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Novel quantitative TaqMan® MGB real-time PCR for sensitive detection of *Vibrio aestuarianus* in *Crassostrea gigas*

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ABSTRACT: Validation of a novel quantitative real-time PCR using TaqMan® minor groove binder (MGB) chemistry is described for sensitive and rapid detection of *Vibrio aestuarianus*, an increasingly important pathogen of Pacific cupped oyster *Crassostrea gigas* aquaculture. Primers and TaqMan® MGB hydrolysis probe were designed to specifically amplify a 58bp DNA fragment of the *V. aestuarianus dnaJ* gene. Real-time PCR selectivity was empirically tested using DNA extracted from isolates of *V. aestuarianus* and a selection of different aquatic bacterial species, including other *Vibrio* spp. Theoretical selectivity was assessed through sequence comparison using the NCBI BLAST similarity tool. Quantitative PCR plasmid standards were generated to test assay linearity, amplification efficiency and the limit of quantitation (LOQ), according to International Organisation for Standardisation ISO 16140 validation recommendations. LOQ ranged between 5 and 10 PCR copies, although the detection range extended beyond this with reduced precision. Applied performance was tested using *C. gigas* samples taken from a selection of Irish aquaculture sites. Increasing levels of *V. aestuarianus*, accompanied by the development of tissue pathology in examined oysters, were found at 1 site that was sampled repeatedly in 2013. Rapid, sensitive and reproducible detections of *V. aestuarianus* from *C. gigas* tissue samples were attained during this validation study with a small sample size, and a practical application for disease management is described.

KEY WORDS: *Vibrio aestuarianus* · Vibriosis · Pacific oyster · Real-time PCR · TaqMan® MGB

INTRODUCTION

Worldwide production of the Pacific cupped oyster *Crassostrea gigas* currently surpasses that of most other aquatic animals, making it a leading species in global aquaculture. Aquaculture production of *C. gigas* was estimated to be worth in excess of 3.3 billion USD per annum (FAO 2012). Almost the entire production (>80%) takes place in China, with reported production of 3.5 million t annually, although other countries including Japan, Korea, and France all produce in excess of 100 000 t yr^{-1} (FAO 2012). In Europe, France is the biggest producer, followed by Ireland and the United Kingdom, with the latter 2 countries collectively producing less than 10 000 t yr^{-1} (FAO 2012).

Recent worldwide *C. gigas* mortality events have significantly impacted upon global production, despite the continued overall growth in *C. gigas* aquaculture. The emergence in 2008 of a highly virulent new genotype of the species *Ostreid herpesvirus* (OsHV-1), named the OsHV-1 micro-variant (OsHV-1 µVar) caused catastrophic mortalities in spat animals across the world (Segarra et al. 2010, Martenot et al. 2011, Jenkins et al. 2013, Morrissey et al. 2015). *Vibrio aestuarianus*, a marine bacterium, has also emerged recently as an increasingly harmful pathogen to *C. gigas* aquaculture. Owing to the ubiquitous

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nature of vibrios in marine ecosystems, it also carries the potential to impact *C. gigas* aquaculture globally. *V. aestuarianus* has already become increasingly associated with large aquaculture mortality events in France (Gay et al. 2004, Saulnier et al. 2010), and also appears to be implicated with *C. gigas* mortalities in Ireland. Furthermore, empirical studies have already proven that the *V. aestuarianus francensis* isolated in France, causes high mortality rates when used for challenges in the laboratory (Labreuche et al. 2006, Garnier et al. 2007, 2008). The potential economic impacts caused by *V. aestuarianus* to *C. gigas* aquaculture are considerably high; although detected in spat, juvenile and market size oysters characterise *V. aestuarianus*-related mortality events (Saulnier et al. 2009, 2010).

The requirement for rapid, sensitive and highly specific diagnostics for aquaculture is paramount to limiting infection and disease transmission. Classical diagnostic approaches used for vibrio identification can be labour intensive and time consuming. Typically, such methods involve isolation of vibrios with selective growth media (DiSalvo et al. 1978, Tison & Seidler 1983) and characterisation of metabolic pathways using API® analysis (Smith et al. 1972). The presence of a highly divergent phenotype, as found in *V. aestuarianus*, can limit the effectiveness of these traditional identification methods (Garnier et al. 2007). Furthermore, the variable size of oyster production animals can make diagnostic sampling from oyster haemolymph for pure selective microbial culture extremely problematic, particularly when dealing with small oysters.

