A multiplex PCR for the detection of *Vibrio vulnificus* hazardous to human and/or animal health from seafood

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ARTICLE INFO

ABSTRACT

Vibrio vulnificus is a zoonotic pathogen linked to aquaculture that is spreading due to climate change. The pathogen can be transmitted to humans and animals by ingestion of raw shellfish or seafood feed, respectively. The aim of this work was to design and test a new procedure to detect *V. vulnificus* hazardous to human and/or animal health in food/feed samples. For this purpose, we combined a pre-enrichment step with multiplex PCR using primers for the species and for human and animal virulence markers. *In vitro* assays with mixed DNA from different *Vibrio species* and *Vibrio* cultures showed that the new protocol was 100 % specific with a detection limit of 10 cfu/mL. The protocol was successfully validated in seafood using artificially contaminated live shrimp and proved useful also in pathogen isolation from animals and their ecosystem. In conclusion, this novel protocol could be applied in health risk studies associated with food/feed consumption, as well as in the routine identification and subtyping of *V. vulnificus* from environmental or clinical samples.

1. Introduction

Vibrio vulnificus is a multi-host pathogen native to brackish waters located in temperate, tropical and subtropical areas that, in recent years, is spreading to traditionally cold areas due to global warming (Amaro et al., 2020; Baker-Austin et al., 2012; Baker-Austin et al., 2017). The pathogen causes disease (hereafter referred to as vibriosis) in multiple species of aquatic animals (mainly farmed fish) and humans by using two transmission routes; contact or ingestion (Ceccarelli et al., 2019). The most severe form of these vibrioses is a hemorrhagic septicemia with a high probability of death under risk conditions (elevated blood iron levels in humans; water salinity of 1–1.5 % plus temperatures above 25 °C in fish) (Amaro et al., 2015; Ceccarelli et al., 2019). The main natural reservoirs of this pathogen are seawater, farmed fish, farmed shrimps and filter-feeding organisms (mainly oysters) (Amaro et al., 2020), from which the pathogen can infect susceptible hosts. Remarkably, cases of diseased animal-to-human transmission (zoonosis) of the vibriosis have also been reported, all of them linked to fish farms (Dalsgaard et al., 1996; Veenstra et al., 1992).

In humans, *V. vulnificus* is mostly recognized as a foodborne pathogen since it is one of the leading causes of seafood-related mortality (Ceccarelli et al., 2019). Notably, primary septicemia associated with ingestion of *V. vulnificus* by susceptible hosts (mainly those with high

iron levels in blood due to underlying diseases and immunocompromised) carries the highest mortality rate of all foodborne pathogens that have been studied (Ceccarelli et al., 2019; Rippey, 1994), probably due to the short incubation period between the onset of clinical signs and the subsequent clinical outcome (Baker-Austin et al., 2009; Baker-Austin and Oliver, 2018), which usually occurs within 24 h of exposure (Jones and Oliver, 2009). The same is true for fish septicemia, as death of the animals can occur in less than 24 h in fish farms (Amaro et al., 2020). Therefore, considering the rapidity with which animal and human septicemia leads to host death, there is a need for rapid detection methods for the presence of the species in seafood for human or animal (feed derived from seafood) consumption.

V. vulnificus is a genetically variable species that includes virulent and avirulent strains for a wide range of hosts. The species is subdivided into 5 phylogenetic lineages, all of which are potentially virulent to humans, plus a fish virulent pathovar (pv. *piscis*) due to a transmissible virulence plasmid (Lee et al., 2008). The plasmid encodes a fish transferrin receptor (Frbp, fish transferrin-binding protein) and a lipoprotein that protects the bacterium from fish complement and phagocytosis (Fpcrp, fish phagocytosis and complement resistance lipoprotein) (Hernández-Cabanyero et al., 2019; Pajuelo et al., 2015 and unpublished results). Both genes have been transferred to several strains of *V. harveyi* isolated from diseased fish (Carmona-Salido et al., 2021 and

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unpublished results). Therefore, a PCR-based method for rapid detection of fish virulent *V. vulnificus* strains combining one species marker (*vvhA*, a gene that encodes a hemolysin present in all strains of the species) with one plasmid marker was developed and tested in fish farms (Sanjuán and Amaro, 2007). In the case of humans, it appears that no single gene involved in human vibriosis is absent in nonvirulent strains, probably because human vibrioses are multifactorial processes that depend on the presence of an appropriate combination of virulence genes (Baker-Austin and Oliver, 2018). Therefore, some "human virulence" markers have been selected by comparing genomes or fragments of genomes from clinical and environmental strains. Among the "human virulence" genetic markers found, we selected a polymorphism in the *pilF* gene that correlates with the human clinical origin of the isolate and, consequently, with human health hazard with a probability of around 80 % (Dickerson et al., 2021; Roig et al., 2010).

