Development of a Taqman real-time PCR assay for rapid detection and quantification of *Vibrio tapetis* in extrapallial fluids of clams

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ABSTRACT

The Gram-negative bacterium *Vibrio tapetis* is known as the causative agent of Brown Ring Disease (BRD) in the Manila clam *Venerupis* (=*Ruditapes*) *philippinarum*. This bivalve is the second most important species produced in aquaculture and has a high commercial value. In spite of the development of several molecular methods, no survey has been yet achieved to rapidly quantify the bacterium in the clam. In this study, we developed a Taqman real-time PCR assay targeting virB4 gene for accurate and quantitative identification of *V. tapetis* strains pathogenic to clams. Sensitivity and reproducibility of the method were assessed using either filtered sea water or extrapallial fluids of clam injected with the CECT4600[T] *V. tapetis* strain. Quantification curves of *V. tapetis* strain seeded in filtered seawater (FSW) or extrapallial fluids (EF) samples were equivalent showing reliable qPCR efficacies. With this protocol, we were able to specifically detect *V. tapetis* strains down to 1.125×10^1 bacteria per mL of EF or FSW, taking into account the dilution factor used for appropriate template DNA preparation. This qPCR assay allowed us to monitor *V. tapetis* load both experimentally or naturally infected Manila clams. This technique will be particularly useful for monitoring the kinetics of massive infections by *V. tapetis* and for designing appropriate control measures for aquaculture purposes.

Subjects Aquaculture, Fisheries and Fish Science, Environmental Sciences, Marine Biology, Microbiology, Molecular Biology
Keywords *Vibrio tapetis*, virB4 gene, Taqman real-time PCR, Molecular diagnostic, *Venerupis philippinarum*, Marine pathogen, Brown ring disease

INTRODUCTION

The Manila clam *Venerupis philippinarum* (Adam & Reeve 1850) is the second most important aquaculture species in the world, after the oyster *Crassostrea gigas* (*Gosling, 2008*). *V. philippinarum* was introduced to French coasts in 1972 from the Pacific Coast of North America for economic purposes. The species progressively colonized the European coasts as it grows faster than the endemic European clam *Venerupis decussatus* (*Flassch & Leborgne, 1994*). In 1987, mass mortalities of cultured clams occurred in the main French production site located at Landéda (Brittany, France). Mortalities have been attributed to the Brown Ring Disease (BRD) due to the pathogenic Gram-negative bacterium *Vibrio tapetis* (*Paillard & Maes, 1990*).

V. tapetis first colonizes the periostracal lamina of the clam between the mantle and the shell. The proliferation of the pathogen in the periostracal lamina and extrapallial fluids (EF) inhibits the normal shell biomineralization process, resulting in a brown deposit of melanised matrix (conchiolin) on the inner surface of the valves (*Paillard & Maes, 1994*; *Paillard & Maes, 1995a*). The clam's extrapallial fluids have been demonstrated to be a major compartment in the early stage of the defense process against the infection (*Allam, Paillard & Maes, 1996*; *Allam, 1998*). Indeed, extrapallial fluids contain numerous hemocytes (*Allam, Paillard & Auffret, 2000*) which are responsible for phagocytosis of micro-organisms (*Allam & Paillard, 1998*). Accumulation of hemocytes suggests an efficient defense system able to neutralize the pathogen before colonization of the extrapallial cavity and eventually tissues and could lead to septicemia (*Allam & Ford, 2006*). In this case, the penetration of *V. tapetis* into the hemolymph can provoke mortality before the clam exhibits BRD symptoms (*Allam, Paillard & Ford, 2002*; *Paillard, Allam & Oubella, 2004*). In a natural environment, the prevalence range of BRD in Manila clam has been estimated as 10–20% (*Paillard et al., 2014*).

Here, pathogenicity and virulence are defined as proposed in *Casadevall & Pirofski (1999)* and *Sparling (1983)*, that is to say as the capacity of a microbe to cause damage to the, and the degree of pathogenicity respectively. A subtractive bank between two *V. tapetis* strains, i.e., the fish pathogen LP2 and the clam pathogen CECT4600$^{\mathrm{T}}$ revealed that some genes are present only in the genome of the *V. tapetis* strain pathogenic to *V. philippinarum* (G Dias, 2015, unpublished data). Among these genes, *virB4* existed only in the genome of the clam pathogen CECT4600$^{\mathrm{T}}$ (accession number: KT382306). This finding suggests that some of these genes may be specifically associated with pathogenicity in the Manila clam. The genomes of several *V. tapetis* strains, including CECT4600$^{\mathrm{T}}$, were recently sequenced (G Dias, 2015, unpublished data), confirming the presence of one copy of the *virB4* gene in the CECT4600$^{\mathrm{T}}$ chromosome, using the MicroScope genomics platform (*Vallenet et al., 2013*). The *virB4* gene encodes a protein involved in a large complex assigned to type IV secretion systems (T4SSs). In bacteria, secretion is essential for virulence and survival. Bacteria use T4SSs to translocate DNA and protein substrates across the cell envelope (*Juhas, Crook & Hood, 2008*; *Low et al., 2014*; *Christie, Whitaker & González-Rivera, 2014*), which contributes to genome plasticity and the evolution of pathogens through dissemination of antibiotic resistance and virulence genes (*Fronzes, Christie & Waksman, 2009*).

Classical methods currently used to identify *Vibrio* species associated with BRD were based on the cultivability of bacteria on non-selective synthetic medium and biochemical criteria (*Borrego et al., 1996*). Phylogenetic and molecular identification of *Vibrionaceae*, at the family and genus levels, can also be obtained by Multi Locus Sequence Typing using the *rpoA, recA* and *pyrH* genes (*Thompson et al., 2005*) or the *gyrB* gene (*Le Roux et al., 2004*; *Yamamoto et al., 2000*). Likewise, Single Specific Primer-PCR (SSP-PCR) amplification related to 16S rRNA identity (*Paillard et al., 2006*) or molecular typing and fingerprinting methods (*Romalde et al., 2002*; *Rodríguez et al., 2006*) have been developed. Drummond and collaborators (*2006*) showed that the combination of shell valve analysis with the SSP-PCR assay of *Paillard et al. (2006)* proved to be most sensitive but was time-consuming

and not specific enough and sensitive enough to detect *V. tapetis* pathogens in clams. These methods are therefore inadequate for monitoring individual clams and above all, inappropriate to detect asymptomatic infected clams.

The aim of this study was to develop a rapid and accurate detection method for *Vibrio tapetis*, the causative agent of BRD, which is of growing interest due to the increased prevalence of the disease and the high commercial value of clams. Taqman real-time PCR protocols have previously been carried out for *V. aestuarianus* (*Saulnier, De Decker & Haffner, 2009*; *McCleary & Henshilwood, 2015*) and *V. harveyi* (*Schikorski et al., 2013*). In this paper, we developed a Taqman real-time PCR assay for specific and rapid *V. tapetis* detection and quantification from extrapallial fluids of clams, and validated it on both pure cultures and through an experimental infection of *V. philippinarum* by a virulent *V. tapetis* strain.

MATERIALS AND METHODS

Bacterial strains and culture conditions

Bacteria belonging to twelve *Vibrio* species and seventeen strains of *V. tapetis* isolated from clams and fishes were used in this study (Table 1) as positive and negative controls to check species specificity of the designed primers. Some reference strains were purchased from CIP and LMG collections. *V. anguillarum* strain 775 was supplied by JH Crosa (*Frans et al., 2011*; *Crosa, Schiewe & Falkow, 1977*). The Norwegian S2-2 strain was kindly offered by S Mortensen (2009, unpublished data) from the Institute of Marine Research at Bergen in Norway. Finally, the FPC 1121 strain was graciously provided by T Matsuyama (*Matsuyama et al., 2010*). The other strains were available from the LEMAR collection.

The *V. tapetis* CECT4600[T] strain was used for the experimental infection as the reference strain (*Paillard & Maes, 1995b*; *Borrego et al., 1996*).

All bacterial strains were cultured in Zobell medium (pastone 4 g/L, yeast extract 1 g/L, sea salt 30 g/L at pH 7.4) enriched with iron phosphate (0.1 g/L) at 18 °C during 18 h under constant shaking at 180 rpm (Infors HT[®]) (*Balboa et al., 2012*).

Clams, experimental infection and extrapallial fluids collection (EF)

Two year old *Venerupis philippinarum* were provided by Fabien Fonteneau in Marennes-Oleron ("Les Claires de Bonsonge"[®], EARL, brood stock producer). A first health diagnostic was performed *in situ* on 50 clams to ensure absence of BRD, prior to the sampling effort for the bacterial challenge. They were transferred to the Ifremer's facilities (Laboratoire de Physiologie des Invertébrés, LEMAR, Plouzané) for six days quarantine with chloramphenicol (8 mg/L; Sigma Aldrich, St. Louis, MO, USA) at 13 °C.

