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Development of a *mreB*-targeted real-time PCR method for the quantitative detection of *Vibrio harveyi* in seawater and biofilm from aquaculture systems

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Abstract :

Vibrio harveyi is a particularly problematic Gram-negative bacterium because it can form biofilms on aquaculture facility surfaces, leading to resistance of bacteria against antibiotics and water sanitizers. A SYBR Green I quantitative real-time PCR method was developed to detect V. harveyi directly from environmental samples, including seawater and biofilm. Specific primers targeting the mreB gene were designed. The exclusivity and inclusivity of the newly designed primers were evaluated using a panel of 85 bacteria: 58 V. harveyi from multiples origins and 27 non-V. harveyi isolates, and compared with two pairs of primers targeting the topA and toxR genes that were designed previously. All sets of primers were able to distinguish V. harveyi from closely related species belonging to the Harveyi clade. However, the mreB primers showed better inclusivity and were thus used to develop the real-time PCR assay. A quantification curve was obtained from pure culture of V. harveyi and exhibited excellent efficacy with detection levels as low as 5 genome copies in the PCR reaction. After selection of the extraction kit allowing the best DNA quantity and purity, validation was performed on both seawater and biofilm samples collected from a fish farm. The presence of inhibitors in the DNA templates was evaluated and a 10-fold dilution of template DNA was recommended in order to avoid their effects. The assay was able to detect V. harveyi from environmental samples, confirming the validity of the method. This real-time PCR method will help to evaluate the dynamics of V. harveyi in aquaculture facilities. Suitable prophylactic control measures could be designed using this method, instead of the use of curative methods such as antibiotics.

Highlights

► A real-time PCR was developed for quantification of *V. harveyi* in farming systems. ► The method was tested on seawater and biofilm samples from a seabass farm. ► New *mreB* primers were designed and revealed high specificity for *V. harveyi*. ► The method will be useful for the epidemiological investigation of *V. harveyi*.

Keywords : Vibrio harveyi, quantitative detection, mreB gene, seawater, biofilm, aquaculture

1. Introduction

Vibrio are Gram-negative bacteria ubiquitous in aquatic environments (Farmer *et al.*, 2005). Some of them, such as *Vibrio harveyi*, are known to be the causative agents of vibriosis, a fatal hemorrhagic septicemia disease affecting aquatic animals (Austin and Zhang, 2006). Vibriosis caused by *V. harveyi* is a major concern in aquaculture. More particularly, this species is a well-known pathogen of shrimps, mollusks and crustaceans (Sawabe *et al.*, 2007b; Soto-Rodriguez *et al.*, 2012; Travers *et al.*, 2008). Infections have also been reported in fishes such as seabass, *Dicentrarchus labrax* (Pujalte *et al.*, 2003). Recently, Vendramin *et al.* (2016) identified *V. harveyi* as an emerging problem in reared seabass, an economically important fish species in Europe (FAO 2005-2020). Importantly, Bourne *et al.* (2006) highlighted that biofilms on rearing tanks may act as potential reservoirs for *V. harveyi*. Biofilms of *V. harveyi* are even more dangerous to reared animals given that they can enhance the resistance of bacteria to antibiotics or water sanitizers (Karunasagar *et al.*, 1996; Thompson *et al.*, 2004a). This phenomenon leads to persistence of *V. harveyi* in aquaculture tanks. Early diagnosis and monitoring of *V. harveyi* prevalence are therefore major concerns in seabass farming systems.

Conventional culture-based and biochemical methods for the detection and identification of *V. harveyi* are unreliable, laborious, and time-consuming (Bonnin-Jusserand *et al.*, 2017). On the contrary, polymerase chain reaction (PCR)-based methods offer a rapid, reliable, and more specific analysis (Cano-Gomez *et al.*, 2009). A number of conventional PCR methods have already been reported for the detection of *V. harveyi* in farming systems (Conejero and Hedreyda, 2003; Kim *et al.*, 2014; Oakey *et al.*, 2003; Pang *et al.*, 2006). However, to our knowledge, only two studies have described real-time PCR methods for the quantification of *V. harveyi* in seawater coming from an abalone farm (Fukui and Sawabe, 2008; Schikorski *et al.*, 2013). These

studies did not process complex samples such as biofilms, and did not include a wide range of environmental isolates or several closely *V. harveyi*-related species belonging to the Harveyi clade. They also stated that improvements in their protocols remain to be made in order to increase the sensitivity of their methods. Furthermore, Fukui and Sawabe (2008) highlighted the lack of specificity of their designed oligonucleotides, leading to an unreliable estimation of the amount of *V. harveyi* in seawater.

Identification of reliable target genes of *V. harveyi* remains difficult due to genome similarity between species belonging to the Harveyi clade (Gomez-Gil *et al.*, 2004). The standard target 16S rRNA gene, commonly used, is not suitable to distinguish these species from one another. For instance, *V. harveyi, V. campbellii,* and *V. rotiferianus* share more than 99 % sequence similarity in the 16S rRNA gene (Gomez-Gil *et al.*, 2003; Gomez-Gil *et al.*, 2004). Cano-Gomez *et al.* (2011) performed a multilocus sequence analysis (MLSA) based on the 16S rRNA, *mreB, ftsZ, pyrH, rpoA* and *topA* genes, and results showed that the concatenation of both the *mreB* and *topA* loci was sufficiently accurate to differentiate between *V. harveyi*-related species. Therefore, using only the *mreB* (rod shape-determining gene, subunit B) or *topA* (topoisomerase I) gene could potentially enable discrimination by PCR. In addition, Pascual *et al.* (2010) performed an MLSA study based on the 16S rRNA, *gyrB, pyrH, rctB, recA, rpoD* and *toxR* genes. They suggested that the individual *toxR* (transmembrane transcriptor regulator) gene has high discriminatory power and could be suitable to differentiate between *V. harveyi*-related species. Oligonucleotides targeting the *topA* and *toxR* genes have already been designed (Cano-Gomez *et al.*, 2015; Pang *et al.*, 2006). Their specificity still needs to be clarified since the studies did not include a wide range of environmental isolates or several closely *V. harveyi*-related species. However, no oligonucleotide targeting the *mreB* gene of *V. harveyi* has been described so far.

