spp. prevalence is significantly affected by detection methods. To date, the reference methods used to detect *Vibrio* rely mainly on culture-based techniques. Traditional microbiological methods involving the use of selective media were usually considered the gold standard methods for pathogen detection; however, over the past few years, to reduce the detection time, nucleic acid-based assays have been introduced in daily laboratory routines to detect pathogenic bacteria in different foods. For *Vibrio* detection, since June 2017, PCR has been introduced in ISO/TS 21872-1:2017 (ISO, 2017a) as an alternative to traditional methods because of their higher efficiency. However, the method included in ISO/TS 21872-1:2017 (ISO, 2017a), involving the use of three simplex qPCR and different reaction temperatures, still requires time for detection. In the present study, a molecular platform based on multiplex qPCR has been developed to determine the simultaneous presence of different *Vibrio* spp. in mollusks. Because of the presence of known PCR inhibitors in shellfish, to detect *Vibrio* spp., a non-competitive IAC was included in the qPCR assay to avoid false-negative results (Messelhäusser *et al*., 2010).

This qPCR system was based on the detection of the *tdh* and *trh* genes in *V. parahaemolyticus*, *vvhA* in *V. vulnificus,* and *rtx*A in *V. cholerae.* To our knowledge, studies on methods based on multiplex qPCR assays targeting *V. parahaemolyticus*, *V. vulnificus,* and *V. cholerae* are old and limited. In the study of Garrido-Maestu *et al*. (2014), for the simultaneous detection of *V. parahaemolyticus*, *V. vulnificus,* and *V. cholerae,* the *tlh*, *vvhA,* and *ompW* genes were used. Kim and Lee (2014) developed a multiplex qPCR assay for the detection of *V. anguillarum*, *V. parahaemolyticus,* and *V. vulnificus* in fish products. Therefore, although there are several possible targets for the detection of *V. parahaemolyticus*, *V. vulnificus,* and *V. cholerae*., the specific genes used in the present work (*tdh* and *trh* in *V. parahaemolyticus*, *vvhA* in *V. vulnificus*, and *rtxA* in *V. cholerae*) are highlighted in ISO 21872-1:2017 (ISO, 2017a) as essential markers for identifying pathogenic strains within these *Vibrio* species. To our knowledge, the present work describes the first qPCR assay for the simultaneous detection of *tdh, trh, vvhA,* and *rtxA* genes in combination with an internal amplification control in mollusks.

According to ISO/TS 21872-1:2017 (ISO 2017a), for the detection of *V. cholerae,* a traditional PCR (endpoint PCR) must be performed. The qPCR used in the present work could be a promising alternative to endpoint PCR. Even though qPCR technology has been principally developed for the quantification of bacteria, its use in qualitative analysis, as performed in the present work, should not be underestimated. Compared with endpoint PCR, qPCR is more precise and quicker, as it does not require the preparation of gels, and it is safer as ethidium bromide is not needed (Ahmed *et al.*, 2009).

Concerning the LOD_{50}, results are in contrast with other methods previously developed for the detection of single or multiple vibrios. A higher detection limit was determined for *V. parahaemolyticus* by Garrido *et al.* (2012) and Garrido-Maestu *et al.* (2014), and for *V. vulnificus* in the study of Han and Ge (2010) and Garrido-Maestu *et al.* (2014). In particular, in the study of Garrido *et al*. (2012), the detection limit determined for *V. parahaemolyticus* in boiled frozen mussels was 6 CFU/25 g for *tdh*, 11 CFU/25 g for *trh1,* and 8 CFU/25 g for *trh2.* In the study of Han and Ge (2010), the detection limit determined for *V. vulnificus* in spiked oysters without any enrichment was 10^4 CFU/g with RT-LAMP and 10^7 CFU/g by PCR, and in the study of Garrido-Maestu *et al.* (2014), it was 3 CFU/25 g for *V. vulnificus*, 7 CFU/25 g for *V. parahaemolyticus,* and 4 CFU/25 g for *V. cholerae*. Differences may arise from factors such as PCR inhibitors, limitations in primer design, or interference from closely related organisms that can affect sensitivity (Bustin *et al*., 2020). Moreover, the qPCR protocol demonstrated that it could amplify only *V. vulnificus, V. cholerae,* and *V. parahaemolyticus* and not *Salmonella* and *E. coli* experimentally inoculated in the samples.

Conclusions

Globally, *V. parahaemolyticus*, *V. cholerae,* and *V. vulnificus* species represent the most important bacterial pathogens associated with seafood consumption. In several countries, food safety criteria or guidelines have been introduced to limit the risks of exposure of seafood consumers to *Vibrio* spp. For this reason, it is essential to develop methods that are rapid and highly specific for the detection of these food pathogens. In the present study, a rapid method based on the use of multiplex qPCR to detect *V. parahaemolyticus*, including potentially enteropathogenic strains, *V. vulnificus* , and *V. cholerae* in mollusk bivalves has been developed. The overall procedure takes approximately 24 hours, including the enrichment culture period, yielding a method that is faster,

simpler, and less costly than conventional and other molecular methods. Multiplex qPCR proved to be reliable for *V. vulnificus* and *V. cholerae.* Results for *V. parahaemolyticus* are promising, but further analysis is needed. The proposed method could represent a quick monitoring tool and, if used, would allow the implementation of food safety. However, expanding the research scope to encompass a broader range of data is crucial to validating these findings. This would involve conducting experiments on a larger set of bivalve mollusks, including experimentally contaminated and naturally contaminated samples. Additionally, exploring various matrices would be necessary to ensure comprehensive and robust confirmation of the data obtained. This broader approach will strengthen the validity and reliability of the research outcomes.

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