For the duration of the experiment, clams were split randomly into eighteen 100 L-tanks with air-lift systems and a complete water-renewal every two days. During the whole experiment, clams were daily fed an algal ration (maintenance ratio from FAO, 2004) of two algae commonly used in aquaculture (*Isochrysis affinis galbana* and *Chaetoceros calcitrans*) (Figs. 1 and S1A).

Four weeks after algal-conditioning, clams were exposed to air for 12 h in the experimental room and replaced into water just before injection to facilitate valve opening.

Table 1 Bacterial strains used in this study isolated from different hosts and origins, and specificity of Taqman qPCR method for the detection of *virB4* gene in strains pathogenic for clams.

Strain	*Vibrio* species	Source and location isolation	Strain reference	Virulence[a]	qPCR[b]
CECT4600[T]	V. tapetis	Venerupis philippinarum, Landéda, France	Paillard & Maes, 1995b	+[d]	+
FPC 1121	V. tapetis	Venerupis philippinarum, Japan	Matsuyama et al., 2010	+	−
IS 1	V. tapetis	Venerupis philippinarum, Landéda, France	Borrego et al., 1996	+[d]	+
IS 5	V. tapetis	Venerupis philippinarum, Landéda, France	Borrego et al., 1996	+[d]	+
IS 7	V. tapetis	Venerupis philippinarum, Quiberon, France	Borrego et al., 1996	+[d]	+
IS 8	V. tapetis	Venerupis aurea, Quiberon, France	Borrego et al., 1996	+[d]	+
IS 9	V. tapetis	Cerastoderma edule, Quiberon, France	Borrego et al., 1996	+[d]	+
P16B	V. tapetis	Venerupis philippinarum, Morbihan Gulf, France	Allam, Paillard & Ford, 2002	+[d]	+
RD 0705	V. tapetis	Venerupis decussatus, Galicia, Spain	Novoa et al., 1998	+[d]	+
RP 11.2	V. tapetis	Venerupis philippinarum, Landéda, France	Borrego et al., 1996	+[d]	+
RP 2.3	V. tapetis	Venerupis philippinarum, Landéda, France	Borrego et al., 1996	+[d]	+
RP 8.17	V. tapetis	Venerupis philippinarum, Landéda, France	Borrego et al., 1996	+[d]	+
RP 9.7	V. tapetis	Venerupis philippinarum, Landéda, France	Borrego et al., 1996	+[d]	+
UK6	V. tapetis	Venerupis philippinarum, Poole Harbour, UK	Allam, Paillard & Auffret, 2000	+[d]	+
S2-2	V. tapetis	Solea Solea, Norway	S Mortensen, 2009, unpublished data	−[c]	−
LP2	V. tapetis	Symphodus melops, Bergen, Norway	Jensen et al., 2003	−[d]	−
HH6087	V. tapetis	Hippoglossus hippoglossus, Inverailort, UK	Reid et al., 2003	−[d]	−
LMG 20012[T]	V. tasmaniensis	Salmo Salar L., Tasmania Australia	Thompson, Thompson & Swings, 2002	−[d]	−
LMG 4042[T]	V. splendidus	marine fish	Le Roux et al., 2004	−[d]	−
LMG 19703[T]	V. shilonii	Oculina patagonica, Mediterranean sea	Kushmaro et al., 2001	nd	−
LMG 20539[T]	V. kanaloe	Ostrea edulis larvae, France	Thompson, 2003	nd	−
CIP 107166[T]	V. lentus	cultivated oyster, Spain	Macián et al., 2001	nd	−
LMG 16745[T]	V. chagasii	marine fish	Le Roux et al., 2004	nd	−
775 (ATCC 68554)	V. anguillarum	Oncorhynchus kisutch, US Pacific Coast	Crosa, Schiewe & Falkow, 1977	nd	−
02/041	V. aestuarianus	Crassostrea gigas, Argenton, France	Garnier et al., 2007	nd	−
P9	Halomonas sp. 33	Venerupis philippinarum, Marennes, France	Paillard et al., 2006	−[d]	−
CF6	V. splendidus	Crepidula formicata	Choquet, 2004	−[d]	−
GM4	Vibrio. sp.	Venerupis philippinarum, Morbihan Gulf, France	Paillard et al., 2006	−[d]	−
ORM4	V. harveyi	moribund abalone, France	Austin & Zhang, 2006	−[c]	−

Notes.

[a] Virulence *in vivo* on *Venerupis philippinarum*.

[b] Real-time PCR results.

[c] Unpublished.

[d] Published on Choquet thesis *Choquet, 2004*.
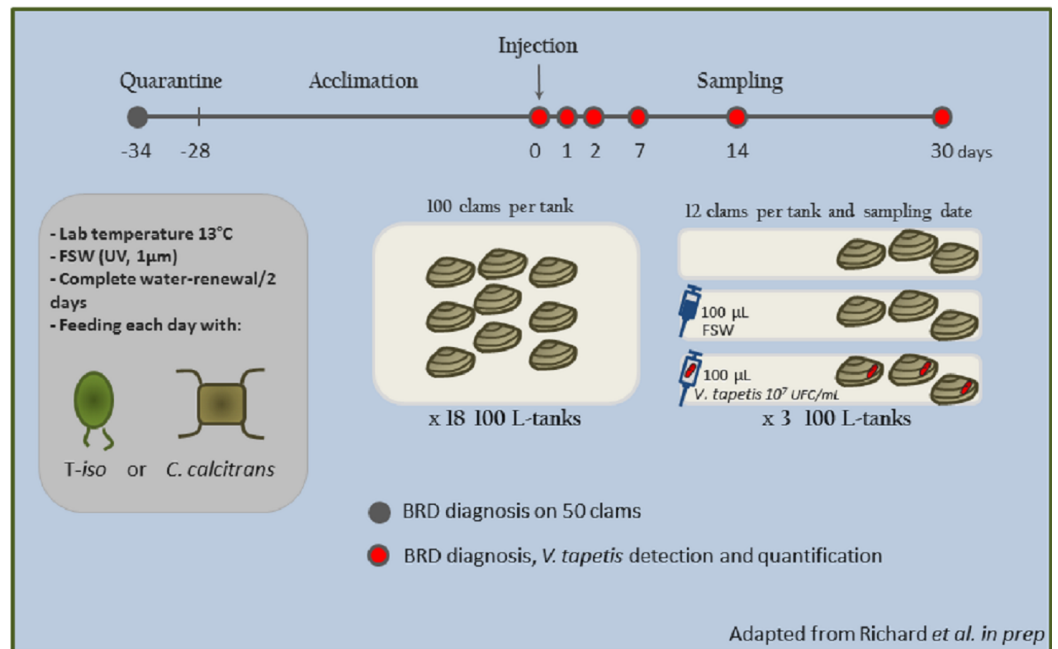
[nd] Not determined.

Figure 1 Schematic view of the infection procedure.

Seventy two clams were injected, in the extrapallial cavity, with 100 µL of Filtered Sea Water (FSW), 72 were injected with 100 µL of *V. tapetis* CECT4600[T] fresh suspension, at a 10^7 cells per mL density and the last 72 clams were not injected (Fig. 1).

Twelve samples from each three different conditions (a total of 36 clams) were sampled at six different time points (216 clams sampled in total): 0d, 1d, 2d, 7d, 14d and 30d (respectively 0—not injected, 1, 2, 7, 14 and 30 days post injection). For each clam, 500 µL of extrapallial fluids were collected close to the shell under mantle, using a syringe fitted with a 25-G needle. The fluids were immediately flash frozen in liquid nitrogen and stored at −80 °C until DNA extraction.

Extrapallial fluids from an additional 40 clams were withdrawn and pooled to constitute the bacteria dilution range. Fifteen mL of the resulting pool were filtered from 80 µm to 0.22 µm, with intermediary filtrations at 10 µm, 1 µm and 0.45 µm, to obtain extrapallial fluids free of bacteria.

The presence of *V. tapetis* CECT4600[T] was determined and quantified in collected samples by real-time PCR using the appropriate standard curve.