The aim of the present work was to develop a multiplex PCR that discriminates whether a seafood or feed sample contains *V. vulnificus* hazardous to human or animal health, respectively. For this purpose, we combined primers for *fpcrp* (fish health hazard marker) and *pilF* (human health hazard marker) with primers for *vvhA* (a species marker encoding a hemolysin) (Hernández-Cabanyero et al., 2019; Hor et al., 1995; Roig et al., 2010). The specificity and sensitivity of this multiplex PCR was tested with pure cultures of *V. vulnificus* strains representative of the genetic diversity of the species, as well as with mixed samples of *V. vulnificus* strains and other vibrios naturally present in seafood. The initial detection limit proved to be high (around 10^5 cfu/mL), so a previous enrichment (pre-enrichment) step was added to reduce it to 10 cfu/mL. To validate the whole protocol, we used one of the animal reservoirs of the pathogen (farmed shrimps) experimentally contaminated with *V. vulnificus* and with water samples from the experimental tanks. We were successful and were able to detect and even isolate *V. vulnificus* from all infected samples and the surrounding water. Consequently, this two-step protocol could be implemented in health risk studies associated with seafood and feed consumption, as well as in the routine identification and subtyping of *V. vulnificus* derived from environmental or clinical samples.

2. Materials and methods

2.1. Bacterial strains and growth conditions

All strains used in this study as well as their main characteristics are shown in Table 1. The strains of *V. vulnificus* were selected as representative of the five phylogenetic lineages described in the species (Roig et al., 2018) and the other *Vibrio* species were selected as representative of the vibrios that share the same habitats with *V. vulnificus* (Khamesipour et al., 2014; Mahbub et al., 2011; Noriega-Orozco et al., 2007).

The strains were maintained in lyophilized stocks at −80 °C in LB-1 (Luria-Bertani 1 % NaCl) plus 20 % (vol/vol) glycerol. Bacteria were routinely grown in LB-1 or on TSA-1 (Tryptone Soy Agar 1 % NaCl) plates for 18–24 h at 28 °C. To isolate *V. vulnificus* from different samples, the selective and differential VVM agar (Cerdà-Cuéllar et al., 2000) was used.

2.2. Bacterial DNA extraction from cultures

Pure cultures from the strains listed in Table 1 were obtained in LB-1 after 18 h of incubation at 28 °C. Volumes of 1 mL of each culture were centrifuged at 12,000 rpm for 4 min at 4 °C. Bacterial pellets were resuspended in 100 μL of PBS (Phosphate Buffered Saline 1 %, pH 7), boiled at 100 °C for 5 min, kept on ice for 5–10 min and, then, centrifuged again at 12,000 rpm for 4 min at 4 °C (Gyrozen 1730R, Controltecnica, Spain). DNA present in the supernatant was stored at −20 °C and used as a PCR template. The concentration and quality of the DNA per sample was analyzed using NanoDrop ND1000 (Thermo Fisher Scientific, Spain) and Qubit (Thermo Fisher Scientific, Spain). Pooled DNA samples were prepared by mixing 0.25 μg/mL of DNA samples from *V. vulnificus*, *V. harveyi*, *V. cholerae* and *V. parahaemolyticus*.

2.3. Design of a new protocol for V. vulnificus detection

2.3.1. Multiplex PCR

In order to develop a new multiplex PCR method, we looked for a

Table 1
Characteristics of the *Vibrio* strains used in the study.

Strain[a]	Isolation data				Subtyping[c]		
	Source	Geographic location/year of isolation	Phylogenetic lineage-clade[b]		pv. *piscis*	*pilF* PCR	*Fpcrp* PCR
V. vulnificus							
CECT 4999	Diseased eel	Spain, 1999	L2-clade E		+	+	+
YJ016	Human blood	Taiwan, 1993	L1		−	+	−
A14	Diseased eel	Spain, 2002	L2-clade A		+	−	+
12	Health tilapia	Israel, 2002	L3		+	+	+
VVyb1	Healthy Tilapia	Israel, 2004	L3		+	+	+
V252	Human blood	Israel, 2004	L5		+	−	+
Riu1	Seawater	Spain, 2003	L4		−	+	−
V. cholerae							
8A (environmental isolate)	Diseased tilapia	Spain, 2019	NA		NA	−	−
V. harveyi							
CECT 8408	Diseased shrimp	Mexico, 2005	NA		NA	−	−
A2[d]	Diseased seabass	Spain, 2015	NA		NA	−	+
CECT 5156	Diseased seabass	Spain. 1998	NA		NA	−	−
V. parahaemolyticus							
CECT 8407	Diseased shrimp	Mexico, 1995	NA		NA	−	−
TW-2[e]	Diseased shrimp	Taiwan, unknown	NA		NA	−	−

NA, not applicable.

[a] CECT, Spanish Type Culture Collection.

[b] Phylogenetic lineage (L) and clade from Roig et al. (2018) and Carmona-Salido et al. (2021), respectively.

[c] Pv. *piscis* identification as well as *pilF* and *fpcrp* PCR results from Carmona-Salido et al. (2021).