Molecular technologies can overcome many of the limitations faced by using traditional diagnostic techniques and facilitate aquaculture with rapid, sensitive, timely warning and monitoring tools for bacterial and other pathogens (Monis & Giglio 2006). Amongst the many molecular technologies available, certain forms of real-time polymerase chain reaction (PCR), albeit relatively expensive and more technically demanding than microbial culture, offer many advantages: assay discrimination capability, assay sensitivity, and importantly, vastly expedited sample test results that can help limit spread of disease. Furthermore, the availability of an extensive molecular database provided by the National Centre for Biotechnology Information (NCBI) greatly enhances comparative sample and isolate analysis, generating a progressive resource for molecular diagnostic assay development. These benefits make the use of molecular technologies, especially PCR amplification techniques, a leading strategy to use in the reaction against the debilitating threat posed by pathogens to the aquaculture of *C. gigas*.

Real-time PCR utilising an oligonucleotide hydrolysis probe enables rapid targeted results with minimal post-assay analysis. The oligonucleotide probe chemistry is reliant upon a proximity quenching principle, whereby a 3′ quenching molecule prevents emission from a 5′ fluorescent reporter molecule, each of which are located on opposite ends of the probe (Heid et al. 1996). Exonuclease hydrolysis by Taq DNA polymerase during PCR amplification releases the 5′ fluorescent reporter molecule from the quencher, resulting in highly specific PCR amplification fluorescence (Andersen et al. 2006). Quenching chemistries also limit non-specific PCR fluorescent amplification results, a significant improvement compared with conventional PCR and real-time PCR that use DNA intercalating dye chemistries such as SYBR green I.

Real-time hydrolysis probes that use TaqMan® minor groove binder (MGB) chemistry have significant performance advancements compared with standard hydrolysis probes. Most important are the increased hybridisation specificity and binding between target DNA and the much shorter TaqMan® MGB oligonucleotide probe, which can also improve PCR amplification efficiency (Kutyavin 2000). Short TaqMan® MGB probes also minimise background fluorescence and amplification signal noise, which improves assay precision, since the reporter molecule is more efficiently quenched by increased proximity to the quencher. These benefits and others, such as robust performance, and the relative ease in assay design and transfer compared with standard hydrolysis probe PCRs, which often require modifications to thermal cycling parameters or reagent chemistry, have meant TaqMan® MGB chemistries are widely used in diagnostic laboratories (Andersen et al. 2006, Monis & Giglio 2006).

In the last 5 years, standard hydrolysis probe real-time PCR has become part of the existing detection methods used in response against *V. aestuarianus*. In particular, the central reference laboratory for mollusc diseases, Ifremer, describes the validation of an assay for detection of the molecular chaperone *dnaJ* gene (Saulnier et al. 2009). Chaperone genes are frequently used in studies of species phylogeny discrimination (Nhung et al. 2007) and can provide useful targets for highly specific PCR assays. The aim of this research was to use the extensive NCBI Genbank data available from the existing molecular chaperone *dnaJ* gene assay, to develop a highly specific and

novel TaqMan® primers and MGB probe assay for detection and quantitation of *V. aestuarianus* in *C. gigas* shellfish. Applied performance of the assay was subsequently validated using samples taken from Irish *C. gigas* aquaculture.

MATERIALS AND METHODS

Bacteria and matrices

Multiple isolates of *Vibrio aestuarianus* and also a number of marine vibrio and bacterial species were used during the validation procedure (Table 1). These were generally taken from the Marine Institute, Ireland, sample and strain archives, and most originate from *Crassostrea gigas* mortality incidents in either Ireland or France. Each isolate identity had previously been confirmed by genetic sequence identification and phylogenetic analysis of the 16S ribosomal DNA (Genbank accession no. AJ845023). *V. aestuarianus francensis* isolate 01/32 (Genbank accession no. AJ845023), *Vibrio splendidus* isolate LGP32 and *Vibrio crassostreae* isolate LGP7 had previously been provided by the European reference laboratory for molluscs diseases (Ifremer, France). Isolates were cultured on Marine agar (BD Difco) for DNA extraction from colony cultures, and in Marine broth (BD Difco) for extraction from suspension. *C. gigas* gill and mantle tissue from juvenile and adult oysters, and whole *C. gigas* spat were used as matrices during the validation process of naturally infected tissue.

Nucleic acid extraction and PCR

Bacterial colonies were picked using a loop and mixed into 200 µl of molecular biology grade water (Sigma Aldrich) and heated using a thermal cycler (DNA Engine, Bio-Rad Laboratories) at 98°C for 10 min and stored at −20°C for PCR testing. Bacterial suspensions were centrifuged at 3000 rpm for 3 min, and the supernatant discarded prior to DNA extraction. The DNA mini kit (Qiagen) was used for DNA extraction from centrifuged bacterial suspension pellets, and also for extraction from 20 mg of dissected shellfish gill and mantle tissue according to manufacturer's guidelines. Whole oyster spat too small for fresh tissue dissections were crushed in molecular grade water (Sigma Aldrich) using a Tissue Lyser II (Qiagen), and the supernatant used for DNA extraction from tissue. Purified DNA was eluted in 200 µl volume and stored at −20°C. All extractions were completed using an automated extraction method with Qiacube robots (Qiagen).