BRD diagnostic method

After fluid collection, clams' shells were retrieved at each sampling in order to diagnose BRD occurrence. Images of inner shells were obtained using a 50 mm Canon® macro-scopic lens and analyzed using image analysis software (Visilog® 6.6) (Richard et al., 2015, unpublished data). Subsequently, clams exhibiting brown ring deposit surfaces were defined as BRD positive (BRD+) in this study (Fig. 2). Those that were visibly healthy were reported as BRD negative (BRD−).
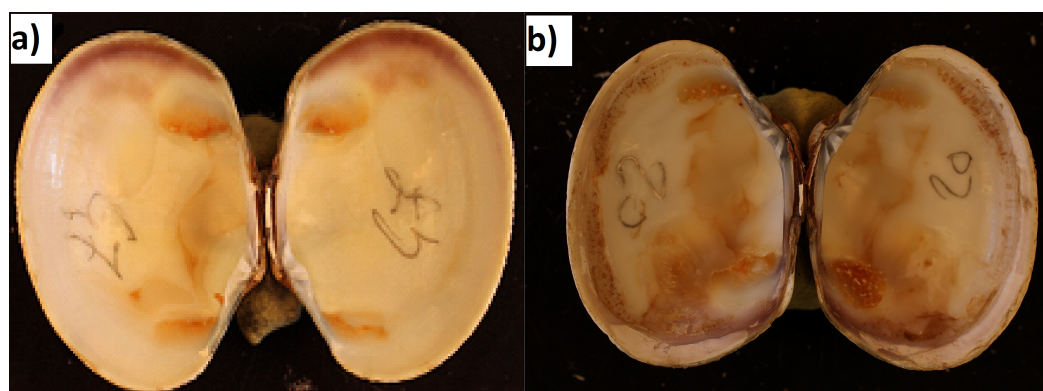
Figure 2 **Photography of (A) BRD- clam and (B) BRD+ clam.** From Richard et al., 2015, unpublished data.

Total DNA extraction from bacterial culture and from EF

DNA extraction was performed using the QIAamp DNA mini kit (Qiagen) for both bacterial cultures and total extrapallial fluids. 450 μL were centrifuged at 10,000 g at 4 °C for 10 min. Pellets were deproteinized in a hot dry bath at 56 °C by addition of 180 μL of ATL Buffer supplemented by 20 μL of proteinase K during one hour. DNA extractions were then performed according to supplier instructions. Finally, DNA were eluted in 200 μL of ultra-pure water and stored at −20 °C until use. DNA yield and purity were determined by spectrophotometry (Quantifluor dsDNA kit; Promega, Madison, WI, USA; and POLARstar Omega microplate spectrophotometer; BMG Labtech, Orgenberg, Germany).

Enumeration of *V. tapetis* by spectrophotometry

In order to accurately inoculate CECT4600$^{\text{T}}$ at the target dilution of 10^7 cells/mL during the experiment, enumeration of bacteria was performed to measure the absorbance at 492 nm on a Multiskan spectrophotometer (Fisher Scientific, Hampton, NH, USA). *Vibrio tapetis* culture density was calculated according to the formula 1.3 $10^9 \times \text{DO} - 3.6\ 10^7$ CFU/mL (*Choquet, 2004*; *Le Bris et al., 2015*).

For the dilution ranges of bacteria (described below), an early stationary phase culture of CECT4600$^{\text{T}}$ was enumerated, and was first diluted in Filtered SeaWater (FSW) to obtain a 10^8 cells/mL suspension. Bacteria were then serially diluted in FSW in a final volume of 500 μL from 2.25 10^7 to 0.565 10^1 cells/mL (ten-fold dilutions until 2.25 10^1 and half dilutions for the last two) to generate the standard curve of bacteria. DNA was extracted from 450 μL of each diluted suspension. For the second standard curve in extrapallial fluids, FSW was replaced by filtered EF prepared previously. DNA extractions were also performed on 450 μL of sterile EF in the same conditions in order to establish a negative control for the standard curve.

PCR primer and fluorogenic probe design

Oligonucleotide sequences used in this study are listed in Table 2. The *virB4* primers and probe were developed to target exclusively the virB4 gene existing in the pathogenic

Table 2 Nucleotide sequences and melting temperatures (T_m) of primers and probe designed for real-time PCR reaction, targeting the *virB4* gene.

Description	Name	Sequence (5′–3′)	T_m (°C)
Forward primer	virB4-F3	TTA-AAA-GTG-GCG-GAG-GAA-TG	58
Reverse primer	virB4-R3	AAG-CTC-TGC-ATC-GGT-TAG-GA	60
Taqman probe	virB4-P1	CGA-GTA-CCA-ACA-TGC-CTT-CCC-GT	53.7

Vibrio tapetis CECT4600^T (Genbank accession number: KT382306). Using Primer-3 software, several primers pairs were designed and tested with DNA samples of different species and strains of the *Vibrio* genus available as positive and negative controls on a 9700 ABI® thermocycler (Applied Biosystems, Foster City, CA, USA) (Table 1). The choice for the best primer pair was determined, with optimal concentration and reaction conditions for PCR amplification compatible with the hydrolysis Taqman probe (Table 2). The *virB4* probe was dually labeled with 5′-reporter dye 6-FAM (wavelength emission at 502 nm) and a downstream 3′-quencher dye TAMRA.

The selectivity of the primers was checked using the BLAST algorithm of the NCBI database (http://www.ncbi.nlm.nih.gov/), assuring their specificity for the *virB4* region, without homology to other known sequences described in GenBank and EMBL databases. Oligonucleotides were also aligned to the CECT4600^T and LP2 genomes to ensure that they selectively amplified only the *virB4* gene. Primers and probe were purchased from Eurogentec (Angers, France). The expected length of the amplicon deduced from nucleotide sequence was 173 bp, and the selectivity was also theoretically assessed by BLAST.

Quantitative real-time PCR (qPCR)

Real-time PCR was performed on a LightCycler 480 Instrument (Roche Diagnostics, Mannheim, Germany) using LightCycler 480 Probe Master Mix based on Taqman detection (Roche Diagnostics). Each real-time PCR experiment included technical triplicates, in a final volume of 15 μL. Each reaction contained 5 μL of DNA template, 0.5 μM of each primer, 0.1 μM of hydrolysis probe virB4-P1 and 7.5 μL of LC480 Probe Master Mix 2X. A single initial denaturation step of 10 min at 95 °C was followed by 45 cycles of 95 °C for 10 s (denaturation), 54 °C for 20 s (annealing) and 72 °C for 1 s to measure the fluorescence signal. Finally, a cooling step at 40 °C during 10 s was included. The results were analyzed with Roche LightCycler 480 software. Threshold cycle (Ct) value corresponds to the PCR cycle number at which an increase in reporter fluorescence above a baseline signal was first detected, after background subtraction. Negative controls with molecular biology grade water as template were performed in each run. For quantitation, dilution ranges of *V. tapetis* were tested in triplicates across multiple orders of magnitude described above. Results were analyzed by linear regression to calculate the slope. The PCR amplification efficiency (E) was measured according to $E = [10^{1/(-slope)}] - 1$, using the Roche LightCycler 480 software. Standard curve data points were used to determine real-time PCR quantification and detection limits through the assessment of the variance (standard deviation), measured at each dilution standard. In conformity with ISO 16140,
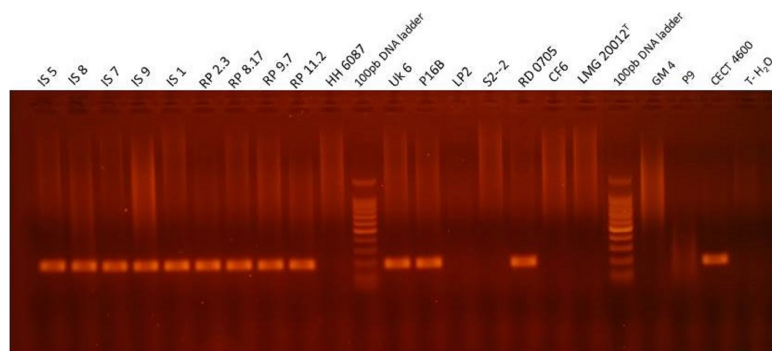
Figure 3 Visualization of the PCR product in agarose gel obtained with qPCR *virB4* assay for representative strains of *Vibrio*, i.e., which were tested positive and negative for BRD development after an infection experiment. Lanes MT corresponds to the BenchTop DNA ladder (Promega, Madison, WI, USA). T-H$_2$O represents the water negative control.

the limit of detection (LOD) was calculated in accordance with 95% of 20 tested replicates giving positive amplification results (*Bustin et al., 2009*). The repeatability (intra-assay variance) was estimated using triplicates of each template to assess the precision of the method. The reproducibility (inter-assay variance) was calculated using standard deviation (SD) of two *virB4* gene standard concentrations used for each run to assess the variation between runs.

