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Development of TaqMan Real-Time PCR assays for monitoring *Vibrio harveyi* infection and a plasmid harbored by virulent strains in European abalone *Haliotis tuberculata* aquaculture

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Abstract:

The Gram-negative bacterium *Vibrio harveyi* is known to be highly pathogenic for the European abalone *Haliotis tuberculata*, which is a gastronomically important marine gastropod with a high commercial value. Since 1998, some particular bacterial strains are described as implicated in recurrent mortality outbreaks in French farm and field stocks of abalone. Recently, a 9.6 kb plasmid named pVCR1, was shown to be harbored by one highly *V. harveyi* virulent ORM4 strain suggesting its involvement in virulence phenotype. Thus, we have developed in the present study two TaqMan real-time PCR assays allowing to (i) rapidly and specifically detect, by a duplex procedure and in less than two hours, both *V. harveyi* and the presence of plasmid pVCR1 from unidentified bacterial colony and (ii) to quantify both *V. harveyi* and the plasmid pVCR1 in the haemolymph of abalone or its surrounding seawater. Quantification curves of *V. harveyi* or ORM4 strain seeded in haemolymph or artificial sea water samples were equivalent showing excellent qPCR efficacies and detection level as low as 18 *V. harveyi* cells-equivalent genomic DNA in a PCR reaction well. This qPCR allowed us to monitor of *V. harveyi* ORM4 strain in experimentally infected *H. tuberculata*. These diagnosis assays could provide powerful and useful tools to better understand the epidemiology of vibriosis caused by *V. harveyi* in different cultured marine species including *H. tuberculata*.

Keywords: *Vibrio harveyi* ; *Haliotis tuberculata* ; Real-time PCR ; Plasmid ; marine pathogen ; molecular diagnostic

1. Introduction

The European abalone *Haliotis tuberculata* is a gastronomically important marine gastropod with a high commercial value but stocks of this mollusk are actually in decline in Europe (Huchette and Clavier, 2004). Numerous events of mortality have been reported in Europe in both farmed and wild populations of abalone in association with the detection of different pathogens, including the protozoan parasite *Haplosporidium montforti*, *Rickettsia*-like bacteria, or the Gram-negative bacterium *Vibrio harveyi* (Azevedo et al., 2006a, Azevedo et al., 2006b, Balseiro et al., 2006, Huchette and Clavier, 2004). This last bacterium has been frequently involved in recurrent mortality outbreaks occurring seasonally, at the end of warm season, since 1998 in French farms and field stocks of *H. tuberculata* (Nicolas et al., 2002). *V. harveyi* has been widely recognized as a common pathogen of many commercially cultured fish and shellfish species worldwide (Aguirre-Guzman et al., 2001, Austin and Zhang, 2006, Lee et al., 2002, Liu et al., 1996, Nishimori et al., 1998, Pass et al., 1987, Zhang and Austin, 2000) including abalone in Australia and Japan (Handlinger et al., 2005, Sawabe et al., 2007a).

Vibriosis outbreaks in *H. tuberculata* cultivated in France were shown to be driven by sea water temperature exceeding a 17°C threshold (Huchette and Clavier, 2004, Travers et al., 2009a) and host physiology such as gametogenesis and reduced immune defense capacities (Travers et al., 2008). Although being non-specific, clinical signs of *V. harveyi* infection encompass a loss of muscular strength occurring concomitantly with the appearance of white pustules on the foot. Subsequently, diseased animals develop a fatal septicemia leading to up to 80% mortality within a few days to 3 weeks (Travers et al., 2009b). Furthermore, reproduction of the disease and mortality could be achieved by experimental infection using either intramuscular injection or bath contamination with ORM4 virulent *V. harveyi* strain. Recent studies focused on the identification of virulence factors displayed by this strain. ORM4 strain was found to inhibit phagocytosis and ROS production (Travers et al., 2009b) by a mechanism presumed by authors to involve the MAP kinase signal transduction pathway displayed by abalone hemocytes. Moreover, ORM4 strain harbored a 9.6kb plasmid, named pVCR1, which was found to date only in pathogenic virulent strains of *V. harveyi* isolated during mortality outbreaks among *H. tuberculata* in France (Travers, 2008).