[d] Strain that contains *ftbp* and *fpcrp* in a plasmid (Fouz et al., unpublished results)

[e] Strain supplied by Dr. Lien-I Hor (National Cheng-Kung University, Tainan, ROC).

proper combination of primers relevant in identification and subtyping of *V. vulnificus* to be used in the same PCR. The selected genes and the designed primers appear in Table 2. Primers were synthesized by Integrated DNA Technology (IDT, Belgium) and were used at a stock concentration of 10 μM. PCR reactions were performed using a MiniAmp Thermal Cycler (Thermo Fisher Scientific). In the first design we followed the recommended conditions for the polymerase of the commercial Master Mix (Speedy Supreme NZYTaq 2× green from NZYtech). Thus, samples for PCR contained 12.5 μL PCR Master Mix, 6–12 μL primers (1–2 μL of each primer), 1 μL DNA and 5.5 μL of water HyPure Molecular Biology Grade Water (Cytiva) to a final volume of 25 μL. The PCR conditions were 55 °C of annealing temperature in 30 cycles.

To adapt the multiplex PCR to natural samples, mixtures of *V. vulnificus* DNA with DNA from several *Vibrio* species were used and PCR parameters were varied (primer volumes; 1 to 2 μL: annealing temperatures; 55 to 67 °C: number of cycles; 25 to 30: and $MgCl_2$ content; 0 to 5.5 μL of $MgCl_2$ 1 mM) until only bands corresponding to the three *V. vulnificus* genes were visualized. A negative control (no template DNA) and a positive control (purified DNA from *V. vulnificus* strain CECT 4999) (Table 1) was used in each assay. A volume of 5 μL of the PCR reaction were loaded in an agarose gel (1.8 % agarose) containing RedSafe Nucleic Acid Staining Solution (Intron Biotechnology) (5 μL/ 100 mL). The ladder Thermo Scientific Gene Ruler 100 bp (Plus DNA 00998333) was used as reference for molecular weights. The amplified products were visualized and photographed under UV light. To calculate the detection limit as cfu/mL, overnight pure cultures were 10-fold diluted and sampled for DNA extraction as described before. In parallel, number of cells of each dilution was estimated by drop plate count on TSA-1 agar (Hoben and Somasegaran, 1982). The detection limit was calculated from the lowest dilution giving a clear positive result for *V. vulnificus* detection with the new multiplex PCR. The best conditions in terms of *V. vulnificus* detection were selected and tested in the following experiments.

2.3.2. Enrichment

The effect of a previous enrichment step in alkaline peptone water (APW; 0.5 % NaCl, pH 8.6) on the detection limit was studied. To this end, samples were incubated in APW (ratio 1:10) at 28 °C for 4, 6 or 8 h. After this amplification step, DNA was extracted and analyzed for *V. vulnificus* detection using the new multiplex PCR. The combination that gave the best results in terms of detection limit and incubation time was selected for the whole protocol.

2.4. Validation of the whole protocol

The new protocol was validated *in vivo* with whiteleg shrimps (*Penaeus vannamei*) (between 1.5 and 1.8 g) provided by the company WhitePanther (Republic of Austria). Shrimps were maintained in the Fish facilities (code ES461900001203) of the SCSIE (University of Valencia; UV) in tanks of 200 L containing 150 L of saline water (30–34 ppt NaCl, pH 8) at 26 °C. A group of 18 shrimps was infected by immersion in a bath containing 3×10^7 cfu/mL of the strain CECT 4999 for 1 h (infected group) and another group of 7 individuals was kept as negative control (control group). Animals were necropsied at 0 (infected and control), 24 (infected and control) and 48 h (infected) post

infection. Hepatopancreas and exoskeleton from each sampled exemplar were homogenized separately in APW 0.5 % (1:5 wt/vol) and samples of water were taken from tanks containing infected shrimps at 48 h post infection. All samples from animals and tank water were subjected to the optimized whole protocol to detect *V. vulnificus*. The extraction of DNA was done following the protocol described for bacterial DNA extraction from cultures but using samples of homogenized tissue. In parallel, each sample was microbiologically analyzed by seeding directly on TSA-1 and VVM plates. The colonies recovered were identified by API20E system (Biomerieux, Spain).

3. Results

3.1. Design and optimization of the new multiplex PCR

The specificity of the new multiplex PCR was tested using *V. vulnificus* strains representative of the five lineages of the species together with strains of other *Vibrio* species that can be co-isolated with *V. vulnificus* from the same marine habitats (Table 1). The expected amplicons were obtained with all *V. vulnificus* DNA samples (Fig. 1A). Thus, the samples from the pv. *piscis* strains that belonged to the zoonotic groups (CECT 4999, 12 and VVyb1) were positive for *vvhA*, *fpcrp* and *pilF* while those from strains not hazardous to human health (A14 and V252) were only positive for *vvhA* and *fpcrp* (Fig. 1A). Likewise, the samples from the strains that did not belong to pv. *piscis* but were hazardous for human health were positive for *vvha* and *pilF* (YJ016 and Riu1) (Fig. 1A). The detection limit of the new multiplex PCR was around 10^3 cfu/mL (Supplementary Fig. 1A).