Real-time PCR

Real-time PCR primers and TaqMan® Minor groove binder probe were designed from a *dnaJ* partial gene sequence published in Genbank (AB263018) using Primer Express 3.0 software (Life Technologies). A 58 bp PCR amplicon sequence was compared with Genbank to theoretically assess primer selectivity. TaqMan® real-time PCR probe and primer details are listed in Table 2.

Table 1. Vibrio and bacterial isolates used for selectivity testing of real-time PCR primers and TaqMan® hydrolysis probe for *Vibrio aestuarianus dnaJ* gene

Isolate	Specimen	Sample details
ATCC®35048™	*Vibrio aestuarianus aestuarianus*	Type strain, validation material
01/32	*Vibrio aestuarianus francensis*	Isolate from France, validation material
02/08; 03/08; S38/12/B; S15/13/C; S15/13/B; S14/14; S15/14	*Vibrio aestuarianus*	*Crassostrea gigas* oyster mortality, Ireland (sampled 2009–2014)
NCIMB 829	*Vibrio anguillarum*	Validation material, Scotland
S4/12/A	*Vibrio splendidus*	*Crassostrea gigas* oyster mortality, Ireland
LGP32	*Vibrio splendidus*	Isolate from France, validation material
S12/12/C	*Vibrio tapetis*	*Crassostrea gigas* oyster mortality, Ireland
F70/12/A	*Yersinia ruckeri*	Atlantic salmon sample, Ireland
F28/11/A	*Aeromonas salmonicida*	Atlantic salmon sample, Ireland
F97/08/A	*Chryseobacterium indologens*	Perch sample, Ireland
DSMZ 19142	*Vibrio tubiashi*	Validation material, Germany
LGP7	*Vibrio crassostreae*	Isolate from France, validation material

Table 2. Real-time PCR primers and TaqMan® hydrolysis probe nucleotide sequences from the *dnaJ* gene of *Vibrio aestuarianus* and the ribosomal 18S gene of *Crassostrea gigas*. Conventional PCR primer sequences from the *dnaJ* gene, used for generation of plasmid quantitative real-time PCR standard

Primers and probe	Sequence (5′-3′)
TaqMan® PCR	
dnaJ f420	GTGAAGGGACGGGTGCTAAG
dnaJ r456	CCATGACAAGTGCCACAAGTCT
dnaJ p441	FAM-AGGGCACGTCGGC-MGB
Ribosomal 18S	
Biva 18S#19.F	ACCTCGGTTCTATTTTGTTGGTTT
Biva 18S#19. R	CCCCCGTCAGTCCCTCTTAA
Biva18S#19.P	VIC-CGGAACACGAGGTAAT-MGB
Conventional PCR primers	
Plasmid standard	
dnaJ f354	TAGAAGAAGCGGTTCGTGGT
dnaJ r484	CCTGACGCATCTGAACTTGA

Preparation of *V. aestuarianus* standards

Primer 3 software (available at http://primer3.ut.ee/) was used to generate primers flanking the real-time PCR 58 bp amplicon of the *dnaJ* partial gene sequence. These primers were used to generate PCR amplicon to use as the quantitative real-time PCR standard (see Table 2 for primer details). PCR was performed in 50 µl reaction volumes using MyTaq polymerase (Bioline) according to the manufacturer's guidelines with a Bio-Rad DNA Engine® thermal cycler. PCR thermal profile consisted of 95°C for 1 min followed by 40 cycles of 95°C for 15 s, 55°C for 30 s, 72°C for 10 s. PCR amplification products were generated from *V. aestuarianus* isolate 01/32 and confirmed using a 2% agarose gel stained with ethidium bromide and visualised under UV light. Successful amplicon bands were excised from the agarose gel and pooled prior to sample clean-up using QIAEX II gel extraction kit (Qiagen). Ligation and Transformation were performed using Promega pGEM®-T Easy Vector System with JM109 high efficiency competent cells. Briefly, purified amplicon DNA was ligated for 18 h at 4°C using T4 DNA Ligase with a thermal cycler (Bio-Rad DNA Engine®). Ligant (2 µl) was added to ice cold JM109 cells and then incubated on ice for 20 min. Heat shock treatment at 42°C for 45 s was applied, followed by 2 min incubation on ice, and incubation with Super Optimal Broth SOC (Sigma Aldrich) at 37°C for 90 min, before finally plating 100 µl of transformants onto LB broth media containing ampicillin, and IPTG and X-gal (Sigma Aldrich) with overnight incubation at 37°C. White and blue/white colonies were screened by colony PCR for vector insert confirmation using standard M13 forward (-20) and M13 reverse sequencing primers. Thermal profile and amplicon visualisation as described already for Bioline MyTaq polymerase. Chosen amplicons of correct molecular weight were confirmed using commercial genetic sequencing (Sequiserve). Suitable colonies were grown overnight at 37°C and harvested from LB broth and purified using GenElute plasmid purification kit (Sigma Aldrich). Purified plasmid DNA was quantified using a Nanodrop (Thermoscientific), and subsequently prepared as serial dilutions 5.7×10^7 to 5.7×10^3, to be used as standard curves for real-time PCR of *V. aestuarianus*. Standards were stored at –20°C.