Rapid and reliable identification of Vibrionaceae, at the family and genus levels, can be obtained through 16S rRNA gene sequencing (Thompson et al., 2005). However, differentiation at the species level remains questionable using 16S rRNA sequence comparisons of closely related sister species sharing nearly identical 16S rRNA sequences and similar phenotype features as among the *Vibrio* core group encompassing at least six species, *Vibrio alginolyticus*, *Vibrio campbellii*, *Vibrio harveyi, Vibrio rotiferianus*, *Vibrio natriegens* and *Vibrio parahaemolyticus* (Gomez-Gil et al., 2004, Thompson et al., 2009). Therefore, the taxonomy of *Vibrio* has been molecularly clarified through the use of diverse genomic tools, including DNA-DNA hybridization (Cho and Tiedje, 2001), amplified fragment length polymorphism (AFLP), repetitive extragenic palindromic element polymerase chain reaction (REP-PCR), random amplified polymorphic DNA (RAPD), multi-locus sequence analysis (MLSA) and whole genome sequencing (Gurtler and Mayall, 2001, Rademaker et al., 2000, Thompson et al., 2009, Thompson et al., 2004, Versalovic et al., 1991). However, these techniques are time consuming and appear not well adapted to perform rapid diagnostic tests in the context of disease surveillance and prevention in aquaculture facilities.

The goal of the current study was the development and evaluation of two TaqMan PCR assays that could provide rapid, specific, sensitive and quantitative methods for the detection of *V. harveyi* strains, harboring or not the suspected virulent pVCR1 plasmid, in either pure cultures or in the hemolymph of the European abalone *H. tuberculata* and its surrounding

seawater. For this purpose, two pairs of primers and probes were designed, (i) the first one targeting a specific region of the *toxR* gene, a widely studied housekeeping gene, to detect only *V. harveyi* species among the *Vibrio* core group, and (ii) the second one targeting a region of the pVCR1 plasmid sequence corresponding to the *parA* gene which encodes a putative protein implicated in the plasmid repartition and supposed to be well conserved (Davis et al. 1992, Edgar et al., 2006). The real-time PCR protocol quantifying *V. harveyi* cells was used to monitor the dynamics of infection of *H. tuberculata* abalones that were previously experimentally infected with a virulent ORM4 strain.

2. Material and methods

2.1. Abalone and hemolymph collection

Juveniles (40 mm ± 1.0, 2 years old) of *Haliotis tuberculata* were purchased from the hatchery of France Haliotis (Plouguerneau, France) and transferred in the Ifremer's facilities (Laboratoire de Génétique et Pathologie, La Tremblade, France). Animals were acclimated in 50 L polyethylene tanks with an open seawater circuit at 14-15°C under continuous aeration. During this period, abalones were fed *ad libitum* with marine algae diet of *Palmaria palmata*.

Two weeks before to be used for the different experimental assays, groups of 10 abalone were transferred in small aquaria filled with 5 liters of 1 µm filtered natural seawater. Abalone were slowly acclimated at 19°C (0.5°C per day) in absence of feeding. Seawater was continuously aerated and daily renewed. Abalone (control and experimentally infected animals) were previously anesthetized by bathing during 10 min in seawater containing 3% of absolute ethanol. In time relaxed, hemolymph was withdrawn from the cephalic arterial sinus located at the anterior extremity of the pedal muscle using a 1 mL syringe fitted with a 20-G needle.

2.2. Bacterial strains and culture conditions

Seventeen reference strains of *Vibrio* species phylogenetically closed to *V. harveyi* LMG 4044 type strain and eight *V. harveyi* strains isolated from *Haliotis sp.* were used in this study (Table 1). References strains were purchased from DSMZ, CIP and LMG collections, two isolates S35 and S20 were generously provided by T. Sawabe (Sawabe et al., 2007a). Other strains were obtained from samples collected at Larmor Baden in Brittany (France). They were identified by sequencing of 16S rRNA (Thompson et al., 2005) and *toxR* gene detection by PCR (Conejero and Hedreyda, 2003). All bacterial strains were cultured in Luria-Bertani (LB) Broth medium complemented with 2% NaCl (w/v) salt (LBS) at 28°C during 18h under constant shaking. They originated from bacterial stock culture stored at −80 °C and prepared as 15% (v/v) glycerol suspensions in LBS.