No amplicon corresponding to species (*vvhA*) and health hazard markers (*fpcrp* and *pilF*) was obtained from DNA samples from the strains belonging to the other *Vibrio* species (*V. cholerae*, *V. harveyi* and *V. parahaemolyticus*) with the exception of the DNA from *V. harveyi* A2 strain, which was positive for *fpcrp* gene (Carmona-Salido et al., 2021) (Fig. 1B, Table 1). This strain harbors a plasmid that contains the fish virulence gene *fpcrp* (Carmona-Salido et al., 2021; Hernández-Cabanyero et al., 2019; Pajuelo et al., 2015 and unpublished results). Unexpectedly, multiple bands corresponding to non-specific amplifications were observed in the gels when mixtures of DNA from the selected *Vibrio* spp. were analyzed (Fig. 2A and C). Therefore, the protocol was optimized to eliminate this non-specific amplification by making conditions more stringent. The final selected conditions were: initial denaturation; 94 °C for 5 min, denaturation cycling; 27 cycles at 94 °C for 2 s, primer annealing at 65 °C for 5 s: and a first extension at 72 °C for 5 s followed by a final extension at 72 °C for 10 min. The final PCR reaction used:

[12.5 μL of 2xPCR master mix, 6 μL of primers, 1 μL of template DNAs, 5.5 μL of 1 mM $MgCl_2$].

With the new multiplex PCR, all the mixed samples gave the expected amplification products without false positive or non-specific amplification (Fig. 2B and D).

Subsequently, the minimal quantity of bacterial cells necessary for reliable detection was determined. The three amplicons were observed in samples containing at least 1.3×10^5 cfu/mL (Fig. 3A), a number too high for routine detection of pathogenic vibrio in seafood. Thus, to reduce the limit of detection, a previous step of pre-enrichment in APW was added (Fig. 3B and C). The detection limit after 4, 6 and 8 h of pre-

Table 2
Primers, expected amplicon size, annealing temperature and reference.

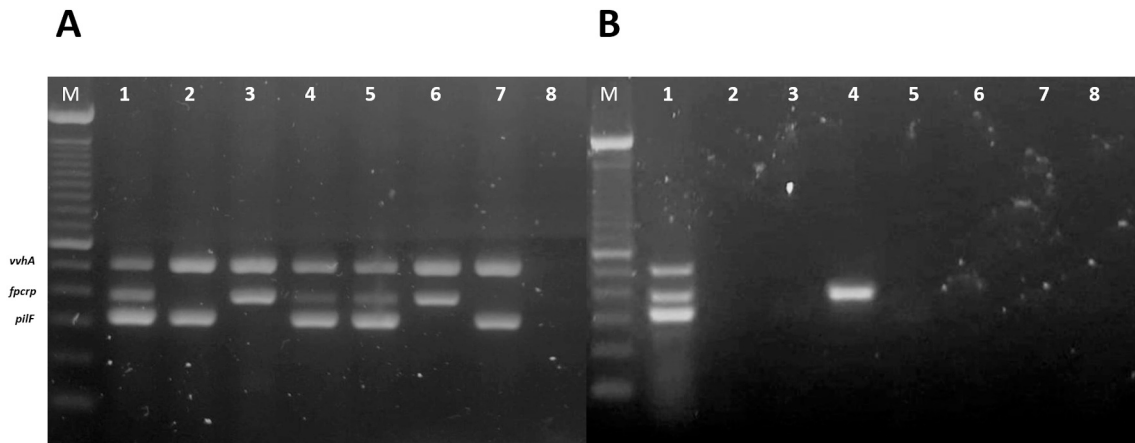| Primer	Sequence (5′ → 3′)	Amplicon size (bp)	Annealing Tª (°C)	Reference
vvha-F	CGGCACCCACTTTCGGGCC	519	63	Sanjuán and Amaro, 2004
vvha-R	CCGCGGTACAGGTTGGCGC			
fpcrp-F	AGTTTGCGGAAAAAGCACAG	392	54	Carmona-Salido et al., 2021
fpcrp-R	CATTGATCGTCGTCTGAACC			
pilF-F	TGTCGGTGAAAACGGCAAAGCTG	338	47	Roig et al., 2010
pilF-R	GGTATCGATTTCCAACTTAGCGAG GTTGAGCACC			