This study reports the development of a specific SYBR Green I real-time PCR method for the detection and quantification of *V. harveyi* in both seawater and biofilm samples from aquaculture tanks. The optimized method provides a common protocol to extract *V. harveyi* DNA from both of these complex environmental samples, while minimizing the impact of PCR inhibitors. The specificity of the previous *topA* and *toxR* and the newly designed *mreB* oligonucleotides was assessed. Thereafter, in view of the results obtained, the real-time PCR protocol was established and optimized using the *mreB* target gene. Finally, the detection and quantification of indigenous *V. harveyi* in seawater and biofilm from a fish farm were performed.

2. Materials and methods

2.1. Bacteria strains and culture methods

A total of 85 *Vibrio* strains were used in this study (Table 1): (i) 38 collection strains were obtained from the Belgian Coordinated Collections of Microorganisms (BCCM/LMG), the German Collection of Microorganisms and Cell Cultures (DSMZ), the Spanish Type Culture Collection (CECT), and the Collection of Institut Pasteur (CIP), (ii) 35 isolates were obtained from a fish farm raising seabass *Dicentrarchus labrax*. Bacteria were isolated from the spleen of moribund fish, plated on marine agar (MA), (Difco Laboratories, Detroit, MI, USA), incubated at 25 °C overnight and then identified by matrix-assisted laser desorption ionization - time of flight mass spectrometry (MALDI TOF MS) using the Bruker Biotyper database by a veterinary laboratory, and (iii) 12 isolates were isolated by the French Research Institute for the Exploitation of the Sea (IFREMER) from oysters (*Crassostrea gigas*) and abalone (*Haliotis tuberculata*). All bacteria were maintained in Luria-Bertani broth supplemented with 20 % NaCl (LBS), with a final pH of 7.2 ± 0.2, and with 20 % glycerol and stored at -80 °C until use. Bacteria were plated on thiosulfate- citrate- bile salts- sucrose (TCBS) agar (Biokar Diagnostics, Beauvais, France)

and incubated overnight at growth temperature, in order to check the purity of the isolates. A single colony was then plated on LBS agar and incubated overnight at growth temperature. This temperature was 37 °C, except for LMG 11216^T, LMG 25266^T, Vh2, Vh3, Vt1, Vt2 and Vg1, for which the growth temperature was 25 °C.

2.2. Isolation of bacteria from seawater samples

Seawater volumes of 1 L from aquaculture tanks were collected 20 cm below the water surface. Samples were maintained on ice during transport to the laboratory and processed within 2 h. They were filtered through 0.45 µm- pore- size nitrocellulose filters (Sartorius, Goettingen, Germany). If the samples contained many aggregates and filters clogged, several filters were used. They were then immersed in 25 mL of LBS with 20 % glycerol and stored at -80 °C until analysis. Subsequently, samples were defrosted at ambient temperature and vortexed for 3 min in order to free bacteria from filters. Filters were removed and the liquid was centrifuged at 8000×*g* for 10 min. The cell pellet was resuspended in 25 mL of physiological water (0.9 % NaCl) and centrifuged at 8000×*g* for 10 min. Supernatant was removed and DNA was extracted from cell pellets.

2.3. Isolation of bacteria from biofilm samples

Pieces of concrete (5 cm × 2.5 cm) were made with the same materials as the walls of aquaculture tanks and immersed 20 cm below the water surface in these tanks. The objective was to mimic the biofilm formation occurring on the walls of aquaculture tanks, on pieces of concrete that can easily be retrieved. The concrete pieces, still immersed in seawater, were maintained on ice during transport to the laboratory and processed within 2 h. Bacteria from biofilm were collected by swabbing 2.34 cm^2 of concrete surface using a sterile stainless-steel jig. The procedure used to remove biofilm from concrete has previously been described by Mougin *et al.* (2019), and was

modified slightly. Briefly, two swabs (150C, Copan, Brescia, Italy) were used twice for each piece of concrete and immersed into 3 mL of LBS with 20 % glycerol. The tube containing the two swabs was then stored at -80 °C until analysis. Subsequently, samples were defrosted at ambient temperature and vortexed for 3 min in order to free bacteria from swabs. Swabs were removed and the liquid was centrifuged at 8000×*g* for 10 min. The cell pellet was resuspended in 3 mL of physiological water (0.9 % NaCl) and centrifuged at 8000×*g* for 10 min. Supernatant was removed and DNA was extracted from cell pellets.

2.4. DNA extraction

DNA templates were extracted from pure culture, isolated from LBS agar, using the DNeasy Blood&Tissue kit (Qiagen, Hilden, Germany) and an automated nucleic acid extractor QIAcube Connect (Qiagen, Hilden, Germany), according to the manufacturer's instructions. The final elution volume was 100 µL. The extracted DNA was then stored at -20 °C until use.

In order to prepare DNA templates from environmental samples, five DNA extraction kits were tested: the DNeasy PowerWater kit (no. 1), the DNeasy PowerBiofilm kit (no. 2), the DNeasy Blood&Tissue kit (no. 3) (Qiagen, Hilden, Germany), the Extracta DNA Prep for PCR-Tissue kit (no. 4) (Quantabio, Beverly, MA, USA), and the Arcis DNA Sample Prep kit (no. 5) (Arcis Biotechnology, Daresbury, United Kingdom). For each kit, the protocol was carried out according to the manufacturer's instructions. Extracted DNA was resuspended in a final volume of 100 µL and stored at -20 °C.

2.5. Design of primers, specificity, and annealing temperature

Three *V. harveyi*-specific pairs of primers were tested in this study (Table 2): two pairs of primers targeting the *topA* and *toxR* genes (Cano-Gomez *et al.*, 2015; Pascual *et al.*, 2010), and one newly designed pair of primers targeting the *mreB* gene (Table 2).