2.3. Bacterial template DNA

Two methods were used to extract bacterial DNA as previously described (Saulnier et al., 2009). The boiling method was used for specificity evaluation of real-time PCR assay using a single bacterial colony as DNA template for qPCR. In contrast, for the detection and quantification of *toxR* and *parA* bacterial genes, total DNA was extracted from samples constituting ten-fold serial dilutions of artificially spiked *V. harveyi* ORM4 cells performed either in Sterile Artificial Sea-Water (SASW, 2.3% NaCl, 20 mM KCl, 5 mM $MgSO_4$, 2 mM $CaCl_2$) or in freshly collected abalone hemolymph pool (see section, below). DNA extraction was carried out using the QIAamp DNA mini kit (QIAgen) combined with the use of the QIAcube automate according to the manufacturer's recommendations. Briefly, samples of 200 µL were centrifuged at 10,000 g at 4°C during 5 min. Pellets, containing bacteria and/or

haemocytes, were digested overnight on a rocking platform at 56°C by addition of 180 µL of ATL buffer supplied with 20 µL of proteinase K. Lysates were transferred into a 2 mL microcentrifuge tube. DNA extraction with QIAamp Mini spin columns was carried out using a QIAcube automate. Final elution of DNA was performed with 200 µL of ultra pure water, and samples were then stored at -20°C. DNA yield and purity were determined by spectrophotometry (Eppendorf).

2.4. PCR primers and fluorogenic probes design

In order to target specifically *V. harveyi* species, the *toxR* oligonucleotide and probe sequences were designed in variable regions deduced from the sequence alignment analysis of numerous *Vibrionaceae toxR* genes available in GenBank database. Based on the common sequence of 22 *toxR* genes of *Vibrio harveyi*, two primers and one probe were designed.

The *parA* oligonucleotide sequences and probe were designed to target exclusively the *parA* like gene of pVCR1 plasmid (Accession number KC306506), encoding a putative plasmid partition protein sharing 59% of identities with ParA family protein of *Acinetobacter radioresistens* (ZP_05362210). Primer-3 software was used to design real-time PCR probes and primers, taking into account optimal thermal and chemical conditions for PCR amplification compatible with probe hydrolysis based qPCR (Table 2). The *toxR* probe was dually labeled with 5'-reporter dye Texas Red (wavelength emission at 602 nm) and a downstream 3'-quencher dye BHQ-2. The parA probe consists of an oligonucleotide dually labeled with 5'-reporter dye 6-FAM (wavelength emission at 502 nm) and a downstream 3'-quencher dye Tamra. The specificity of the probes and primers was assessed by using a BLAST (Basic Local Alignment Search Tool) to ensure that they amplify only *parA* and *toxR* genes from pVCR1 plasmid and *V. harveyi* respectively with no homology to other known sequences found in GenBank and EMBL databases. The two probes emitting light at two distinct wavelengths and the two couples of primers were purchased from Eurogentec.

Real-time PCR assays were conducted on a MX3000 Thermocycler (Stratagene) using Brilliant® qPCR Core Reagent Kit (Stratagene). Each reaction was run in duplicate in a final volume of 25 µL containing various concentrations of DNA sample (5 µL), 5 mM MgCl2, 200 µM of each dNTP, 300 nM of each primer, 200 nM of oligonucleotidic probe and 1.25 units of hot start Sure Taq polymerase (Stratagene). The thermal cycle was performed with a two step PCR protocol: 1 cycle at 95°C for 10 min followed by 40 cycles at 95°C for 15°C and 60°C for 90 s. Fluorescence intensity was expressed in delta reporters (dR) after background saturation. Threshold Cycle (Ct) value corresponds to the PCR cycle number at which an increase in reporter fluorescence above a baseline signal was first detected.

2.5. Quantification of *V. harveyi* in artificially spiked samples

An early stationary phase centrifuged pure culture of *V. harveyi* ORM4 strain was centrifuged (10 min at 3200 g), and the bacterial pellet suspended in a determined volume of SASW in order to obtain the desired concentration of bacterial cells. Bacterial cells were enumerated in a *Malassez* counting chamber and ten-fold serial dilutions (from 10^{-3} to 10^{-8}) in SASW were performed to generate the first standard curve. For the second standard curve, SASW was replaced by abalone hemolymph. Each DNA extract was tested in triplicate by real-time PCR. Quantification of bacterial cells by real time PCR assays in spiked samples was compared with enumeration of bacterial cells using a Malassez counting chamber.