RESULTS

Selectivity of the real-time PCR protocol

Sequence alignments of the real-time Taqman PCR amplicon showed no cross reactivity with others species when compared using the BLAST analysis program. Only one theoretical partial cross reactivity with *V. tasmaniensis* (KP795691.1, 78% of identity with differences in 3′ end terminals of both forward primer and Taqman hydrolysis probe) appeared with BLAST analysis when comparisons were extended (more dissimilar sequence search settings). The species selectivity of the real-time PCR assay developed for the identification of *V. tapetis* was empirically evaluated in this study on 17 *V. tapetis* strains (Table 1), including two reference strains CECT4600[T] and LP2, isolated from the Manila clam *Venerupis philippinarum* and the fish *Symphodus melops*, respectively, and 12 marine pathogens, mostly *Vibrio*, except for one (*Halomonas sp.*). Real-time PCR results obtained showed that all virulent *V. tapetis* strains tested, i.e., able to reproduce BRD after injection into the pallial cavity (*Choquet et al., 2003*; *Paillard, Allam & Oubella, 2004*), were positive (except for FPC1121 strain, a Japanese *V. tapetis*), whatever the geographical origin of the bacterial strain. For example, UK6 and IS9 strains were clearly positives though they were isolated from *V. philippinarum* in United Kingdom and from *C. edule* in France, respectively. Moreover, the LP2 reference strain which is a pathogen for *S. melops* (*Jensen et al., 2003*) but not for the Manila clam (*Paillard, Allam & Oubella, 2004*), did not show any signal for the *virB4* gene (Fig. 3). No amplification was detected with bacterial strains belonging to the other *Vibrio* species (*n* = 12), even for the closest phylogenetic neighbors

to *V. tapetis*, among tested strains (*V. tasmaniensis*, *V. spendidus*, *V. lentus*) (*Thompson, Thompson & Swings, 2002*; *Le Roux et al., 2004*; *Sawabe et al., 2013*; *Al-saari et al., 2015*), in agreement with the empirical and theoretical results generated by BLAST.

Positive signals obtained by real-time PCR experiments for bacterial strains were checked by electrophoresis. The amplicon observed at 173 bp, corresponds to the expected size, calculated from the nucleotide sequence of the *virB4* gene (Fig. 3).

Sensitivity of the real-time PCR assay

Standard curves for *virB4* gene quantification were generated in parallel in filtered sterile water (FSW) and total extrapallial fluids (EF) with pure bacterial suspension of *V. tapetis* CECT4600^T strain of known concentrations, determined by spectrophotometry and checked by the Malassez counting method. Ten-fold bacterial dilutions ranging from $2.25 \ 10^7$ to $2.25 \ 10^2$ cells mL^{-1}, and two last half dilutions to $0.565 \ 10^1$ cells mL^{-1} were prepared in these two diluents. Since only one copy of the chromosomal *virB4* gene is present per bacteria, the threshold cycle (Ct) values deduced from real-time PCR amplifications on purified DNA extracts were plotted to the number of bacteria initially present in PCR templates.

Because similar slopes were achieved for quantification curves of each diluent in this study (data not shown), the standard curve in EF was chosen for analyzing the experimental infection samples as this diluent corresponded to the *in situ* conditions of the samples, namely *V. tapetis* in EF.

The standard curve reliably showed linearity across 8 orders of magnitude, from $2.25 \ 10^7$ to $1.125 \ 10^1$ cells mL^{-1}. The final dilution ($0.565 \ 10^1$ cells mL^{-1}) produced less consistent results, and was not retained for the calculation of the standard curve. After extractions, genomic DNA was checked by spectrophotometry and corresponded to values ranging from 900 pg μL^{-1} to 156 pg μL^{-1}. According to the Ct values obtained in triplicates, quantification curve exhibited an excellent linear regression with an r^2 correlation coefficient of 0.99 and a PCR efficacy of 103% (Fig. 4) calculated in the linear zone according to the MIQE guidelines (*Bustin et al., 2009*). The intra-assay variance was evaluated from 0.01 and 0.32 from all the samples tested in triplicate. The reproducibility, calculated using the standard deviation of Ct values generated from two standard concentrations of $2.25 \ 10^2$ cells mL^{-1} and $2.25 \ 10^6$ cells mL^{-1} from different runs, corresponded to 0.14 and 0.10 respectively.

The threshold sensitivity of this method in targeting the presence of *virB4* gene and quantification is given by the lower bacterial concentration detected in the linear zone (at least 95% of 20 tested replicates), and corresponds to the limit of detection (LOD) of *V. tapetis* of $1.125 \ 10^1$ bacteria mL^{-1}.

Specificity of the method: kinetics of infection during Manila clams challenge

The quantification of CECT4600^T DNA in extrapallial fluids sampled from infected clams was estimated by reporting Ct values to the standard quantification curve of the *virB4* gene
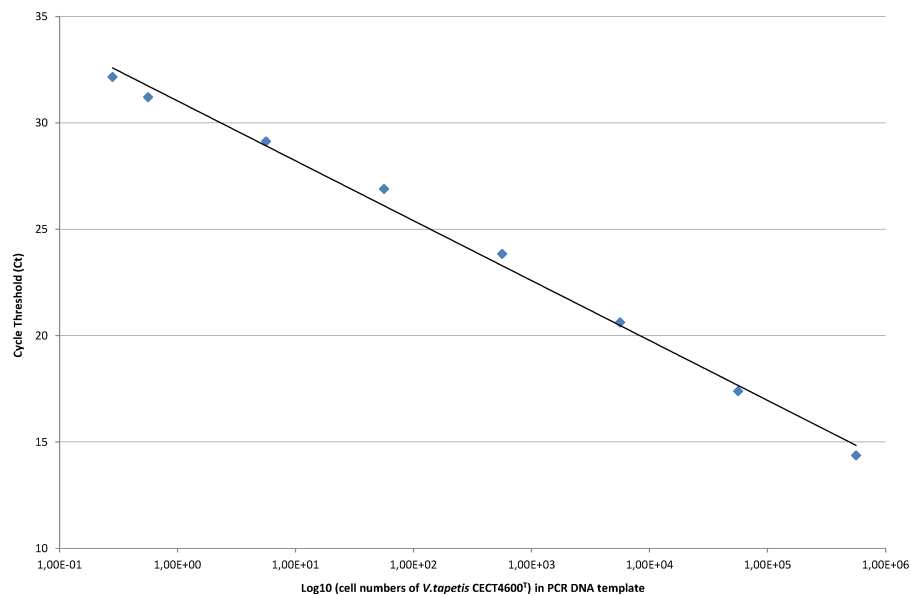
Figure 4 Standard curve for the detection and quantification of the virB4 gene by Taqman real-time PCR, in dilution range of EF samples artificially spiked with CECT4600[T] bacterial strain. Standard curve was generated by plotting the log cell number of bacteria present in PCR DNA template against Ct values.

previously established for EF. The limit of detection established previously corresponded to a cell density of $1.125\ 10^1$ bacteria per mL of extrapallial fluids.

Overall during the experiment, each sampling time of CECT4600[T] injected clams exhibited at least two positive individuals by the real-time PCR assay (Fig. 5). Only 24 h after injection, nine individuals among twelve were spotted with the *virB4* detection protocol. At the same time, all of them were BRD−. It was noticeable that at 7 days post-injection only two real-time PCR positive animals were detected, but with a high *V. tapetis* load, at nearly 10^7 cells per mL in EF ($6.53\ 10^6$ cells mL^{-1}). As regards the BRD among CECT4600[T]-injected clams identified as positive by real-time PCR, one individual at 2 days and one at 7 days were classified as BRD+. At 14 and 30 days post-injection, four clams at each time were positive according to the qPCR assay and they were all BRD+. All these *V. tapetis*-positive individuals showed a bacterial load above 10^2 cells per mL in extrapallial fluids, with a maximum of $1.11\ 10^4$ cells per mL in extrapallial fluids.

The *virB4* gene was also detected in several non-injected individuals: three animals on the day of infection; and one each at 2, 7 and 30 days during the experiment (Fig. 6 and the points 0d on Figs. 5 and 7). The animals were BRD− at 0 and 30 days of the experiment whereas the two positive *virB4* clams (2d and 7d on Fig. 6) displayed clinical signs of BRD.