Fig. 1. Specificity of the new multiplex PCR. Agarose gel electrophoresis of the multiplex PCR products obtained from pure DNA samples (approx. 2.5×10^{-4} μg/well) of *V. vulnificus* strains (A) and *Vibrio* spp. strains (B). A) DNA from *V. vulnificus* CECT 4999 (1), YJ016 (2), A14 (3), 12 (4), Vvyb1 (5), V252 (6) and Riu1 (7) and negative control (Hypure molecular biology grade water from Cytiva, Spain) (8). B) DNA from *V. vulnificus* strain CECT 4999 (1), *V. cholerae* 8A (2), *V. harveyi* CECT 8408 (3), *V. harveyi* A2 (4), *V. harveyi* CECT 5156 (5), *V. parahaemolyticus* CECT 8407 (6), *V. parahaemolyticus* TW-2 (7) and negative control (Hypure molecular biology grade water from Cytiva, Spain). Lane M: molecular weight DNA ladder (Thermo Scientific).
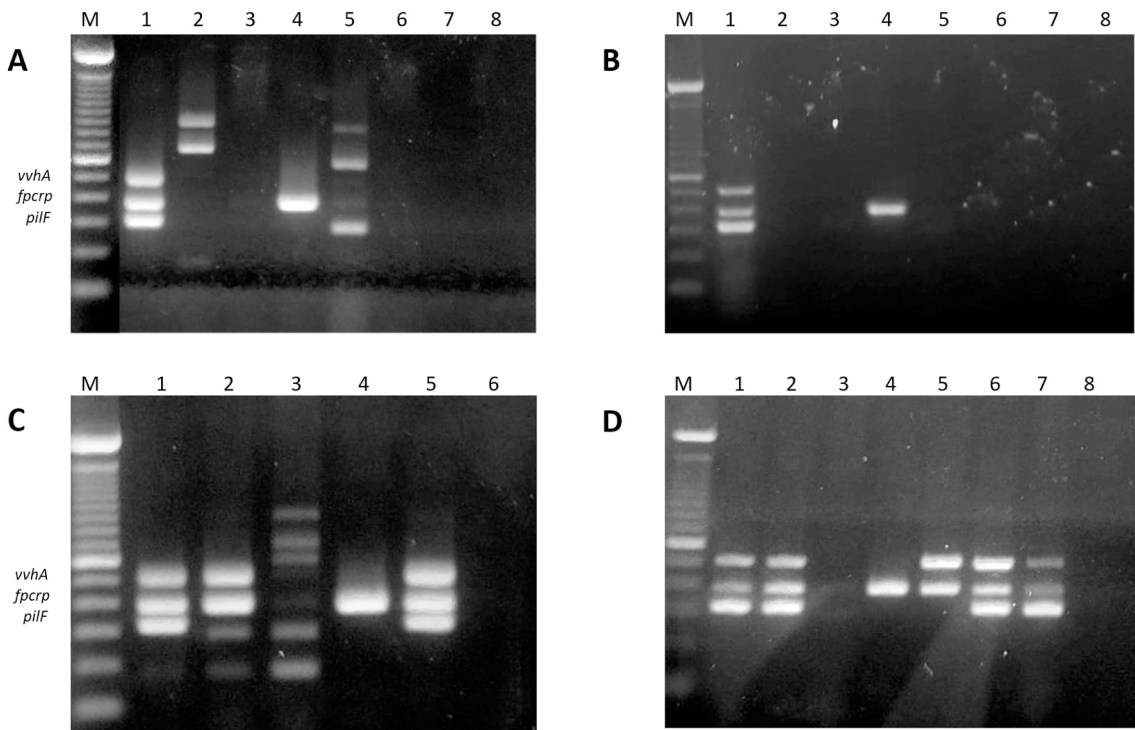

Fig. 2. Optimization of the new multiplex PCR. Agarose gel electrophoresis of the multiplex PCR products obtained from DNA samples (approx. 2.5×10^{-4} μg/well) before (A, C) and after (B, D) optimization. Line 1: A and B) DNA from *V. vulnificus* CECT 4999 (1), *V. cholerae* 8A (2), *V. harveyi* CECT 8408 (3), *V. harveyi* A2 (4), *V. harveyi* CECT 5156 (5), *V. parahaemolyticus* CECT 8407 (6), *V. parahaemolyticus* TW-2 (7) and negative control (hypure molecular biology grade water from Cytiva, Spain) (8). C) Mixture of DNA from *V. vulnificus* CECT 4999, *V. cholerae* 8A, *V. harveyi* CECT 8408 and *V. parahaemolyticus* TW-2 (1), *V. vulnificus* CECT 4999 and *V. cholerae* 8A (2), *V. harveyi* CECT 8408 and CECT 5156 (3), *V. cholerae* 8A, *V. harveyi* A2 and *V. parahaemolyticus* CECT 8407 (4), *V. vulnificus* A14 and *V. parahaemolyticus* TW-2 (5) and negative control (hypure molecular biology grade water from Cytiva, Spain) (6). D) Mixture of DNA from *V. vulnificus* CECT 4999, *V. cholerae* 8A, *V. harveyi* CECT 8408 and *V. parahaemolyticus* TW-2 (1), *V. vulnificus* CECT 4999 and *V. cholerae* 8A (2), *V. harveyi* CECT 8408 and CECT 5156 (3), *V. cholerae* 8A, *V. harveyi* A2 and *V. parahaemolyticus* CECT 8407 (4), *V. vulnificus* A14 and *V. parahaemolyticus* TW-2 (5), *V. vulnificus* A14, *V. vulnificus* Vvyb1 and *V. vulnificus* V252 (6), *V. vulnificus* CECT 4999, *V. cholerae* 8A, *V. harveyi* CECT 8408, *V. harveyi* A2 and *V. harveyi* CECT 5156, *V. parahaemolyticus* CECT 8407 and *V. parahaemolyticus* TW-2 (7) and negative control (hypure molecular biology grade water from Cytiva, Spain) (8). Lane M: molecular weight DNA ladder (Thermo Scientific).

enrichment was 1×10^4, 1×10^2 and 1.3×10^1 cfu/mL, respectively (Fig. 3B–C and data not shown). From these results, a 6 h of pre-enrichment was chosen for the *in vivo* validation.