In order to design the *mreB* primers, two alignments were performed. The first alignment allowed the identification of conserved regions shared by *V. harveyi* strains: 69 *mreB* gene sequences of *V. harveyi*, obtained from the GenBank database (National Center for Biotechnology Information, NCBI), were aligned using Mega X software version 10.0.1 (supplemental data, appendix A). The second alignment allowed the identification of divergent regions between *V. harveyi* and *V. harveyi*-related species: 9 *mreB* gene sequences of *V. harveyi*-related species were aligned with one *mreB* gene sequence of *V. harveyi* using MultiAlin (Corpet, 1988) (supplemental data, appendix B). The target sequence was chosen from the conserved region of *V. harveyi* and the divergent region between *V. harveyi* and *V. harveyi*-related species. *In silico* analyses for melting temperature (Tm) estimation, secondary structures, and potential dimer formation of *mreB*, *topA* and *toxR* were performed using Oligo Calculator version 3.27 (Kibbe, 2007) and Primer3Plus (Rozen and Skaletsky, 2000).

The annealing temperatures (Ta) of all sets of primers were optimized using a temperature gradient ranging from 55 °C to 65 °C with the iCycler™ Thermal Cycler (Bio-Rad Laboratories, Hercules, CA, USA) (data not shown).

The *in silico* specificity of the primers was verified using the nucleotide BLAST search program with the GenBank database (NCBI). The experimental specificity was evaluated using conventional PCR. Exclusivity and inclusivity tests were performed in triplicate with a panel of *V. harveyi* (n=58) and non-*V. harveyi* organisms (n=27) (Table 1). Exclusivity was validated by a lack of PCR products, and inclusivity was validated by a single band and expected product sizes on gel electrophoresis.

All primers were synthetized by TIB MOLBIOL (TIB MOLBIOL Syntheselabor GmbH, Berlin, Germany) and were suspended in nuclease-free water to reach a final concentration of 10 µM and stored at -20 °C.

2.6. Conventional PCR conditions

The PCR reaction mixture contained 2.5 µL of 10X PCR buffer, 0.5 µL of 10 mM dNTP, 0.5 µL of each primer (10 µM), 0.125 µL of 5 units.µL^{-1} HotStarTaq DNA polymerase (Qiagen, Hilden, Germany), 2 µL of template DNA (equilibrated to 25 ng.µL^{-1}), and 18.875 µL of nuclease-free water to a final volume of 25 µL. The PCR reaction was run on a Thermal Cycler (Applied Biosystems, Forster City, CA, USA), under the following conditions: 5 min at 94 °C, followed by 30 cycles of 1 min at 94 °C, 1 min at the optimized Ta, and 1 min at 72 °C. The final cycle was followed by an additional 7 min of extension at 72 °C. All PCR experiments were carried out in triplicate and contained a positive control (LMG 4044 *V. harveyi* DNA), a negative control (CIP 70.67 *V. campbellii* DNA), and a no template control (NTC), DNA-free. The size of the PCR product was verified by ethidium bromide agarose gel electrophoresis (2 %).

2.7. Real-time PCR conditions

The reaction mixture contained 10 µL of 2X Master Mix LightCycler® 480 SYBR Green I Master (Roche Diagnostics, France), 1 µL of each *mreB* primer (10 µM), 5 µL of template DNA, and 3 µL of water to a final volume of 20 µL. The quantitative PCR reaction was run on a LightCycler® 480 (Roche Diagnostics, France), under the following conditions: 5 min at 95 °C, followed by 40 cycles of 10 s at 95 °C, 20 s at 55 °C, and 10 s at 72 °C. The melting curve analysis was then performed under the following conditions: 5 s at 95 °C, 1 min at 65 °C, and an increase of the temperature from 65 °C to 97 °C at 0.3 °C.s^{-1}. All quantitative PCR experiments were performed in triplicate and included a positive control (LMG 4044 *V. harveyi* DNA), a negative control (CIP

70.67 *V. campbellii* DNA), and a no template control (NTC), DNA-free. Lack of specific amplification was defined for C_T values ≥ 35. The baseline of PCR was automatically set by the system, and data analysis was carried out with LightCycler® 480 SW 1.5.1 software.

2.8. Sequencing conditions

PCR amplification products were generated for three pairs of primers targeting the *ftsZ* (cell division protein FtsZ gene), *recA* (recombination and DNA repair protein gene) and *gyrB* (uridylate kinase gene) genes (Table 2), screened from the literature (Sawabe *et al.*, 2007a; Teh *et al.*, 2010). The PCR reaction mixture contained 25 µL of 2X Platinum™ Green Hot Start PCR Master Mix (Invitrogen, Carlsbad, CA, USA), 1 µL of each primer (10 µM), 5 µL of template DNA (equilibrated to 25 ng.µL^{-1}), and 18 µL of nuclease-free water to a final volume of 50 µL. The PCR reaction was run on a Thermal Cycler (Applied Biosystems, Forster City, CA, USA), under the following conditions: 5 min at 95 °C, followed by 30 cycles of 1 min at 95 °C, 2 min 15 s at the optimized Ta, and 1 min 15 s at 72 °C. The final cycle was followed by an additional 7 min of extension at 72 °C. The PCR products of expected size were then sequenced from both sides (forward and reverse), using Sanger sequencing by GenoScreen (Lille, France). Analysis was performed using the nucleotide BLAST search program with the GenBank database (NCBI). For whole-genome sequencing, organisms were sent to GenoScreen on LBS agar after incubation at growth temperature. Analysis was performed with CLC Genomics Workbench version 20.0.2. Raw sequences were paired and trimmed using default parameters and then assembled into contigs using the default parameters of the Genome Finishing Module. A distance tree was generated using the neighbor joining algorithm, with these contigs and different genome sequences of *Vibrio* spp. available on NCBI.

2.9. Real-time PCR validation

For the sensitivity of the DNA detection assay, pure *V. harveyi* DNA was extracted and serially diluted in nuclease-free water. The purity and the quantity of the extracted DNA were determined using a DeNovix DS-11 spectrophotometer (Clinisciences, Nanterre, France) and a Qubit 3.0 fluorometer (dsDNA high-sensitivity assay), (Invitrogen, Carlsbad, CA, USA), respectively. The number of genome copies was estimated from the DNA quantity measured, considering the genome size of *V. harveyi* equal to 5.67×10^6 bp (Brankatschk *et al.*, 2012; Urbanczyk *et al.*, 2013). The calibration curve was plotted using the means of C_T values of 20 replicate series versus the logarithm of each dilution factor.