Regarding the detection of *virB4* gene by real-time PCR assay on animals injected with FSW (Fig. 7), one individual tested positive on the day of the infection. Six animals were identified as positive 24 h after the injection of FSW. Two days post-injection, two clams among 12 were positive according to the Taqman protocol and at later times, only one clam was tested positive at 7, 14 and 30 days post-injection.
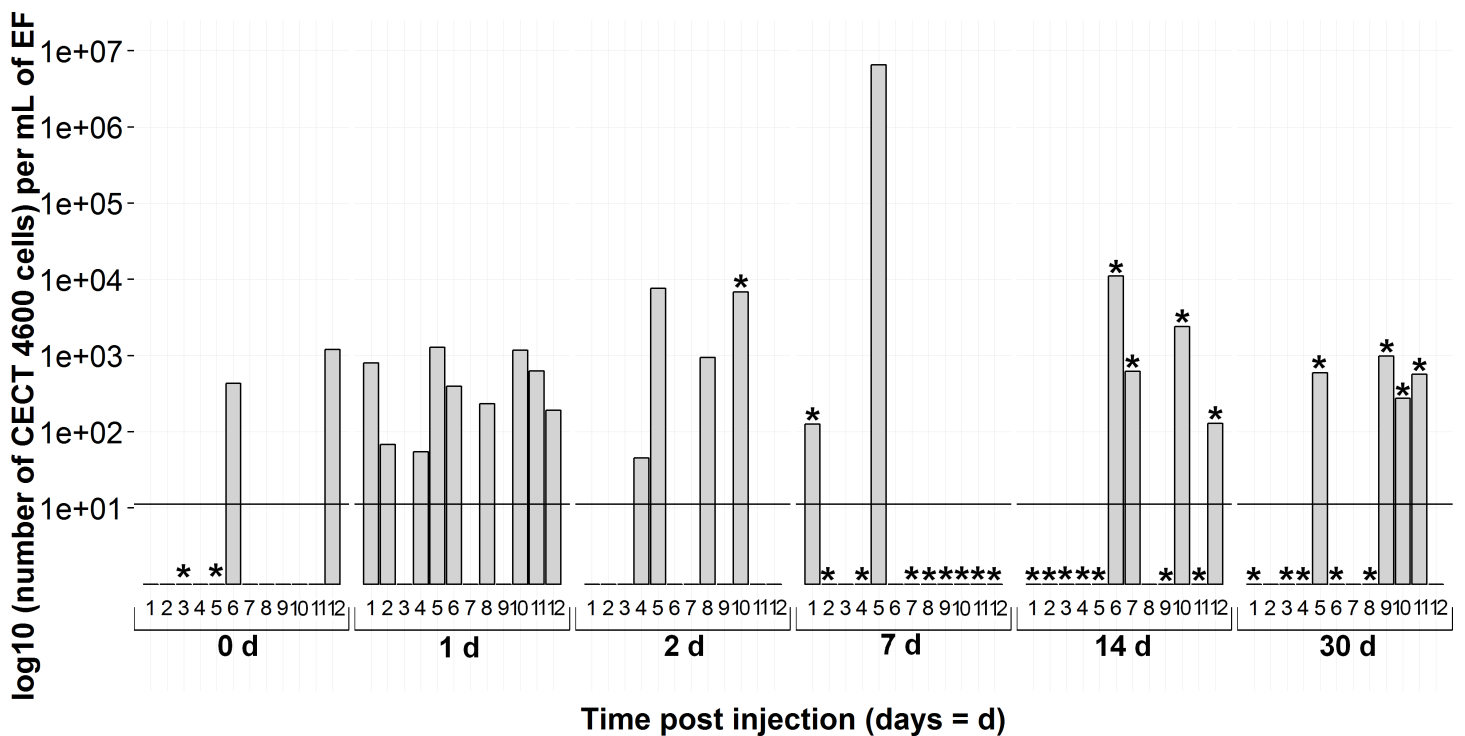
Figure 5 **Kinetics of clam infection by CECT4600[T] *V. tapetis* strain by *virB4* real-time PCR in extrapallial fluids sampled at 0, 1, 2, 7, 14 and 30 days post-injection.** 0d means not injected. * corresponds to BRD+ clam.

DISCUSSION

In this study, we developed a rapid and accurate real-time PCR process for the detection and quantification of *V. tapetis* pathogens for clams in extrapallial fluids of the Manila clam. This protocol was designed using a pair of primers and a Taqman probe targeting the *virB4* gene of *V. tapetis*, encoding a protein engaged in a large complex of type IV secretion systems.

Several publications dealing with rapid and specific molecular identification of *Vibrio* species use PCR techniques. Various PCR assays have been published (*Thompson et al., 2005*; *Paillard et al., 2006*; *Rodríguez et al., 2006*) that have investigated *Vibrio tapetis*. These methods, based on 16S rRNA sequences are less reliable and can be time-consuming when associated with the BRD diagnostic necessary to achieve *Vibrio tapetis* identification (*Drummond et al., 2006*). Moreover, these protocols do not allow quantification of the bacteria.

According to our knowledge, most real-time PCR assays targeting detection and quantification of *Vibrio* species have been developed in *Vibrio* spp. impacting public health risk, e.g., *Vibrio cholerae* (*Gubala, 2006*; *Mehrabadi et al., 2012*), *Vibrio vulnificus* (*Garrido-Maestu et al., 2014*). Only rarely have studies dealt with marine pathogenic *Vibrio*, e.g., *V. aestuarianus* and *V. harveyi* (*Saulnier, De Decker & Haffner, 2009*; *Schikorski et al., 2013*). In this work, we have performed for the first time a real-time PCR protocol to detect *V. tapetis* bacteria.
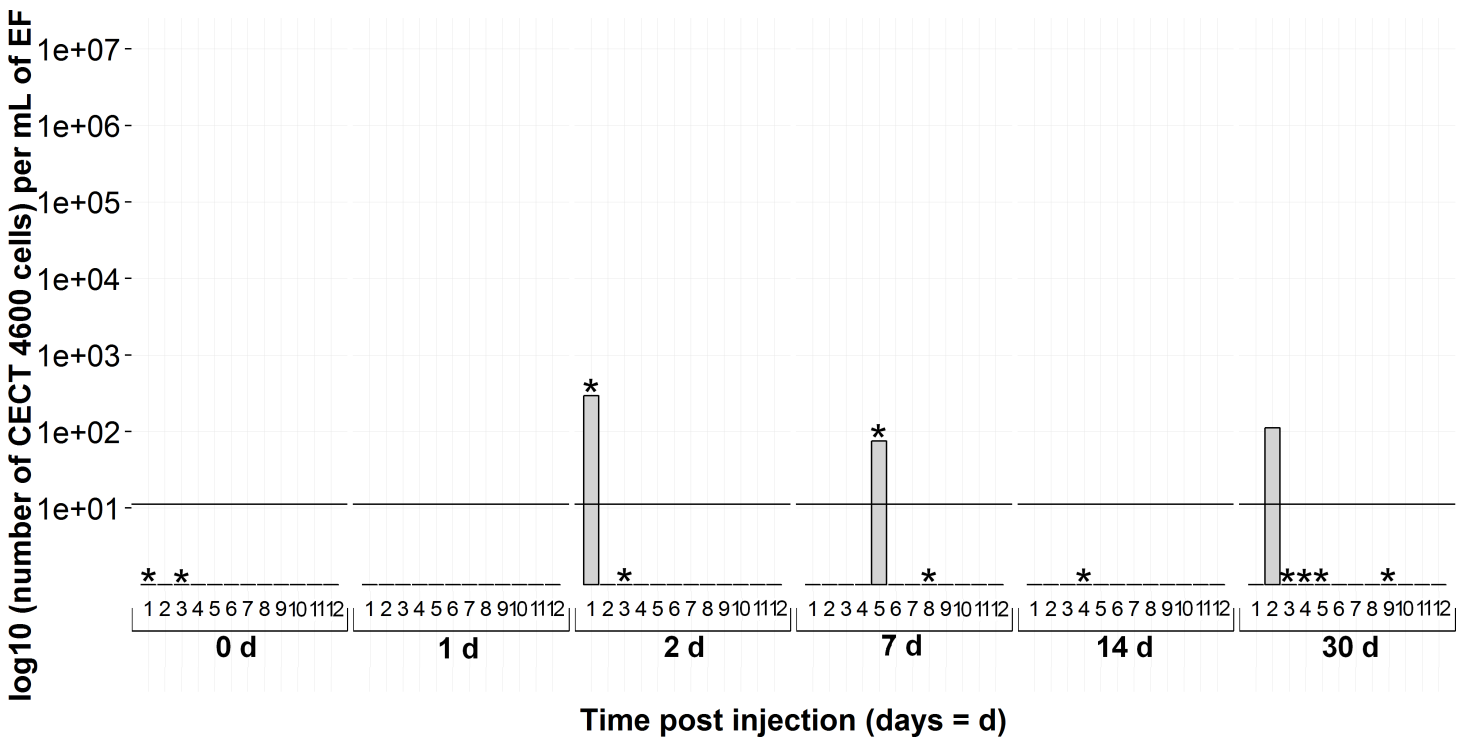
Figure 6 Kinetics of non-injected clams by *virB4* real-time PCR in extrapallial fluids sampled at 0, 1, 2, 7, 14 and 30 days of sampling during the experiment. * corresponds to BRD+ clam.