3.2. Validation of the whole protocol

Artificially cultured shrimps are one of the main reservoirs for *V. vulnificus* (Amaro et al., 2020). Therefore, the accuracy of the two-step
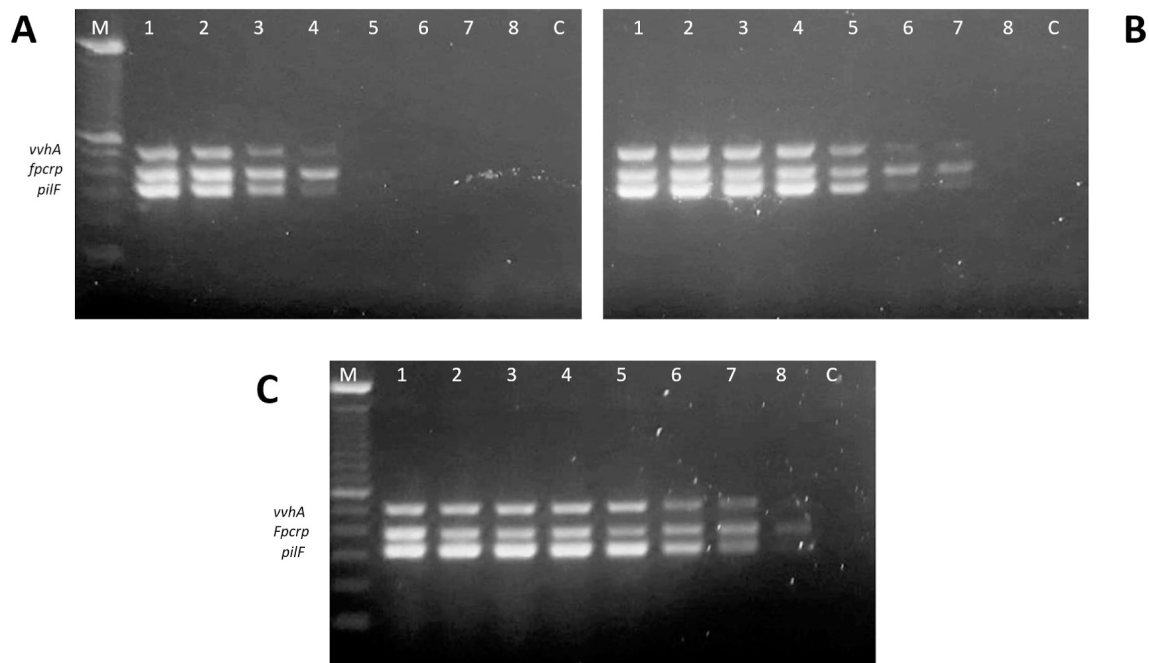
Fig. 3. Detection limits without and with pre-enrichment. Agarose gel electrophoresis of the multiplex PCR products obtained from DNA extracted from serial 10-fold dilutions of an overnight culture of the strain CECT 4999 without (A) and with a pre-enrichment in APW 0.5 % NaCl for 6 (B) or 8 h (C). Lane M: molecular weight DNA ladder (Thermo Scientific). Lane 1 (1.3×10^8 cfu/mL); Lane 2 (1.3×10^7 cfu/mL); Lane 3 (1.3×10^6 cfu/mL); Lane 4 (1.3×10^5 cfu/mL); Lane 5 (1.3×10^4 cfu/mL); Lane 6 (1.3×10^3 cfu/mL); Lane 7 (130 cfu/mL); Lane 8 (13 cfu/mL). Line C (hypure molecular biology grade water from Cytiva, Spain).

PCR protocol was validated in non-contaminated and artificially contaminated shrimps with *V. vulnificus*. First we analyzed the microbiological content of non-contaminated shrimps. No colony grew on VVM agar after enrichment in APW while several colony types were recovered on TSA-1 plates. These colony types were identified after purification as *V. fluvialis* or *V. fluvialis*-like species using the phenotypic API20E system (profiles 2042124 and 3042025, with a probability of identification 98,6–98,9 %, and 3044125 with a probability of identification 57.5 %). Secondly, we analyzed non-contaminated and contaminated shrimp samples by using the new two-step protocol. All samples taken from contaminated shrimps were positive at 0, 24 and 48 h post-challenge (Fig. 4). Further, even samples taken at time 0 were positive without the enrichment step (Fig. 4). As expected, no amplification products were obtained from non-contaminated shrimps as well as tank water from all the groups, even after 6 h of pre-enrichment in APW (Fig. 4 and data not shown). Microbiological analyses supported these results. Thus, *V. vulnificus* was recovered on VVM plates from all the contaminated-shrimp samples at 0, 24 and 48 post-challenge while no *V. vulnificus* colony grew on the selective medium VVM from any sample from non-contaminated shrimps. This last result was quite interesting because it showed that *V. vulnificus* could be easily isolated by using a pre-enrichment of 6 h followed by isolation on VVM agar.

4. Discussion

Vibrios are native inhabitants of aquatic ecosystems where they coexist with different animal species establishing from symbiotic to pathogenic relationships, depending on the species involved and the specific genetic content of the strain (Ceccarelli and Colwell, 2014). In particular, *V. vulnificus* is capable of causing diseases in different species of aquatic animals as well as humans, and, in all cases, both by contact and ingestion (Amaro et al., 2020; Ceccarelli et al., 2019). This bacterial species has reached fish farms, which have accelerated its evolution, favoring the emergence of two new phylogenetic groups and several clades and subgroups belonging to previously established lineages (Carmona-Salido et al., 2021). Further, it is a pathogen that can kill its

human and animal hosts very quickly (Jones and Oliver, 2009), which, together with its geographical expansion to new habitats due to global warming (Amaro et al., 2020; Baker-Austin et al., 2012 and Hernández-Cabanyero and Amaro, 2020), makes necessary to develop new detection methods and control measures to ensure its absence in animals of marine origin destined to human or animal (feed containing processed seafood) consumption.