The presence of PCR inhibitors in the extracted DNA templates was verified by adding an amount of 125 ng of pure *V. harveyi* DNA into template DNA obtained from 3 different seawater and 3 different biofilm samples, in order to reach a final concentration of 10^6 genome copies per reaction. A 10-fold dilution series of inoculated DNA template and pure DNA, prepared in nuclease-free water, were then amplified by real-time PCR.

In order to verify whether the assay enabled detection and quantification of indigenous *V. harveyi* from environmental samples, real-time analysis of 4 seawater and 4 biofilm samples collected from a fish farm was performed. Genome copy equivalent (GE) concentrations were estimated by standard curve, taking into account the dilution factor of the assay.

3. Results

3.1. Specificity of the real-time PCR assay

First, the exclusivity of the *mreB*, *topA* and *toxR* primers was confirmed by performing conventional PCR on a panel of 23 non-*V. harveyi* collection strains including, at least one reference strain for each of the 10 species, and 4 non-*V. harveyi* (Table 1). These organisms were

mostly species in the Harveyi clade, genetically close to *V. harveyi*. Second, the inclusivity of the three pairs of primers was determined by testing a panel of 15 *V. harveyi* collection strains, including the reference strain LMG 4044^{T}. Then, 43 *V. harveyi* isolates obtained from reared seabass (n=31), abalone (*H. tuberculata*) (n=7), and oyster (*C. gigas*) (n=5) were tested. Among these 58 organisms, only 53, 52 and 48 showed positive results for the *mreB*, *topA* and *toxR* primers, respectively (Table 3.A). In fact, for all the primers, no PCR product was detected from the LMG 10946 and LMG 10947 collection strains and from the Vh2, Vh15 and Vh28 isolates. Moreover, the results diverged between the *mreB*, *topA* and *toxR* primers for 5 isolates. PCR products were detected from Vh5, Vh8, Vh22 and Vh29 isolates using the *mreB* and *topA* primers, while no PCR product was detected using the *toxR* primers. PCR products were detected from the Vh25 isolate using the *mreB* primers, while no PCR product was detected using the *topA* and *toxR* primers. As misidentifications could have occurred, identification of these 10 organisms required confirmation.

In order to clearly identify these 10 organisms (LMG 10946, LMG 10947, Vh2, Vh5, Vh8, Vh15, Vh22, Vh25, Vh28 and Vh29), Sanger sequencing was performed targeting three reference genes: *ftsZ*, *recA*, and *gyrB*. The non-*V. harveyi* isolates Vt1, Vt2, Vg1 and Vp1 were also sequenced. When the sequencing results were divergent between the three genes, whole-genome sequencing was performed. Analysis of Vh5, Vh8, Vh22, Vh25 and Vh29 sequences showed high identity to *V. harveyi* (supplemental data, appendix C). However, LMG 10946, LMG 10947, Vh2, Vh15, Vh28, Vt1, Vt2, Vg1 and Vp1 sequences exhibited high identity to non-*V. harveyi* species (supplemental data, appendix C and D). PCR results obtained with the *mreB* primers fitted with these data, whereas those obtained with *topA* and *toxR* did not (Table 3.B).

Ultimately, the *mreB*, *topA* and *toxR* primers exhibited 100 %, 98.1 % and 90.6 % inclusivity, respectively. The lack of PCR product detected from all the non-*V. harveyi* strains (n = 27) revealed 100 % exclusivity for all primers. The specificity of the *mreB* primers was confirmed by sequencing PCR products obtained from the LMG 4044^T reference strain. Moreover, analysis of the melting curve, obtained from real-time PCR experiments, highlighted only one peak with a melting temperature (Tm) of 83.70 ± 0.10 °C (data not shown).

Detection of PCR products using 16S rRNA primers from all tested strains confirmed that DNA extraction was properly performed. The inclusion of blank samples with no template DNA confirmed non-specific reactions between the components.

3.2. Sensitivity and quantification limits of the assay

Assay sensitivity was determined using 10 independent dilution series of purified LMG 4044^T *V. harveyi* DNA, ranging from 2, 5, and 10 to 10^7 genome copies per reaction, in duplicate. The lowest number of genome copies detected in at least 95 % of the 20 replicates was 5 genome copies per reaction, corresponding to the limit of detection (LOD) for pure DNA of *V. harveyi*. The linear regression showed accurate correlation (R=0.9907) between the logarithm of genome copies per reaction and the threshold cycle (C_T) value (Fig. 1). The correlation was linear over a range of 5 to 10^7 genome copies per reaction, confirming that the limit of quantification (LOQ) corresponded to 5 genome copies per reaction. Real-time PCR efficiency was calculated from the slope of the linear portion of the calibration curve, according to the formula: $E = 10^{-1/slope} - 1$ and was equal to 99.9 %.

3.3. Quantitative detection of *V. harveyi* in environmental samples

In order to obtain DNA templates with optimal quantity and purity from both seawater and biofilm samples, five extraction kits were compared (Table 4). To check the integrity of extracted

DNA and the potential presence of PCR inhibitors, two conventional PCRs were carried out. The first targeted the 16S rRNA V4V5 hypervariable region found in most bacteria and archaea, and the second targeted 16S rRNA found in most *Vibrio* species. Both PCR analyses indicated the potential presence of PCR inhibitors when using no. 3 and no. 1 kits. In fact, no PCR product was detected from seawater and biofilm DNA templates extracted with the no. 3 kit. PCR products were only detected from seawater DNA templates extracted with the no. 1 kit using 16S rRNA V4V5 primers. These results highlighted that the no. 3 kit was not suitable for DNA extraction from either of our samples, and the no. 1 kit was not suitable for DNA extraction from biofilm samples. The seawater and biofilm DNA templates extracted with no. 4 and no. 5 kits were cloudy solutions. That is why the PCR analyses were not carried out and the two kits were not selected. PCR products were detected from seawater and biofilm extracted DNA templates with the no. 2 kit, using both primers. Moreover, the highest quantity of extracted DNA was obtained with the no. 2 kit. These results indicated that the no. 2 kit – DNeasy PowerBiofilm kit (Qiagen) – is suitable for the extraction of *Vibrio* DNA from both our environmental samples and was therefore selected.