The specificity of primers and Taqman probe have been demonstrated here with the real-time PCR assays carried out on DNA samples extracted from pure cultures of various bacterial strains belonging to the *V. tapetis* group. No signal was obtained with other species close related to *V. tapetis* (belonging to *V. splendidus* or *V. tasmaniensis*), even at high threshold cycle values (Ct > 45). Positive fluorescence signals were acquired with all *V. tapetis* strains virulent for *V. philippinarum*, except the FPC1121 Japanese strain (Table 1). We suggest that this strain lacks the *virB4* gene and we could speculate that this Japanese strain uses a different secretion system from the type IV to translocate substrate for its virulence (G Dias, 2015, unpublished data).

The standard curves generated by real-time PCR in filtered sea water and extrapallial fluids demonstrated excellent coefficients of correlation for the primers and Taqman probe used. Furthermore, both standard curves were stackable indicating that the DNA extraction procedure for extrapallial fluids samples was satisfactory. Taking into account the dilution factor employed in this study to extract DNA from biological samples, we established the calculated threshold sensitivity of the method at 2.8×10^{-1} cells per well. Thus, the limit of detection of the *virB4* real-time PCR assay corresponds to 11.25 bacteria per mL of FSW or EF of clam. This limit of detection is very low compared to *V. harveyi* and *V. aestuarianus* detections methods, which are at 18 and 1.6 bacteria per well respectively (*Schikorski et al., 2013*; *Saulnier, De Decker & Haffner, 2009*) and suggests our technique is useful for detecting both a weak and massive infections of *V. tapetis* in Manila clam.
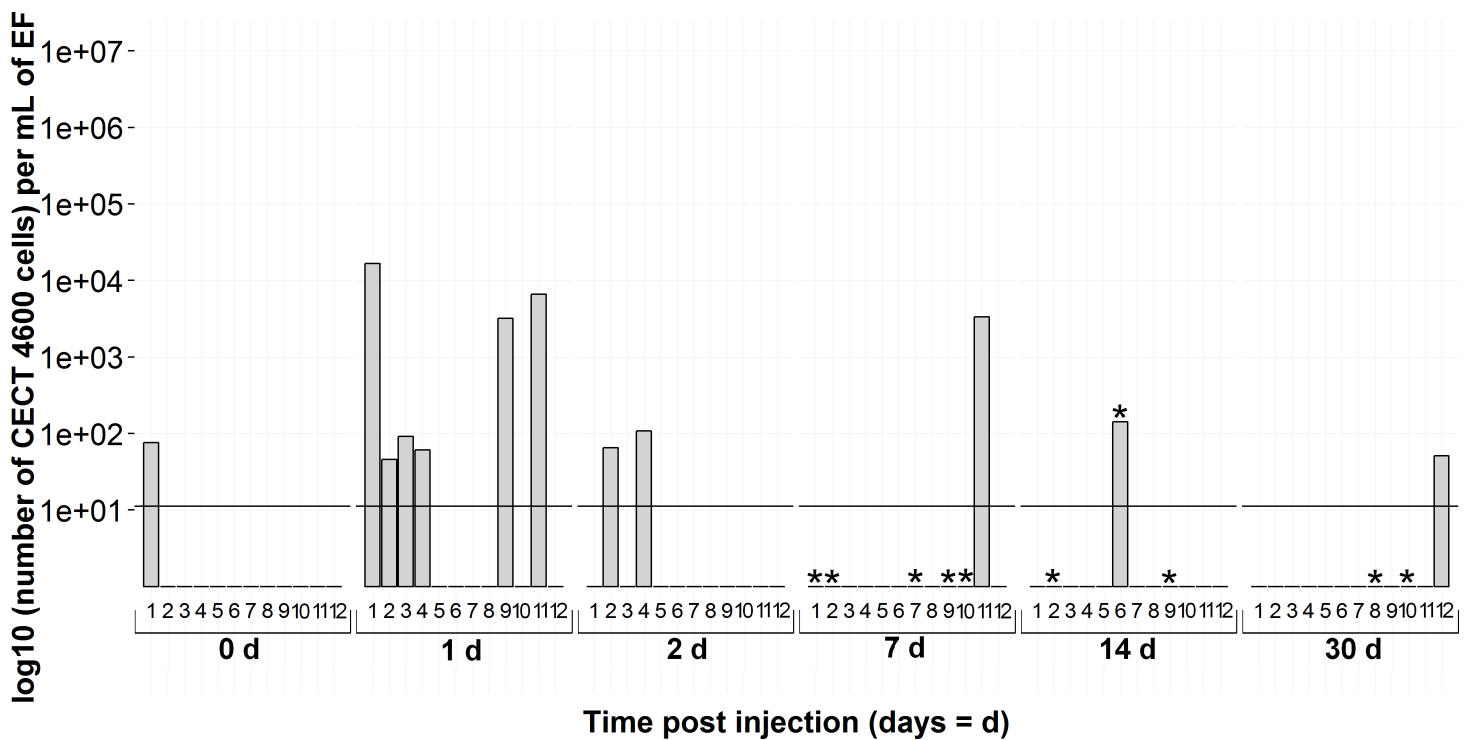
Figure 7 Kinetics of FSW-injected clams by *virB4* real-time PCR in extrapallial fluids sampled at 0, 1, 2, 7, 14 and 30 days of sampling during the experiment. 0d means not injected. * corresponds to BRD+ clam.

We used the real-time PCR developed in this study to monitor the kinetics of *V. tapetis* experimental infection in clams. During bacterial challenge, early stages of the infection showed a high number of individuals infected and high bacteria load (until 7 days post-injection), confirming that extrapallial fluids are involved in the first steps of infection (*Allam, Paillard & Maes, 1996*), where hemocytes contribute to defense against *V. tapetis* by phagocytosis (*Allam & Paillard, 1998*). At 7 days post-injection, only two individuals were detected as positive by the real-time PCR assay, suggesting that animals had efficiently defended against the pathogen. This hypothesis is corroborated by Richard and collaborators (2015, unpublished data). This result also agrees with *Paillard & Maes (1994)*, who showed that the first BRD symptoms appeared in almost all the clams seven days post-injection, bearing out the reaction of hosts facing a pathogen injection. Indeed, the conchiolin matrix was developed to trap encompassing bacteria into the inner surface of the shell, inducing a decrease of circulating bacteria in the EF, and thus a lower concentration detected by the Taqman assay. Then, 14 and 30 days after injection, CECT4600[T] was again found in extrapallial fluids but the load was less than 7 days post-injection. Paillard and collaborators (*2014*) argue that an increase of *V. tapetis* in the host's fluids in advanced stages of the disease is due to the weakening of the clam, despite food intake and shell repair.

Moreover, regarding individuals detected as positive among animals injected with FSW, we observed that they were mainly detected 1 day after injection. These results imply

an effect of injection on animals which were supposedly infected by *V. tapetis*, present in their environment before sampling, despite the quarantine stage. It has already been shown that injection or handling could stimulate bacterial proliferation (*Le Bris et al., 2015*; *Jean et al., in press*). Focusing on non-injected clams, i.e., at 0d for *V. tapetis-* and FSW-injected clams and all the sampling times for non-injected clams, the interpretation that *V. tapetis* was present in individuals before sampling is corroborated: 3 individuals among 36 were already carrying bacteria on the day of injection. During the remaining time, 3 non-injected animals were detected as positive by real-time PCR protocol, while 11 animals displayed clinical signs of BRD among 72 sampled (15%), suggesting that the host has already mounted a defense response against pathogens before the experimental challenge. This corresponds to the natural prevalence of the disease described in literature (*Paillard et al., 2014*).

To conclude, we developed in this study a rapid, specific and individual method to detect and quantify *V. tapetis*. This protocol is based on a real-time PCR assay and is suitable for field and hatchery animals. Indeed, this *virB4* real-time PCR assay is easy to implement because it does not require crushing and grinding and allows individual assays considering inter-individual variability. This protocol allows for early detection of the disease, especially to assess visibly healthy clams (BRD−), bearing in mind the threshold of detection, and will be very useful in helping prevent massive infection in clams, notably in clam aquaculture.

ADDITIONAL INFORMATION AND DECLARATIONS

Bidault et al. (2015), *PeerJ*, DOI 10.7717/peerj.1484

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Competing Interests

The authors declare there are no competing interests.

Author Contributions

- Adeline Bidault conceived and designed the experiments, performed the experiments, analyzed the data, wrote the paper, prepared figures and/or tables.
- Gaëlle G. Richard performed the experiments, analyzed the data, prepared figures and/or tables, reviewed drafts of the paper.
- Cédric Le Bris performed the experiments.
- Christine Paillard contributed reagents/materials/analysis tools, reviewed drafts of the paper.