2.6. Experimental infection of *H. tuberculata*

After being anesthetized by bathing during 5 min in 5% absolute ethanol-seawater, 25 mature abalones were experimentally infected by injection of 100 µL of *V. harveyi* ORM4 bacterial suspension, at 1.10^5 cells per mL concentration, in the foot of animals. Inoculated abalones were randomly distributed in five 5 L aquaria supplied in filtered (1 µm) seawater at 14-15°C without food supply and under continuous aeration. Seawater was renewed every day. Negative control was constituted a batch of 5 individuals injected with 100 µL of SASW. In order to evaluate the kinetics of infection, one individual was collected in each aquarium at 24, 48, 72, 96 and 120 h. A volume of 200 µL of hemolymph per animal was withdrawn, then centrifuged at 10,000 g for 2 min and DNA extracted. The presence of *V. harveyi* ORM4 was determined and quantified in collected samples by real-time PCR using the appropriate standard curve.

3. Results

3.1. Specificity of the duplex real-time PCR

Real-time PCR assays carried out for the detection of *V.harveyi* and pVCR1 plasmid DNAs were carried out simultaneously on 17 different bacterial strains (Table 1) thanks to a duplex TaqMan procedure adding the two couples of primers and the two differently labeled-fluorogenic probes in the PCR reaction mixture targeting *toxR* and *parA* genes.

The species specificity of the real-time PCR assay developed for the identification of *V. harveyi* was evaluated on nine bacterial strains phylogenically related to *V. harveyi*, including two reference strains, LMG4044^T and LMG7890, isolated from the amphipod *Talorchestia sp* and the brown shark *Carcharhinus plumbeus*, *r*espectively and 8 bacterial strains, isolated from the European abalone *Haliotis tuberculata* or closest abalone environment (algae) (n=6) and from the Japanese abalone *Haliotis discus hannai* (n=2).

Real-time PCR results obtained showed that all tested *V. harveyi* strains were positive (n=10) for the detection of the *toxR* gene, including the LMG4044^T and LMG7890 *V. harveyi* strains and the different *V. harveyi* strains isolated from *H. tuberculata* or algae closed to moribund abalone and *H. discus* during mortality outbreaks (Table 1). In contrast, no positive amplification signals were obtained with other bacterial reference strains phylogenically closed to *V. harveyi*, as for *V. campbellii* bacteria. Results of real-time PCR for the detection of pVCR1 plasmid revealed two positive bacterial strains related to *V. harveyi* species including the virulent ORM4 strain (Table 1). Amplified PCR product sizes obtained from *V. harveyi* ORM4 DNA templates were verified by gel electrophoresis visualizing an attempted size deduced from nucleotidic sequence of 162-bp and 168-bp amplicons for *toxR* and *parA* genes, respectively (Figure 1).

3.2. Sensitivity of the real-time PCR assays

Standard curves for the *toxR* gene quantification were carried out in SASW or hemolymph samples artificially spiked with pure bacterial suspension of *V. harveyi* ORM4 strain of known concentration as determined by Malassez counting method. Ten fold bacterial dilutions ranging from 3.7×10^3 to 3.7×10^8 cells mL^{-1} were prepared in these two diluents. Taking into account the different DNA extraction steps and the use of 5 µL of DNA sample for real-time PCR, genomic DNA extracted from 1.8×10^1 to 1.8×10^6 bacteria was added to each PCR reaction mixture. The threshold cycle (Ct) values deduced from real-time PCR amplifications on purified DNA extracts were plotted to the number of bacteria initially presents in PCR templates.

Both quantification curves achieved for each gene were similar and exhibited excellent correlation coefficients and high PCR reaction efficacies. For the *toxR* gene, correlations of r^2=0.988 and r^2=0.999 and efficacies of 100 % and 103.9 % were obtained in SASW and hemolymph spiked samples, respectively (Figure 2). Concerning the *parA* gene, correlations of r^2=0.998 and r^2=1 and efficacies of 98 % and 100.2 % were obtained in SASW and hemolymph spiked samples, respectively (Figure 3).

Comparison between the different curves revealed that the nature of sample, SASW versus hemolymph, had no effect on the detection levels of *V. harveyi* in the range of cell dilutions used in this study. The threshold sensitivity of these quantification methods targeting either *toxR* or *parA* genes, is given by the lower bacterial concentration tested, and corresponds to a minimal *V. harveyi* cell number-equivalent genomic DNA of 18 in the PCR reaction.

3.3. Kinetics of infection during abalone challenge

The quantification of bacterial DNA amounts present in hemolymph samples from abalone was estimated by reporting Ct values to the standard quantification curve of the *toxR* gene previously established for hemolymph. Real-time PCR assay results showed that four, three, three, five and five individuals among five were detected as positive at 24h, 48h, 72h, 96 and 120h post infection respectively (Figure 4). Bacterial loads of seven infected abalones sampled at 96h and 120h post infection ranged from 3.8×10^3 cells mL^{-1} to 5×10^6 cells mL^{-1} of hemolymph. Nevertheless several bacterial concentrations were not calculated because obtained Ct values were above the linear working range of the standard curve. During all the time course of the experiment, no positive signal was detected in control animals and no mortality was recorded.