Even though this chosen kit seemed to reduce the amount of co-extracted inhibitors, some of them could still subsist in the environmental extracted DNA templates. This is why the effect of these inhibitors was evaluated. A known amount of pure *V. harveyi* DNA was added to 3 different seawater and 3 different biofilm DNA templates. These DNA templates were extracted with the no. 2 kit, and previously identified as negative for the target gene. A series of 10-fold dilutions, ranging from 10^2 to 10^6 genome copies per reaction of pure DNA template, was prepared and used as a standard. Series of 10-fold dilutions (10^2 to 10^6 genome copies per reaction) of inoculated seawater and biofilm DNA templates were then prepared and amplified by

real-time PCR. The results showed a significant increase in the C_T value for the less diluted seawater and biofilm DNA templates (10^6) compared to the C_T value of pure DNA (Fig. 2). The data obtained confirmed that seawater and biofilm samples contained inhibitors affecting assay efficiency. Nevertheless, the results indicated that the effect of inhibitors was avoided by 10-fold dilution since no increase in C_T value was observed for the 10-fold diluted DNA template (10^2 to 10^5). Therefore, the DNA templates extracted from both samples must be 10-fold diluted in order to avoid the influence of real-time PCR inhibitors.

The real-time PCR assay was validated by detecting and quantifying indigenous *V. harveyi* by real-time PCR from 4 seawater and 4 biofilm samples, collected from a seabass farm. The results showed specific amplification from both seawater and biofilm samples (Table 5). However, a negative result was obtained from one biofilm sample, meaning that the quantity of *V. harveyi* was below the limit of detection of the assay. The concentration of indigenous *V. harveyi* ranged from 1.23 to 2.68×10^2 GE.mL^{-1} in seawater, and from 2.84×10^2 to 5.00×10^3 GE.cm^{-2} in biofilm samples.

4. Discussion

As *Vibrio harveyi* is a major pathogen in aquaculture, its early detection and quantification are a key focus for aquaculture farms. Development of reliable identification tools is complex since species belonging to the Harveyi clade are genetically and phenotypically similar. Whole-genome and fingerprinting techniques exhibited high discriminatory power. Nevertheless, these later are expensive, time-consuming and thus not suitable to provide a rapid diagnosis in a context of vibriosis outbreaks (Cano-Gomez *et al.*, 2009). On the contrary, real-time PCR is a rapid and reliable method that is commonly used. Previous studies have highlighted the need for more

V. harveyi-specific primers and accurate detection sensitivity in order to prevent vibriosis in aquaculture facilities (Fukui and Sawabe, 2008; Schikorski *et al.*, 2013).

In this work, a *V. harveyi*-specific quantitative real-time PCR was developed. The fluorescent reporter SYBR Green I was used since it has the advantage of not involving the design of an oligonucleotide probe. The protocol was established with a newly designed pair of primers targeting the *mreB* gene, which is a single-copy gene that is stable in the genome (Cano-Gomez *et al.*, 2011). The present real-time PCR allowed for discrimination between *V. harveyi* and closely related species belonging to the Harveyi clade, such as *V. alginolyticus*, *V. azureus*, *V. campbellii*, *V. jasicida*, *V. mytili*, *V. natriegens*, *V. owensii* (synonym of *V. communis*), *V. parahaemolyticus*, and *V. rotiferianus* (Hoffmann *et al.*, 2012; Sawabe *et al.*, 2007a; Urbanczyk *et al.*, 2013).

Additional information was found regarding the exclusivity and inclusivity of the *topA* and *toxR* primers previously designed by Cano-Gomez *et al.* (2015) and Pang *et al.* (2006), respectively. The lack of inclusivity of both sets of primers suggested sequence heterogeneities in the *topA* and *toxR* genes of epidemiologically distinct strains within *V. harveyi* species. Importantly, *Vibrio* species are known for high genomic plasticity (Rowe-Magnus *et al.*, 2006). This is consistent with the study carried out by Conejero and Hedreyda (2003), who developed a *V. harveyi*-specific conventional PCR targeting the *toxR* gene. The validation experiments revealed false-negative results for two *V. harveyi* isolates: STD 3-101 and VIB 391. Both these organisms were isolated from shrimps while other organisms that showed positive results were isolated from fishes. This shows that the high genomic plasticity of *V. harveyi* can lead to false-negative results. Nevertheless, in the present study, the *mreB* gene appeared relatively well conserved since no false-negative results were recorded for our isolates.

Discriminating between species belonging to the Harveyi clade is complex and misidentifications can occur. Our results showed that the *V. harveyi*-LMG 10946 and LMG 10947 collection strains have previously been identified incorrectly. According to our data, LMG 10946 was identified as *V. campbellii* and LMG 10947 as *V. owensii*. Thompson *et al.* (2001) had studied both strains, among others, using fluorescent amplified fragment length polymorphism (FAFLP) genotyping. The strains were distributed in the same cluster as the LMG 4043 and LMG 11659 collection strains identified as *V. owensii*. Therefore, these results indicated genome similarities with non-*V. harveyi* species, suggesting potential misidentification. Furthermore, Hoffmann *et al.* (2012) reported previous incorrect identifications for several collection strains, by performing an MLSA study based on the *ftsZ, mreB, rctB, rpoD, topA* and *toxR* genes. They confirmed that strains LMG 16862 and LMG 16863, first identified as *V. harveyi*, were actually *V. campbellii*, and LMG 4043 was *V. owensii*. They also demonstrated that *V. communis* and *V. owensii* are synonyms. In our study, negative PCR results were obtained using the *mreB, topA* and *toxR* primers for all the collection strains previously mentioned, confirming the accurate exclusivity of all sets of primers.