Patent Disclosures

The following patent dependencies were disclosed by the authors:

V. tasmaniensis genbank accession number KP795691.1.

DNA Deposition

The following information was supplied regarding the deposition of DNA sequences:

GenBank accession number: KT382306.

Data Availability

The following information was supplied regarding data availability:

This work did not generate any raw data.

Supplemental Information

Supplemental information for this article can be found online at http://dx.doi.org/10.7717/peerj.1484#supplemental-information.

REFERENCES

Allam B. 1998. Rôle des fluides extrapalléaux des bivalves dans la défense immunitaire. Cas de la maladie de l'anneau brun chez la palourde d'élevage, *Ruditapes philippinarum*. Thèse de Doctorat de Biologie Thesis. Brest: Université de Bretagne occidentale-Brest.

Allam B, Ford SE. 2006. Effects of the pathogenic *Vibrio tapetis* on defence factors of susceptible and non-susceptible bivalve species: I. Haemocyte changes following *in vitro* challenge. *Fish & Shellfish Immunology* **20**:374–383 DOI 10.1016/j.fsi.2005.05.012.

Allam B, Paillard C. 1998. Defense factors in clam extrapallial fluids. *Diseases of Aquatic Organisms* **33**:123–128 DOI 10.3354/dao033123.

Allam B, Paillard C, Auffret M. 2000. Alterations in hemolymph and extrapallial fluid parameters in the manila clam, *Ruditapes philippinarum*, challenged with the pathogen *Vibrio tapetis*. *Journal of Invertebrate Pathology* **76**:63–69 DOI 10.1006/jipa.2000.4940.

Allam B, Paillard C, Ford SE. 2002. Pathogenicity of *Vibrio tapetis*, the etiological agent of brown ring disease in clams. *Diseases of Aquatic Organisms* **48**:221–231 DOI 10.3354/dao048221.

Allam B, Paillard C, Maes P. 1996. Localization of the pathogen *Vibrio* P 1 in clams affected by brown ring disease. *Diseases of Aquatic Organisms* **27**:149–155 DOI 10.3354/dao027149.

Al-saari N, Gao F, Rohul AAKM, Sato K, Sato K, Mino S, Suda W, Oshima K, Hattori M, Ohkuma M, Meirelles PM, Thompson FL, Thompson C, Filho AGM, Gomez-Gil B, Sawabe T, Sawabe T. 2015. Advanced microbial taxonomy combined with genome-based-approaches reveals that *Vibrio* astriarenae sp. nov., an agarolytic marine bacterium, forms a new clade in *Vibrionaceae*. *PLoS ONE* **10**:e0136279 DOI 10.1371/journal.pone.0136279.

Austin B, Zhang X-H. 2006. Under the microscope. *Vibrio harveyi*: a significant pathogen of marine vertebrates and invertebrates. *Letter of Applied Microbiology* **43**:119–124 DOI 10.1111/j.1472-765X.2006.01989.x.

Balboa S, Dieguez AL, Doce A, Barja JL, Romalde JL. 2012. Evaluation of different culture media for the isolation and growth of the fastidious *Vibrio tapetis*, the causative agent of brown ring disease. *Journal of Invertebrate Pathology* **111**:74–81 DOI 10.1016/j.jip.2012.06.007.

Borrego JJ, Castro D, Luque A, Paillard C, Maes P, Garcia MT, Ventosa A. 1996. *Vibrio tapetis* sp. nov., the causative agent of the brown ring disease affecting cultured clams. *International Journal of Systematic Bacteriology* **46**:480–484 DOI 10.1099/00207713-46-2-480.

Bustin SA, Benes V, Garson JA, Hellemans J, Huggett J, Kubista M, Mueller R, Nolan T, Pfaffl MW, Shipley GL, Vandesompele J, Wittwer CT. 2009. The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. *Clinical Chemistry* **55**:611–622 DOI 10.1373/clinchem.2008.112797.

Casadevall A, Pirofski L. 1999. Host-pathogen interactions: redefining the basic concepts of virulence and pathogenicity. *Infection and Immunity* **67**:3703–3713.

Choquet G. 2004. Caractérisation et pathogénie des isolats de *Vibrio tapetis*, bactérie responsable de la maladie de l'anneau brun chez la palourde japonaise. Doctorat Microbiologie Thesis. Brest: Université de Bretagne occidentale-Brest.

Choquet G, Soudant P, Lambert C, Nicolas J-L, Paillard C. 2003. Reduction of adhesion properties of *Ruditapes philippinarum* hemocytes exposed to *Vibrio tapetis*. *Diseases of Aquatic Organisms* **57**:109–116 DOI 10.3354/dao057109.

Christie PJ, Whitaker N, González-Rivera C. 2014. Mechanism and structure of the bacterial type IV secretion systems. *Biochimica et Biophysica Acta (BBA)—Molecular Cell Research* **1843**:1578–1591 DOI 10.1016/j.bbamcr.2013.12.019.

Crosa JH, Schiewe MH, Falkow S. 1977. Evidence for plasmid contribution to the virulence of fish pathogen *Vibrio anguillarum*. *Infection and Immunity* **18**:509–513.

Drummond LC, O'reilly P, Mulcahy MF, Culloty SC. 2006. Comparison of techniques for diagnosis of Brown Ring Disease and detection of *Vibrio tapetis* in the Manila clam, *Venerupis* (*Ruditapes*) *philippinarum*. *Journal of Shellfish Research* **25**:1043–1049 DOI 10.2983/0730-8000(2006)25[1043:COTFDO]2.0.CO;2.

Flassch J-P, Leborgne Y. 1994. Introduction in Europe, from 1972 to 1980, of the Japanese Manila clam (Tapes philippinarum) and the effects on aquaculture production and natural settlement In: *Introductions and transfers of aquatic species: selected papers from a symposium held in Halifax, Nova Scotia, 12–13 June 1990*. Cogenhagen: International Council for the Exploration of the Sea. *Available at http://archimer.ifremer.fr/doc/00037/14871/*.

Frans I, Michiels CW, Bossier P, Willems KA, Lievens B, Rediers H. 2011. *Vibrio anguillarum* as a fish pathogen: virulence factors, diagnosis and prevention: pathogen profile of *Vibrio anguillarum*. *Journal of Fish Diseases* **34**:643–661 DOI 10.1111/j.1365-2761.2011.01279.x.

Fronzes R, Christie PJ, Waksman G. 2009. The structural biology of type IV secretion systems. *Nature Reviews Microbiology* **7**:703–714 DOI 10.1038/nrmicro2218.

Garnier M, Labreuche Y, Garcia C, Robert M, Nicolas JL. 2007. Evidence for the involvement of pathogenic bacteria in summer mortalities of the Pacific oyster *Crassostrea gigas*. *Microbial Ecology* **53**:187–196 DOI 10.1007/s00248-006-9061-9.

Garrido-Maestu A, Chapela M-J, Peñaranda E, Vieites JM, Cabado AG. 2014. In-house validation of novel multiplex real-time PCR gene combination for the simultaneous detection of the main human pathogenic *vibrios* (*Vibrio cholerae*, *Vibrio* parahaemolyticus, and *Vibrio vulnificus*). *Food Control* **37**:371–379 DOI 10.1016/j.foodcont.2013.09.026.

Gosling E. 2008. *Bivalve molluscs: biology, ecology and culture*. Hoboken: John Wiley & Sons.

Gubala AJ. 2006. Multiplex real-time PCR detection of *Vibrio cholerae*. *Journal of Microbiological Methods* **65**:278–293 DOI 10.1016/j.mimet.2005.07.017.

Jean F, Flye-Sainte-Marie J, Oudard C, Paillard C. 2011. Handling enhances the development of brown ring disease signs in *Ruditapes philippinarum*. *Journal of Shellfish Research* In Press. *Available at http://hal.univ-brest.fr/hal-00568169/document*.

Jensen S, Samuelsen OB, Andersen K, Torkildsen L, Lambert C, Choquet G, Paillard C, Bergh Ø. 2003. Characterization of strains of *Vibrio splendidus* and *V. tapetis* isolated from corkwing wrasse Symphodus melops suffering vibriosis. *Diseases of Aquatic Organisms* **53**:25–31 DOI 10.3354/dao053025.

Juhas M, Crook DW, Hood DW. 2008. Type IV secretion systems: tools of bacterial horizontal gene transfer and virulence. *Cellular Microbiology* **10**:2377–2386 DOI 10.1111/j.1462-5822.2008.01187.x.

Kushmaro A, Banin E, Loya Y, Stackebrandt E, Rosenberg E. 2001. *Vibrio* shiloi sp. nov., the causative agent of bleaching of the coral *Oculina patagonica*. *International Journal of Systematic and Evolutionary Microbiology* **51**:1383–1388 DOI 10.1099/00207713-51-4-1383.