4. Discussion

In this work, we developed a rapid and quantitative diagnostic test by real-time PCR allowing the detection of both *V. harveyi* bacteria and pVCR1 plasmidic DNAs. This protocol was elaborated using two pairs of primers and TaqMan probes targeting in one hand the *toxR* gene, a widely used gene in multi-locus sequence typing approach for molecular phylogeny and identification of *Vibrio* (Pang et al., 2006, Rosec et al., 2009, Takahashi et al., 2005a), and in another hand a specific region of the pVCR1 plasmid coding the putative *parA* product. Primers and probes were used simultaneously for duplex real-time PCR detection assays on bacterial colony DNA previously extracted by rapid boiling method or separately for quantification issues. Indeed, for quantification, the use of both probes and couple of primers in the same PCR reaction mixture during our sensitivity assays was shown to adversely impact the efficacy of the TaqMan real-time PCR assay (data not shown).

Most of scientific publications dealing with rapid, sensitive and specific molecular identification of *Vibrio* species use PCR technique. Concerning *Vibrio harveyi*, several PCR assays have been developed (Conejero and Hedreyda, 2003, Fukui and Sawabe, 2008, Pang et al., 2006) aiming to facilitate disease prevention and surveillance in cultured marine animals. More recently Cao et al. (2010) successfully developed a novel nucleic acid amplification diagnostic method based on loop-mediated isothermal amplification (LAMP) to detect *V. harveyi* infection in the cultured gastropod *Babylonia areolata*. Nevertheless all these methods fail to produce quantitative measurements of *V. harveyi* bacterial load in tested samples. According to our knowledge a few number of real-time PCR assays allowing specific detection and quantification of *Vibrio* DNA have been reported in the literature (Saulnier et al., 2009). These reports concern mainly *Vibrio* sp. inducing public health hazard, e.g. *Vibrio vulnificus* (Takahashi et al., 2005a), *Vibrio parahaemolyticus* (Qin et al., 2008, Takahashi et al., 2005b) and *V. cholerae* (Gubala, 2006). Fukui and Sawabe (2008)

developed a real-time PCR assay on *V. harveyi* targeting the 16S rRNA gene. Nevertheless, the high conservation of nucleotidic sequences of this gene in *Vibrionacea* resulted in the failure for some phylogenetically closed *Vibrio* species, *V. rotiferianus* in particular, to find divergent nucleotidic sequence for PCR primer annealing. As a consequence the quantification of *V. harveyi* in mixed cultures of *V. harveyi* and *V. rotiferianus* was underestimated due to competition between both DNA templates during real time PCR even if the specificity of real time PCR was restored by TaqMan probe (Fukui and Sawabe, 2008). In the present study, we have developed a new real-time PCR protocol to detect exclusively *V. harveyi* bacteria and not the closed species belonging to *V. harveyi* group and encompassing at least 5 different species, *e.g. V. rotiferianus, V. campbellii, V. parahaemolyticus, V. alginolyticus, Vibrio mytili*, as revealed by a multi-locus sequence typing study including 8 housekeeping genes (Sawabe et al., 2007b, Thompson et al., 2007). ToxR protein coding gene was targeted because its demonstrated higher inter-species discriminatory power than 16S rRNA gene (Franco and Hedreyda, 2006, Montieri et al., 2010) when sequence of *V. harveyi* and closely related strains genes were aligned and compared.

The specificity of PCR primers and TaqMan probe designed was determined by real-time PCR assays carried out on DNA samples extracted from pure culture of different bacterial strains belonging to the *V. harveyi* group. Only positive fluorescence signals were obtained for DNA samples extracted from the virulent ORM4 reference strain, from the *V. harveyi* reference strains (LMG 7890 and LMG 4044^{T}) or from different bacterial strains belonging to the *V. harveyi,* after 16S rRNA sequencing and *toxR* gene detection by conventional PCR (Conejero and Hedreyda, 2003). In this study, no signal in the other species closed to *V. harveyi*, such as *V. rotiferianus, V. campbellii, V. parahaemolyticus, V. alginolyticus, V. mytili* and reference type strains *Vibrio coralliilyticus and Vibrio brasiliensis* was detected even at high threshold cycle value (Ct>40).