The main objective of the present work was to design and test a protocol to accurately detect food and feed contamination with *V. vulnificus* dangerous for animal and/or human health. To achieve this goal, we first developed a new multiplex PCR based on previous knowledge about the genes or gene fragments that are related to virulence for animals and humans. Therefore, we selected a widely used species marker, *vvhA* (Wright and Morris, 1991) together with two virulence-related gene markers, *fpcrp*, a gene that confers resistance to complement and phagocytosis in fish blood (Hernández-Cabanyero et al., 2019 and unpublished results), and a *pilF*-polymorphism positively correlated with the clinical (human) or environmental origin of the isolate (Roig et al., 2010) The new multiplex PCR showed 100 % specificity and a detection limit of 10^3 cfu/mL when working with DNA samples from pure cultures. However, with samples containing DNA from various *Vibrio* species to simulate natural seafood microbiota, nonspecific amplification products were obtained. Consequently, the PCR conditions were changed to be more stringent, which eliminated the unspecific amplification but significantly increased the detection limit (from 10^3 to 10^5 cfu/mL). To solve this problem, we added a pre-enrichment step that would favor the growth of *V. vulnificus* over other competing *Vibrio* species. Since *V. vulnificus* is a marine bacterium capable of growing at 0.5 % NaCl, we selected APW-0.5 % NaCl as the enrichment medium. In this medium other marine *Vibrio* species grow more slowly than *V. vulnificus* (Ceccarelli and Colwell, 2014). In addition, we analyzed microbiologically the samples, using a selective and differential medium for the recovery of *V. vulnificus* that had previously been shown to be highly effective in the recovery of any strain of the species regardless the phylogenetic group (Ceccarelli and Colwell, 2014). This strategy allowed us to reduce the detection limit of
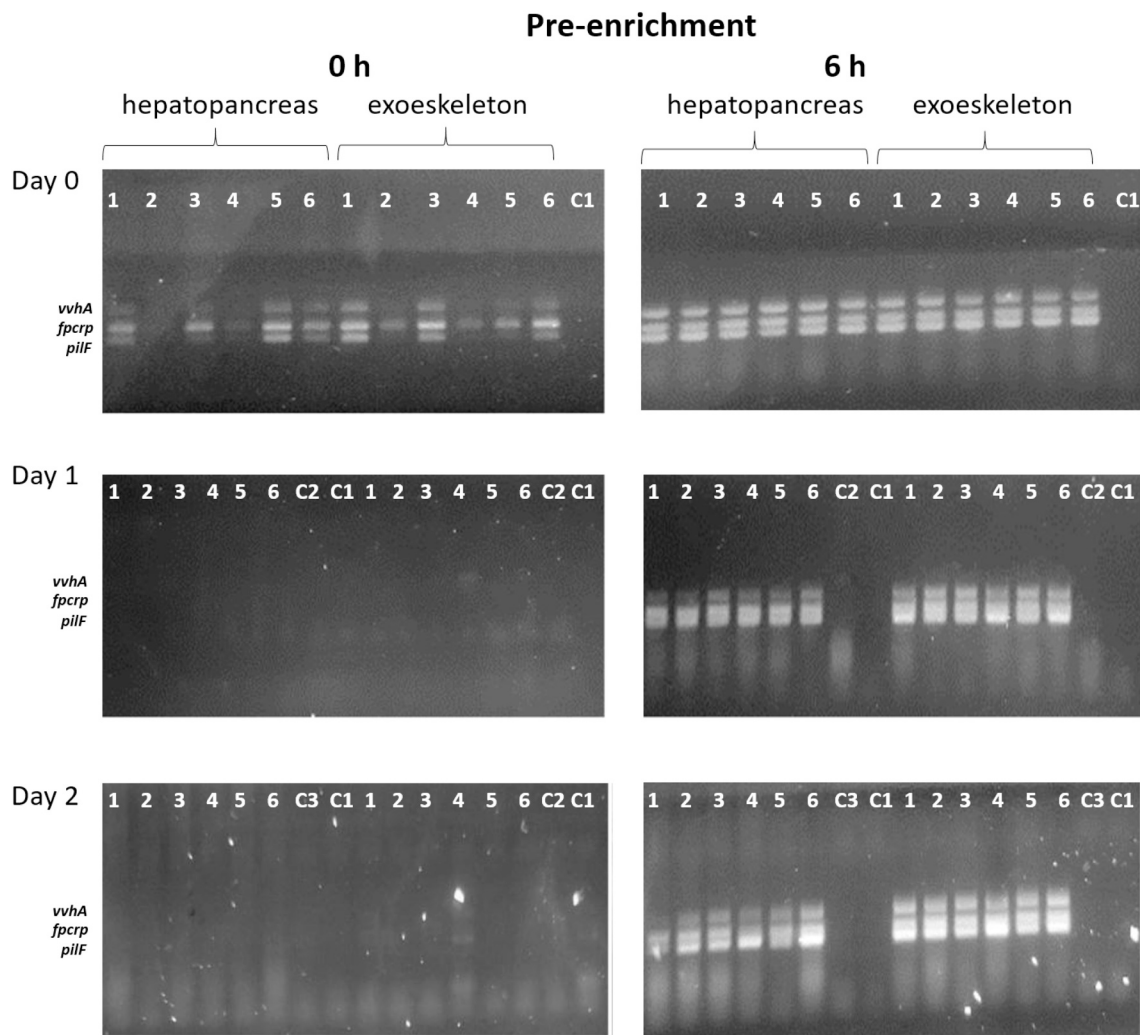
Fig. 4. Validation of the whole protocol with artificially infected shrimps. Agarose gel electrophoresis of the multiplex PCR products obtained with DNA extracted from hepatopancreas and exoeskeleton of shrimps (*Penaeus vannamei*) contaminated with *V. vulnificus* CECT 4999 strain by immersion. Samples were taken immediately after challenge (Day 0), and at 24 (Day 1) and 48 h (Day 2) post-challenge. Lanes with numbers correspond to different samples of shrimps, lane C1 to the negative control of the PCR (hypure molecular biology grade water from Cytiva, Spain), lane C2 to a negative control of a non-infected shrimp and lane C3 to a negative control of tank water.