Le Bris C, Richard G, Paillard C, Lambert C, Seguineau C, Gauthier O, Pernet F, Guérard F. 2015. Immune responses of phenoloxidase and superoxide dismutase in the manila clam *Venerupis philippinarum* challenged with *Vibrio tapetis*—Part I: spatio-temporal evolution of enzymes' activities post-infection. *Fish & Shellfish Immunology* **42**:16–24 DOI 10.1016/j.fsi.2014.10.021.

Le Roux F, Gay M, Lambert C, Nicolas J-L, Gouy M, Berthe FCJ. 2004. Phylogenetic study and identification of *Vibrio splendidus*-related strains based on gyrB gene sequences. *Diseases of Aquatic Organisms* **58**:143–150 DOI 10.3354/dao058143.

Low HH, Gubellini F, Rivera-Calzada A, Braun N, Connery S, Dujeancourt A, Lu F, Redzej A, Fronzes R, Orlova EV, Waksman G. 2014. Structure of a type IV secretion system. *Nature* **508**:550–553 DOI 10.1038/nature13081.

Macián MC, Ludwig W, Aznar R, Grimont PA, Schleifer KH, Garay E, Pujalte MJ. 2001. *Vibrio lentus* sp. nov., isolated from Mediterranean oysters. *International Journal of Systematic and Evolutionary Microbiology* **51**:1449–1456 DOI 10.1099/00207713-51-4-1449.

Matsuyama T, Sakai T, Kiryu I, Yuasa K et al. 2010. First isolation of *V. tapetis*, the etiological agent of brown ring disease (BRD), in manila clam *Ruditapes philippinarum* in Japan. *Fish Pathology* **45**:77–79 DOI 10.3147/jsfp.45.77.

McCleary S, Henshilwood K. 2015. Novel quantitative TaqMan® MGB real-time PCR for sensitive detection of *Vibrio aestuarianus* in *Crassostrea gigas*. *Diseases of Aquatic Organisms* **114**:239–248 DOI 10.3354/dao02869.

Bidault et al. (2015), *PeerJ*, DOI 10.7717/peerj.1484

17/19

Mehrabadi JF, Morsali P, Nejad HR, Imani Fooladi AA. 2012. Detection of toxigenic *Vibrio cholerae* with new multiplex PCR. *Journal of Infection and Public Health* **5**:263–267 DOI 10.1016/j.jiph.2012.02.004.

Novoa B, Luque A, Castro D, Borrego JJ, Figueras A. 1998. Characterization and infectivity of four bacterial strains isolated from brown ring disease-affected clams. *Journal of Invertebrate Pathology* **71**:34–41 DOI 10.1006/jipa.1997.4704.

Paillard C, Allam B, Oubella R. 2004. Effect of temperature on defense parameters in Manila clam *Ruditapes philippinarum* challenged with *Vibrio tapetis*. *Diseases of Aquatic Organisms* **59**:249–262 DOI 10.3354/dao059249.

Paillard C, Gausson S, Nicolas JL, Le Pennec JP, Haras D. 2006. Molecular identification of *Vibrio tapetis*, the causative agent of the brown ring disease of *Ruditapes philippinarum*. *Aquaculture* **253**:25–38 DOI 10.1016/j.aquaculture.2005.03.047.

Paillard C, Jean F, Ford SE, Powell EN, Klinck JM, Hofmann EE, Flye-Sainte-Marie J. 2014. A theoretical individual-based model of Brown Ring Disease in Manila clams, *Venerupis philippinarum*. *Journal of Sea Research* **91**:15–34 DOI 10.1016/j.seares.2014.03.005.

Paillard C, Maes P. 1990. Etiologie de la maladie de l'anneau brun chez Tapes philippinarum: pathogénicité d'un *Vibrio* sp. *Comptes Rendus de l'Académie des Sciences. Série 3, Sciences de la vie* **310**:15–20.

Paillard C, Maes P. 1994. Brown ring disease in the Manila clam *Ruditapes philippinarum*: establishment of a classification system. *Diseases of Aquatic Organisms* **19**:137–146 DOI 10.3354/dao019137.

Paillard C, Maes P. 1995a. The Brown Ring Disease in the Manila clam *Ruditapes philippinarum*, II: microscopic study of the brown ring syndrome. *Journal of Invertebrate Pathology* **65**:101–110 DOI 10.1006/jipa.1995.1016.

Paillard C, Maes P. 1995b. The Brown Ring Disease in the Manila clam *Ruditapes philippinarum*, I: ultrastructural alterations of the periostracal lamina. *Journal of Invertebrate Pathology* **65**:91–100 DOI 10.1006/jipa.1995.1015.

Reid HI, Duncan HL, Laidler LA, Hunter D, Birkbeck TH. 2003. Isolation of *Vibrio tapetis* from cultivated Atlantic halibut (*Hippoglossus hippoglossus* L.). *Aquaculture* **221**:65–74 DOI 10.1016/S0044-8486(03)00060-7.

Rodríguez JM, López-Romalde S, Beaz R, Alonso MC, Castro D, Romalde JL. 2006. Molecular fingerprinting of *Vibrio tapetis* strains using three PCR-based methods: ERIC-PCR, REP-PCR and RAPD. *Diseases of Aquatic Organisms* **69**:175–183 DOI 10.3354/dao069175.

Romalde JL, Castro D, Magariños B, Lopez-Cortes L, Borrego JJ. 2002. Comparison of Ribotyping, randomly amplified polymorphic dna, and pulsed-field gel electrophoresis for molecular typing of *Vibrio tapetis*. *Systematic and Applied Microbiology* **25**:544–550 DOI 10.1078/07232020260517689.

Saulnier D, De Decker S, Haffner P. 2009. Real-time PCR assay for rapid detection and quantification of *Vibrio aestuarianus* in oyster and seawater: a useful tool for epidemiologic studies. *Journal of Microbiological Methods* **77**:191–197 DOI 10.1016/j.mimet.2009.01.021.

Sawabe T, Ogura Y, Matsumura Y, Feng G, Amin AR, Mino S, Nakagawa S, Sawabe T, Kumar R, Fukui Y, Satomi M, Matsushima R, Thompson FL, Gomez-Gil B, Christen R, Maruyama F, Kurokawa K, Hayashi T. 2013. Updating the *Vibrio* clades defined by multilocus sequence phylogeny: proposal of eight new clades, and the description of *Vibrio* tritonius sp. nov. *Frontiers in Microbiology* **4**:414 DOI 10.3389/fmicb.2013.00414.

Schikorski D, Renault T, Paillard C, Bidault-Toffin A, Tourbiez D, Saulnier D. 2013.
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.
Aquaculture **392–395**:106–112 DOI 10.1016/j.aquaculture.2013.02.005.

Sparling PF. 1983. Bacterial virulence and pathogenesis: an overview. *Review of Infectious Diseases*
5:S637–S646 DOI 10.1093/clinids/5.Supplement_4.S637.

Thompson FL. 2003. *Vibrio* kanaloae sp. nov., *Vibrio* pomeroyi sp. nov. and *Vibrio chagasii* sp.
nov., from sea water and marine animals. *International Journal of Systematic and Evolutionary
Microbiology* **53**:753–759 DOI 10.1099/ijs.0.02490-0.

**Thompson FL, Gevers D, Thompson CC, Dawyndt P, Naser S, Hoste B, Munn CB, Swings J.
2005.** Phylogeny and molecular identification of *Vibrios* on the basis of multilocus sequence
analysis. *Applied and Environmental Microbiology* **71**:5107–5115
DOI 10.1128/AEM.71.9.5107-5115.2005.

Thompson FL, Thompson CC, Swings J. 2002. *Vibrio tasmaniensis*, sp. nov., isolated from Atlantic
salmon (*Salmo salar* L.). *Systematic and Applied Microbiology* **26**:65–69
DOI 10.1078/072320203322337326.

**Vallenet D, Belda E, Calteau A, Cruveiller S, Engelen S, Lajus A, Le Fevre F, Longin C,
Mornico D, Roche D, Rouy Z, Salvignol G, Scarpelli C, Thil Smith AA, Weiman M,
Medigue C. 2013.** MicroScope–an integrated microbial resource for the curation and
comparative analysis of genomic and metabolic data. *Nucleic Acids Research* **41**:D636–D647
DOI 10.1093/nar/gks1194.

Yamamoto S, Kasai H, Arnold DL, Jackson RW, Vivian A, Harayama S. 2000. Phylogeny of the
genus Pseudomonas: intrageneric structure reconstructed from the nucleotide sequences of
gyrB and rpoD genes. *Microbiology* **146**:2385–2394 DOI 10.1099/00221287-146-10-2385.