For the *parA* gene amplification, positive signals were detected for DNA samples extracted from only two bacterial strains of *V. harveyi,* the virulent reference strain ORM4 isolated from moribund *H. tuberculata* abalone and the *V. harveyi* strain LEM/07/014 isolated from algae closed to moribund abalone. Contrarily to S35, S20 and LEM/07/001, LEM/07/004 bacterial strains, strain LEM/07/014 is able to induce high mortality after abalone experimental infection (C. Paillard, pers. comm.). No real-time PCR signals were generated with DNA samples extracted either from other *V. harveyi* strains isolated from algae closed to moribund abalone, LEM/07/0012 and LEM/07/0013, or from other tested *Vibrio* reference strains. This result demonstrated the specificity of PCR primers and TaqMan probe designed to target the *parA* gene of the pVCR1 plasmid, with no detectable background signals that could be generated by the presence of bacterial genomic DNA in the tested total DNA extracts.

The standard curves generated by real-time PCR in 10-fold dilutions of SASW or abalone hemolymph samples artificially spiked with the virulent ORM4 strain of *V. harveyi* demonstrated excellent coefficients of correlation for the PCR primers and TaqMan probe used. Moreover, both standard curves were perfectly stackable, indicating that presence of complex DNA matrix in hemolymph DNA samples had no effect on both real time PCR efficiency and sensitivity and that DNA extraction procedure for hemolymph samples was satisfactory. The comparison between Ct values recorded for standard curves generated with each couple of PCR primers and TaqMan probes allowed us to estimate the copy number of plasmid per bacteria. Indeed, for a same sample and whatever the considered dilution, Ct values generated for single-copy *toxR* gene are less than 2 cycles more than Ct values obtained for *parA* gene. Thus, the copy number of pVCR1 per *V. harveyi* cell can be consequently estimated around 2-4 copies per bacteria cell, which make pVCR1 in ORM4 as a very low copy plasmid. A large range of plasmids is often maintained at very low copy numbers per cell such as for the pJM1 plasmid in *Vibrio anguillarum* cells (Di Lorenzo et al., 2003).

Whatever the nature of tested samples (SASW or hemolymph), the detection limits of the real-time PCR assays were estimated at 1.8 x 10^1 bacteria per well. Taking into account the dilution factor employed in this study to extract DNA of biological samples, the detection limit corresponds to a minimal bacterial concentration of 3.7x10^3 mL^{-1} of SASW or hemolymph. Greater detection limits have been reported in the literature concerning other real-time TaqMan PCR protocols targeting other *Vibrio* species in mollusks, such as for the detection methods for *Vibrio aestuarianus* or *V. vulnificus* showing detection limits estimated at 2,3x10^2 and 1.6x10^2 bacteria mL^{-1} which were extrapolated to correspond to 6 and 1.6 cells per PCR well, respectively (Campbell and Wright, 2003, Panicker and Bej, 2005, Saulnier et al., 2009). Thus, practical modifications to increase sensibility of our real time PCR would consist in developing procedures to concentrate bacteria of samples prior to DNA extraction.

Using an invasive way of infection, by experimental injection of ORM4 cells in apparently healthy *H. tuberculata* abalone, we showed in the present study that hemolymph of abalone is a good target tissue to monitor infection, this tissue having the advantage to be not destructive for *H. tuberculata*, allowing broodstock screening to check the presence or absence of virulent *V. harveyi* in indoor breeding structures. The number of infected animals as well as ORM4 bacterial loads in positive animals increased over time, with values ranging from 3.8 x 10^3 *V. harveyi* cells mL^{-1} to 5 x 10^6 cells mL^{-1}. Furthermore 5 abalones among 15 tested were heavily infected by more than 1 x 10^4 *V. harveyi* cells mL^{-1} between 72h and 120h post infection, whereas all the ten abalones tested for the first 48 h of our survey were either found not infected (n=3) or infected at bacterial load lower than 3.7 x 10^3 cells mL^{-1} during our survey.

In conclusion, the developed protocol constitutes a useful molecular tool allowing to detect and to quantify rapidly virulent *V. harveyi* strains in the European abalone *H. tuberculata* that could contribute to safety measures in the production of virulent *V. harveyi*-free broodstock in indoor facilities.

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Tables

Table 1: Specificity of the Taqman real-time PCR assays for the detection of toxR and parA genes according to bacterial strains isolated from different origins and hosts.