The present study clearly demonstrates the complexities of processing environmental samples from aquaculture systems. Our method was optimized to quantify the population of indigenous *V. harveyi* in complex environmental samples: seawater and biofilm from aquaculture. Detection of target DNA in tanks was even more problematic due to the high diversity and abundance of organic matter. The challenge was to provide accurate DNA yield, while preserving DNA quality. Filtration is a common method used to process aquatic samples (Staley *et al.*, 2015). This method makes it possible to concentrate bacteria and thereby increase DNA yield, without previous enrichment steps, which can be time-consuming (Akkermans *et al.*, 1995). Likewise,

swabbing has been widely used to collect environmental biofilm samples from concrete (De Muynck *et al.*, 2010). However, environmental samples often contain various PCR-inhibitors leading to false-negative results or inaccurate quantification (Wilson, 1997). That is why our study reports the DNA extraction efficiency for five distinct DNA extractions kits. We found that the DNA extraction kit allowing the best DNA yield and purity was the DNeasy PowerBiofilm kit (Qiagen), which was therefore selected to perform the assay. Nevertheless, this optimization was not sufficient and a few inhibitors remained in extracted template DNA. The effects of these inhibitors can be avoided by a simple widely used method, which consists in diluting template DNA of environmental samples (Wilson, 1997). However, the target DNA is also diluted, and this strategy reduces detection sensitivity. The real-time PCR detection limit for *V. harveyi* derived from pure culture was 5 genome copies per PCR reaction. Although complex environmental matrices can affect sensitivity, the assay detection limit for environmental samples could not be determined due to background *V. harveyi* levels in seawater and biofilm samples. However, performing this assay in aquaculture systems did enable the detection and quantification of indigenous *V. harveyi* in unseeded samples. The present method is therefore applicable in aquaculture facilities.

The concentration of indigenous *V. harveyi* detected in seawater samples ranged from 1 to 10^2 GE.mL^{-1}. Higher bacteria abundances were obtained in the study carried out by Zhou *et al.* (2007). They quantified the abundance of *V. alginolyticus* in environmental seawater by real-time PCR. The concentration ranged from 10^2 to 10^3 CFU.mL^{-1}. Likewise, Saulnier *et al.* (2009) quantified the abundance of *V. aestuarianus* in seawater by real-time PCR. The concentrations found ranged from 10^1 to 10^2 cells.mL^{-1}. The volume of the treated sample could explain this difference. Importantly, in these previous studies, volumes of 1 mL or less of seawater were

analyzed by real-time PCR, while in the present study, 1 L of seawater was first concentrated and then analyzed. In this way, the concentration of bacteria by filtration allowed us to improve the detection limit. Nevertheless, the concentration of large volumes of water can lead to a significant loss of the target organism during the filtration procedure (Akkermans *et al.*, 1995; Fukui and Sawabe, 2008). The concentration of indigenous *V. harveyi* detected in biofilm samples ranged from 10^1 to 10^3 GE.cm^{-2}. This value makes sense when compared to the findings reported by Shikuma and Hadfield (2010). They quantified the abundance of *V. cholerae* in seawater and biofilm samples isolated from harbors by real-time PCR. High abundance of *V. cholerae* was detected and the concentration obtained was around 10^3 GE.cm^{-2}. They also highlighted that the concentration in seawater samples was lower than in biofilm, and was approximately 10^1 GE.mL^{-1}. Thus, our method allows for accurate quantification of bacteria in environmental samples.

5. Conclusion

To conclude, the present method is a useful molecular tool, allowing for direct quantification of *V. harveyi* in seawater and biofilm samples from aquaculture. The *mreB* primers designed showed high specificity for *V. harveyi* strains isolated from various organisms, achieving discrimination between *V. harveyi* and closely related species belonging to the Harveyi clade. This tool will be used in a forthcoming study with the aim of monitoring seasonal changes in *V. harveyi* abundance in aquaculture facilities.

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Table 1. List of collection strains and bacterial isolates used in this study