V. vulnificus to only 10 cfu/mL after a pre-enrichment in APW for 8 h.

To validate the whole protocol, the procedure was tested in shrimp samples with very good results, even though *V. fluvialis*-like bacteria were part of the natural microbiota of non-contaminated animals (these vibrios are one of the main groups of the genus in shrimp microbiota,

Noriega-Orozco et al., 2007). Further, we were also able to recover *V. vulnificus* from all the samples that were positive in the PCR-detection, showing that the microbiological protocol was very efficient in the recovery of this species from seafood. Remarkably, 6 h of enrichment were proven to be enough for the identification and recovery of *V. vulnificus* in

Table 3

Comparison of different PCR methods for the detection of *V. vulnificus.*[a]

Method	Limit of detection	Time	Genes	Detection level	Reference
Multiplex PCR	1.3×10^5 cfu/mL	1 h	*vvha, fpcrp, pilF*	Species, zoonotic potential, pathogenicity for humans potential	This work
Multiplex PCR and previous broth enrichment (8 h)	13 cfu/mL	9 h	*vvha, fpcrp, pilF*	Species, zoonotic potential, pathogenicity for humans potential	This work
Multiplex PCR	NA	1 h	*PRXII, nanA, 16S rRNA-type B allele, toxR, manIIA, vcg-type C allele*	Species, virulence factors	Bier et al., 2015
Real time PCR	120 cfu/g	2 h	*vvha*	Species	Wang and Levin, 2006
Real time PCR	1 cfu/mL	7 h	*vvha*	Species	Wang and Levin, 2006
Real time recombinase polymerase amplification	120 cfu/mL	3 h	*vvha*	Species	Zhu et al., 2021

[a] The time includes an estimation of the time for the DNA extraction, enrichment and PCR amplification.

living samples, with a 100 % of specificity. Consequently, the enrichment step combined with isolation on agar VVM could be useful in the isolation of *V. vulnificus* from carrier and diseased animals and even for diagnostic and epidemiological/epizootiological purposes.

Table 3 shows a comparison of our method with previous similar methods. Although previous multiplex PCR for *V. vulnificus* already existed (Bier et al., 2015), the present method is the only one that in a single PCR it can be determined whether a sample contains *V. vulnificus* hazardous to human health, animal health or even zoonotic. In addition, the combination of this multiplex PCR with a relatively short pre-enrichment step allows sensitivity levels similar to those achieved with real-time PCRs (Wang and Levin, 2006; Zhu et al., 2021), with the advantage that this method does not require the expensive equipment or materials (fluorophores) that are necessary for real-time PCR.

In addition, the results of this work also suggest that this two-step protocol combined with seeding on VVM agar could also be useful in the recovery of this species for further analysis in epidemiological and/or epizootiological studies.

5. Conclusions

We have developed a new highly specific multiplex PCR that, combined with a pre-enrichment step and isolation on VVM agar, allows the identification of *V. vulnificus* in seafood/feed and the simultaneous discrimination of samples hazardous to human and/or animal health at the same time as the recovery of the bacterium. The new multiplex PCR can be used with pure cultures to identify and subtype *V. vulnificus*, while the whole protocol can also be used in epidemiological studies as well as in fish farms to control vibriosis in fish and thus reduce the likelihood of transmission of the pathogen to humans. The developed protocol could be considered a new tool for the identification of *V. vulnificus* in shellfish and/or animals, with special interest in farmed fish. This tool would represent an advance for *V. vulnificus* diagnosis and food safety assessment.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijfoodmicro.2022.109778.

Declaration of competing interest

Neither of the authors have any conflict of interest, and that the manuscript is not under consideration by any other publication.

Acknowledgment

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