Strain	Vibrio species	Source and context of isolation	Real-time PCR results (+/-)	
			***toxR* gene**	***parA* gene**
ORM4	*V. harveyi*	Isolated from moribund abalone, France 1998 (Nicolas et al., 2002)	+	+
LEM/07/001	*V. harveyi*	Isolated from moribund abalone no 7, France 30/07/2007	+	-
LEM/07/004	*V. harveyi*	Isolated from moribund abalone no 9, France 30/07/2007	+	-
LEM/07/012	*V. harveyi*	Isolated from algae closed to moribund abalone 01/08/2007	+	-
LEM/07/013	*V. harveyi*	Isolated from algae closed to moribund abalone France (Morbihan) 01/08/2007	+	-
LEM/07/014	*V. harveyi*	Isolated from algae closed to moribund abalone France (Morbihan) 01/08/2007	+	+
S35	*V. harveyi*	Isolated from dead abalone (*Haliotis discus hannai),* Japan	+	-
S20	*V. harveyi*	Isolated from dead abalone (*Haliotis discus hannai),* Japan	+	-
LMG 4044^T	*V. harveyi*	Isolated from dead amphipod (*Talorchestia sp.*), Massachusetts USA	+	-
LMG 7890	*V. harveyi*	Isolated from brown shark (*Carcharhinus plumbeus*) kidney, Baltimore, USA	+	-
DSMZ 17184^T	*V. brasiliensis*	Bivalve larvae (*Nodipecten nodosus*), Brazil	-	-
DSMZ 17186^T	*V. rotiferianus*	Rotifer *Brachionus plicatilis* culture, Belgium	-	-
DSMZ 19137^T	*V. mytili*	Mussels (Mytilus edulis), Spain	-	-
LMG 11216^T	*V. campbellii*	Isolated from seawater collected at a depth of 800 m (20°30'N, 157°30'E)	-	-
LMG 4409^T	*V. alginolyticus*	Isolated from spoiled horse mackerel (*Trachurus trachurus*) causing food poising, Japan	-	-
LMG 2850^T	*V. para-haemolyticus*	Isolated from a patient suffering from "Shirasu" food poising, Japan	-	-
CIP 107925^T	*V.coralliilyticus*	Bleached tissue of coral, *Pocillopora damicornis,* Zanzibar	-	-

Table 2: Nucleotide sequences and melting temperature (Tm) of primers and probes designed for real-time PCR reaction.

Gene	Function	Name	Sequence (5'-3')	Tm (°C)
toxR	forward primer	*ToxR* F1	cca-ctg-ctg-aga-caa-aag-ca	60
	reverse primer	*ToxR* R1	gtg-att-ctg-cag-ggt-tgg-tt	60
	taqman probe	*ToxR* P1	cag-ccg-tcg-aac-aag-cac-cg	66
parA	forward primer	*ParA* F1	aag-gag-gct-cag-gaa-aaa-gc	60
	reverse primer	*ParA* R1	caa-tgc-aag-gga-tct-gag-gt	60
	taqman probes	*ParA* P1	cag-cag-aat-ggg-cag-cgg-aa	64

Figures

Figure 1: Visualization of qPCR products in agarose gel using DNA from one ORM4 bacterial colony and specific primers and probes for parA (lane 1; 168 bp) and toxR (lane 2; 162 bp) genes. Lane MT corresponds to a size standard DNA ladder.

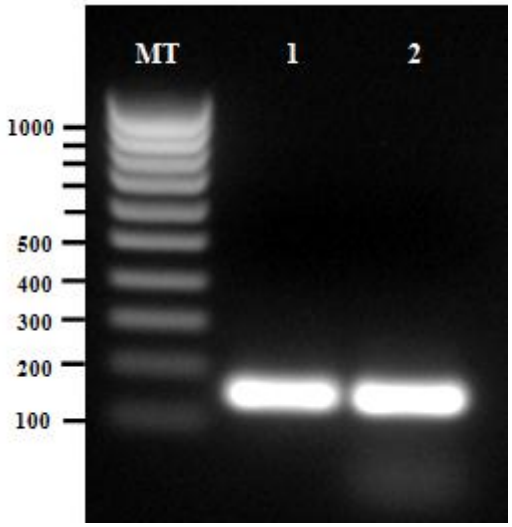

Figure 2. Standard curves for the detection and quantification of the *toxR* gene by real-time PCR in 10-fold dilution range of SASW (solid line) or abalone haemolymph samples (dash line) artificially spiked with the virulent *V. harveyi* ORM4 bacterial strain. Standard curves were generated by plotting the log cell number of bacteria presents in PCR DNA template versus threshold cycle (Ct) values.