Strain/isolate ID	Assigned species	Host/Origin	Isolation	
			Place	Date
LMG 4044^{T}	*Vibrio harveyi*	Dead amphipod (*Talorchestia* sp.)	United States	/
LMG 7890	*Vibrio harveyi*	Brown shark (*Carcharhinus plumbeus*) kidney	United States	/
LMG 23680	*Vibrio harveyi*	Seawater	Japan	2005
LMG 19643	*Vibrio harveyi*	Japanese horse mackerel (*Trachurus japonicus*)	Japan	2002
LMG 23442	*Vibrio harveyi*	Dead abalone	Japan	2002
LMG 18299	*Vibrio harveyi*	Seabream (*Sparus aurata*)	Spain	1990
LMG 11755	*Vibrio harveyi*	Shark, mouth	Bahamas	/
LMG 22894	*Vibrio harveyi*	Shrimp, hemolymph	Mexico	1995
LMG 16832	*Vibrio harveyi*	Black tiger prawn	Thailand	/
LMG 22895	*Vibrio harveyi*	Shrimp, hepatopancreas	Mexico	1999
LMG 23678	*Vibrio harveyi*	Breeding seawater	Japan	2004
LMG 11226	*Vibrio harveyi*	/	/	/
LMG 23688	*Vibrio harveyi*	Abalone, internal organ	Japan	/
LMG 10946[a]	*Vibrio harveyi*[a]	Prawn (*Palaemon indicus*)	/	/
LMG 10947[a]	*Vibrio harveyi*[a]	Seawater	Red Sea	/
LMG 11216^{T}	*Vibrio campbellii*	Seawater (800 meters)	Hawaii	/
LMG 21363	*Vibrio campbellii*	Diseased *Penaeus monodon* juvenile	Philippines	/
LMG 11256	*Vibrio campbellii*	Seawater	Hawaii	/
CIP 70.67	*Vibrio campbellii*	/	/	/
LMG 16862	*Vibrio campbellii*	Oyster	Spain	1989
LMG 16863	*Vibrio campbellii*	Oyster	Spain	1989
DSMZ 23055^{T}	*Vibrio owensii*	Spiny lobster (*Panulirus ornatus*) Aquaculture	Australia	
LMG 25430^{T}	*Vibrio owensii*	*Mussismilia hispida*	Brazil	/
LMG 20370	*Vibrio owensii*	White shrimp (*Litopenaeus vannamei*)	Ecuador	/
LMG 4043	*Vibrio owensii*	Seawater	Israel	/
LMG 11659	*Vibrio owensii*	Seawater	Hawaii	/
LMG 21456	*Vibrio rotiferianus*	Rotifer (*Brachionus plicatilis*) culture	Belgium	/
DSMZ 17186^{T}	*Vibrio rotiferianus*	Rotifer (*Brachionus plicatilis*) culture	Belgium	/
LMG 2850^{T}	*Vibrio parahaemolyticus*	Human, "Shirashu" food poisoning	Japan	/
LMG 16838	*Vibrio parahaemolyticus*	*Penaeus orientalis*	China	/
LMG 11650	*Vibrio alginolyticus*	Seawater	/	/
LMG 4409^{T}	*Vibrio alginolyticus*	Spoiled horse mackerel (*Trachurus trachurus*) causing food poisoning	Japan	/
LMG 10950	*Vibrio natriegens*	Oyster	United States	/
CECT 526^{T}	*Vibrio natriegens*	Salt marsh mud	United States	
LMG 25266^{T}	*Vibrio azureus*	Seawater	Japan	
CECT 8524	*Vibrio jasicida*	*Lutjanus guttatus*	Mexico	2004
DSMZ 19137^{T}	*Vibrio mytili*	*Mytilus edulis*	Spain	2007
CECT 7298^{T}	*Vibrio sinaloensis*	*Lutjanus guttatus*	Mexico	2003
Vh1 to Vh33 (Vh27 and Vh30 do not exist)	*Vibrio harveyi*	Sea bass (*Dicentrarchus labrax*) spleen, fish farm	France	2017
Vh34 and Vh35	*Vibrio harveyi*	*Haliotis tuberculata*, juvenile, hatchery (Manche)	France	2004
Vh36	*Vibrio harveyi*	*Haliotis tuberculata*, adult, hatchery (Manche)	France	2004
Vh37	*Vibrio harveyi*	*Haliotis tuberculata*, adult (Fermanville)	France	2005
Vh38	*Vibrio harveyi*	*Haliotis tuberculata*, adult (Omonville)	France	2005
Vh43	*Vibrio harveyi*	*C. gigas*, spat (Arcachon)	France	2007
Vh45	*Vibrio harveyi*	*C. gigas*, juvenile (Thau)	France	2007
Vh47 and Vh48	*Vibrio harveyi*	*Haliotis tuberculata*, juvenile (Aber Wrac'h)	France	2014

Vh51	*Vibrio harveyi*	*C. gigas*	France	2007
Vh52 and Vh53	*Vibrio harveyi*	*C. gigas*, larva, hatchery (Vendée)	France	2008
Vt1 and Vt2	*Vibrio tasmaniensis*	Sea bass (*Dicentrarchus labrax*) spleen, fish farm	France	2017
Vg1	*Vibrio gigantis*	Sea bass (*Dicentrarchus labrax*) spleen, fish farm	France	2017
Vp1	*Vibrio rotiferianus*	Sea bass (*Dicentrarchus labrax*) spleen, fish farm	France	2017

[a]LMG 10946 and LMG 10947 strains were initially identified as *Vibrio harveyi* species but our study revealed misidentification

[T] Type strain

Table 2. Nucleotide sequences and characteristics of primers used for conventional and real-time PCR amplification

Gene	Target organism	Primer name	Primer sequence (5'-3')	Annealing temperature (Ta)	Product length (bp)	Reference
mreB	*Vibrio harveyi*	mreB11F mreB9bisR	TGAAGCTGTGATCAACTACG CCTGACAGTGGCTCTTGTAA	55 °C	215	/
topA	*Vibrio harveyi*	Vh.topA-F Vh.topA-R	TGGCGCAGCGTCTATACG TATTTGTCACCGAACTCAGAACC	55 °C	121	(Cano-Gomez *et al.*, 2015)
toxR	*Vibrio harveyi*	toxRF1 toxRR1	GAAGCAGCACTCACCGAT GGTGAAGACTCATCAGCA	55 °C	382	(Pang *et al.*, 2006)
ftsZ	*Vibrio* spp.	VftsZ75F VftsZ800R	GCTGTTGAACACATGGTACG GCACCAGCAAGATCGATATC	50 °C	750	(Sawabe *et al.*, 2007a)
recA	*Vibrio* spp.	VrecA130F VrecA720R	GTCTACCAATGGGTCGTATC GCCATTGTAGCTGTACCAAG	50 °C	600	(Sawabe *et al.*, 2007a)
gyrB	*Vibrio* spp.	gyrB 1 gyrB 2	AGCCAAACNAAAGAYAARYT CGYARYTTRTCYGGRTTRTRYTC	55 °C	493	(Teh *et al.*, 2010)
16S rRNA	*Vibrio* spp.	567F 680R	GGCGTAAAGCGCATGCAGGT GAAATTCTACCCCCTCTACAG	60 °C	113	(Thompson *et al.*, 2004b)
16S rRNA V4V5	High coverage of bacteria and archaea	515FY 926R	GTGYCAGCMGCCGCGGTAA CCGYCAATTYMTTTRAGTTT	50 °C	411	(Parada *et al.*, 2016; Quince *et al.*, 2011)

Table 3. Specificity of the *mreB*, *topA* and *toxR* primers according to collection strains and bacterial isolates used in the present study. A. PCR results of the 85 organisms studied. B. Detail of the PCR and sequencing results of the 10 *V. harveyi* strains that showed divergence with initially assigned species identification

A.

Species	Number of strains	PCR results			
		215 bp *mreB*	121 bp *topA*	382 bp *toxR*	113 bp 16S rRNA
V. harveyi	58	+ (53)	+(52)	+(48)	+(58)
V. campbellii	6	-	-	-	+(6)
V. owensii	5	-	-	-	+(5)
V. rotiferianus	3	-	-	-	+(3)
V. parahaemolyticus	2	-	-	-	+(2)
V. alginolyticus	2	-	-	-	+(2)
V. natriegens	2	-	-	-	+(2)
V. azureus	1	-	-	-	+(1)
V. jasicida	1	-	-	-	+(1)
V. mytili	1	-	-	-	+(1)
V. sinaloensis	1	-	-	-	+(1)
V. tasmaniensis	2	-	-	-	+(2)
V. gigantis	1	-	-	-	+(1)

B.