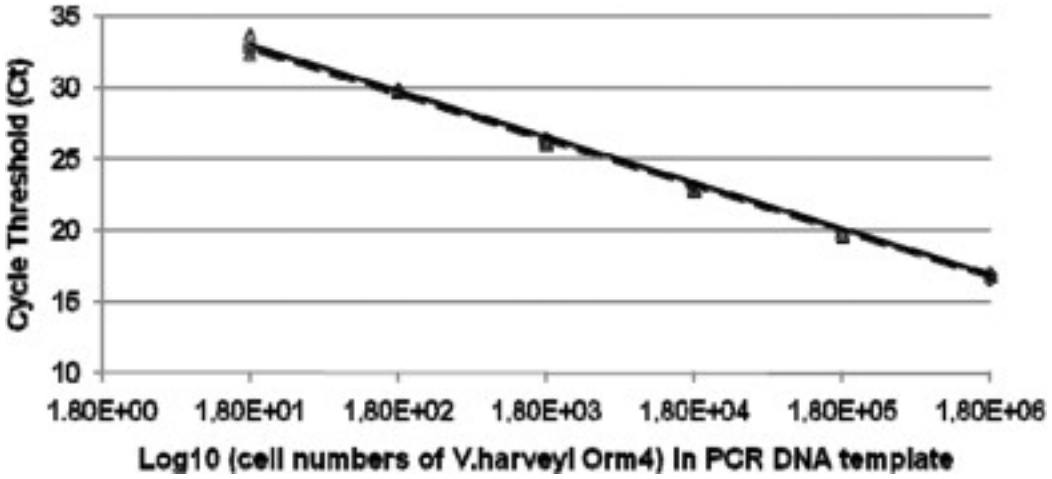

Figure 3: Standard curves for the detection and quantification of the parA plasmid gene by real-time PCR in 10-fold dilution range of SASW or abalone hemolymph samples artificially spiked with the virulent V. harveyi ORM4 bacterial strain. Standard curves were generated by plotting the log cell number of bacteria present in PCR DNA template versus threshold cycle (Ct) values.

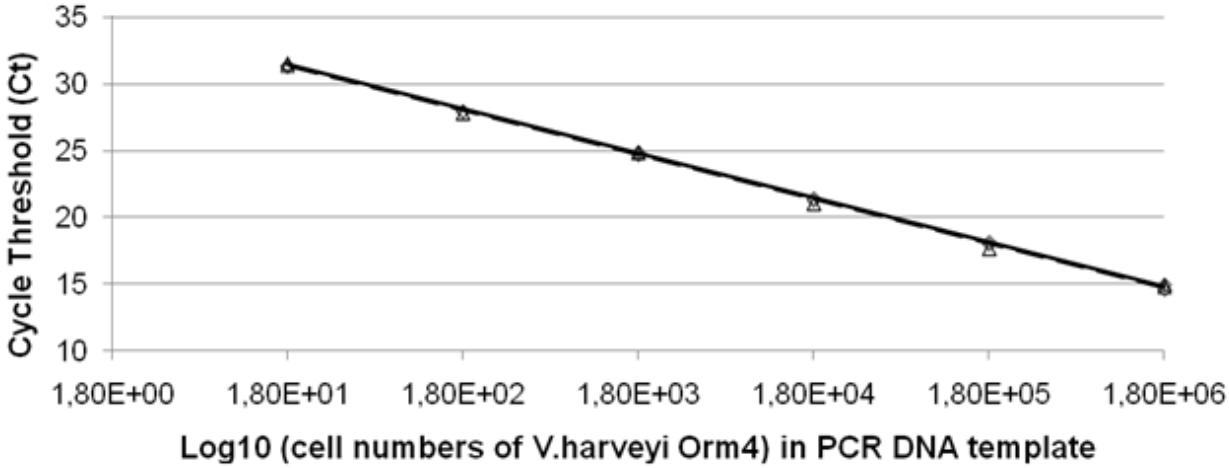

Figure 4: Kinetic of abalone infection by ORM4 V. harveyi strain by toxR real-time PCR in hemolymph samples withdrawn at 24, 48, 72, 96 and 120 post experimental challenge. The sensitivity threshold is indicated by the minimum value of the ordinate axis (3.7x103 cells mL-1). Non-quantifiable ORM4-infected animals (data out of standard curve dilution limits) are represented by an asterisk.