Assigned species	Strain/ isolate ID	Sequencing identification results				
V. harveyi	LMG 10946	*V. campbellii*	-	-	-	+
V. harveyi	LMG 10947	*V. owensii*	-	-	-	+
V. harveyi	Vh2	*V. jasicida*	-	-	-	+
V. harveyi	Vh5	*V. harveyi*	+	+	-	+
V. harveyi	Vh8	*V. harveyi*	+	+	-	+
V. harveyi	Vh15	*V. rotiferianus*	-	-	-	+
V. harveyi	Vh22	*V. harveyi*	+	+	-	+
V. harveyi	Vh25	*V. harveyi*	+	-	-	+
V. harveyi	Vh28	*V. owensii*	-	-	-	+
V. harveyi	Vh29	*V. harveyi*	+	+	-	+

'+' Positive, '-' Negative

Table 4. Comparison of the DNA extraction methods for both matrices seawater and biofilm samples. Values are the means of two independent replicates.

No.	DNA extraction kit	Matrix	Qubit	DeNovix			PCR	
			DNA ng.µL^{-1}	DNA ng.µL^{-1}	OD 260/230	OD 260/280	16S rRNA V4V5 All bacteria	16S rRNA *Vibrio* spp.
1	DNeasy PowerWater kit Qiagen	Seawater	3.73	10.65	0.54	1.10	+	-
		Biofilm	2.82	6.87	0.34	0.67	-	-
2	DNeasy PowerBiofilm kit Qiagen	Seawater	10.56	17.75	0.80	1.45	+	+
		Biofilm	6.15	8.90	0.24	1.07	+	+
3	DNeasy Blood&Tissue kit Qiagen	Seawater	2.07	11.04	0.5	2.18	-	-
		Biofilm	2.73	8.25	0.27	1.49	-	-
4	Extracta DNA Prep for PCR-Tissue kit Quantabio	Seawater	Too low	231.75	0.59	1.35		
		Biofilm	Too low	242.07	0.59	1.63		
5	Arcis DNA Sample Prep kit Arcis Biotechnology	Seawater	3.74	208.07	0.14	0.68		
		Biofilm	3.83	216.88	0.14	0.69		

OD, optic density

Table 5. Quantification of *V. harveyi* from seawater and biofilm samples collected from a seabass farm

Date	Sample	Estimated quantity in samples
May 3, 2018	Seawater	5.82×10^{1} GE.mL^{-1}
	Biofilm	ND
Jul. 11, 2018	Seawater	2.10×10^{2} GE.mL^{-1}
	Biofilm	1.04×10^{3} GE.cm^{-2}
Aug. 22, 2018	Seawater	2.68×10^{2} GE.mL^{-1}
	Biofilm	5.00×10^{3} GE.cm^{-2}
Oct. 17, 2018	Seawater	1.23×10^{0} GE.mL^{-1}
	Biofilm	2.84×10^{2} GE.cm^{-2}

ND, not detected
GE, genome equivalent

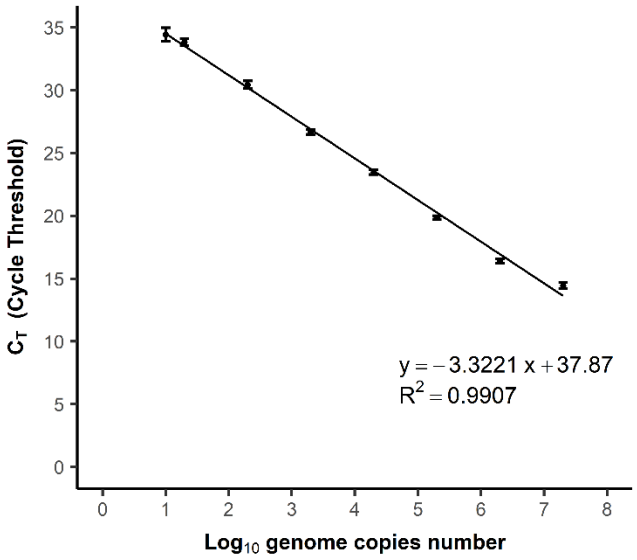

Fig. 1. Standard curve representing the detection and quantification of *V. harveyi* DNA by real-time PCR targeting the *mreB* gene. Means of 20 replicates are represented with corresponding standard deviation

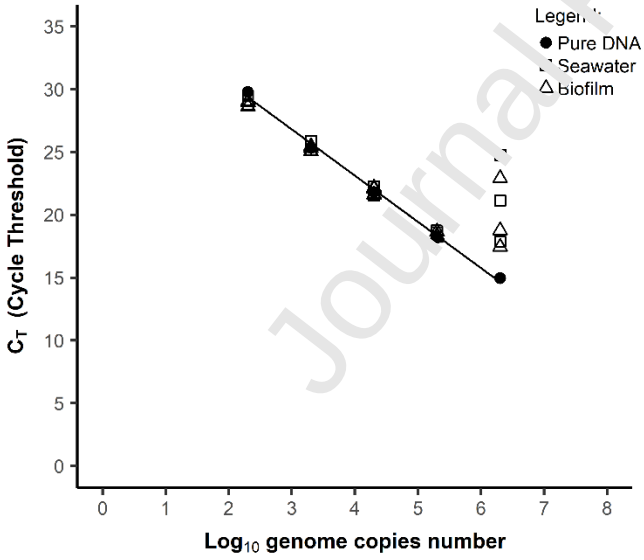

Fig. 2. Plot of 10-fold dilution series representing the detection and quantification of *V. harveyi* DNA by real-time PCR from pure culture of *V. harveyi* and from inoculated seawater and biofilm DNA templates

Highlights

A real-time PCR was developed for quantification of *V. harveyi* in farming systems.

The method was tested on seawater and biofilm samples from a seabass farm.

New *mreB* primers were designed and revealed high specificity for *V. harveyi*.

The method will be useful for the epidemiological investigation of *V. harveyi*.

Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.