Quantitation, sensitivity and matrix testing of *V. aestuarianus* DNA by real-time TaqMan® PCR

Each real-time PCR consisted of 5 µl of *V. aestuarianus* DNA added at a range of concentrations to 20 µl Universal TaqMan® Master Mix (2x) AmpErase® UNG (Life Technologies) containing 300 nm primers, 150 nm probe using a 7500 thermal cycler (Life Technologies). Real-time PCR was completed using a thermal profile of 50°C for 2 min and 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 30 s. Negative template control consisting of molecular biology grade water was included in each run. For quantitation, prepared *V. aestuarianus* standards were tested in triplicate across multiple orders of magnitude as previously stated. Results were interpreted by linear regression for slope analysis, and the PCR amplification efficiency (E) calculated according to: $E = [10^{1/(-slope)}]-1$, using ABI software SDS 7500 v. 2.0.6 (Applied Biosystems). Standard curve data points were used to determine real-time TaqMan® PCR quantitation and detection limits through the assessment of variance (standard deviation) calculated at each dilution standard. To further assess PCR sensitivity and ensure it was consistent with ISO 16140 guidelines for limit of detection, required that 95% of 20 tested replicates returned positive amplification results. This work was completed following the assessment of quantitation limit described above, which helped to identify the dilution standards nearing or exhibiting a drop-off in amplification Ct value. This particular dilution standard was then tested 20 times and the process repeated from 2 other

prepared sets of *V. aestuarianus* standards. Oyster matrix testing was carried out by spiking healthy oyster tissue DNA extract with *V. aestuarianus* DNA standard, and testing a dilution series of spiked oyster tissue samples by real-time TaqMan® PCR. This process was carried out using spikings of 3 different oyster samples and the results were interpreted as per those described for amplification efficiency.

Selectivity and reproducibility validation of real-time TaqMan® PCR

To empirically assess the selectivity of real-time PCR primers for *V. aestuarianus dnaJ*, nucleic acid from *V. aestuarianus* and other marine vibrio and bacterial species that originated from previous (in-house) aquaculture sample isolations or diagnostics were tested (see Table 1 for the list of strains and isolates included). DNA extractions from each isolate were verified by PCR amplification of part of the 16S ribosomal DNA, accession no. stated previously, to control against potential failed extractions.

Reproducibility of TaqMan® PCR for *V. aestuarianus dnaJ* was evaluated by testing 6 different *C. gigas* oysters in triplicate. These samples were selected from a number of aquaculture sites in Ireland with previous *V. aestuarianus* real-time PCR detections that had been confirmed by microbiological analysis. Samples were tested across 3 independent real-time PCR assays on consecutive days. Intra-assay variance occurring within sample triplicates, and also inter-assay variance, i.e. the variance in each of the sample triplicates when compared between the different PCR assays, were determined using SD.

Shellfish sample testing analyses

DNA extracts from 2009–2013 archive samples of *C. gigas*, sourced from shellfish aquaculture sites operating in Ireland, were screened for *V. aestuarianus* by TaqMan® real-time PCR. Sampled animals were inclusive of spat, juveniles and adult *C. gigas* oysters. All samples had previously been extracted for DNA using an automated version of the Qiagen DNA mini kit following the standard protocol for tissues. Quantitative real-time TaqMan® PCR was carried out as described above. Samples were tested in batches of approx. 20 individual oysters, but sometimes this number was not always possible. PCR reactions were carried out in singular with a negative process control, *V. aestuarianus* positive and negative amplification controls in each assay. Ribosomal 18S extraction control (M. Engelsma pers. comm.) (Table 2) and TaqMan® exogenous internal positive control for inhibition were tested for approximately 10% of samples in each batch, since pre-existing control data from these samples following previous real-time PCR diagnostics had already been attained in-house. Samples from this survey were deemed positive for *V. aestuarianus* by real-time PCR providing that Ct values were below an empirical cut off range (limit of quantitation; LOQ) and when control assay criteria were acceptable.

RESULTS

Selectivity of real-time TaqMan® PCR

Sequence alignments of the real-time TaqMan® PCR amplicon showed no cross reactivity with other species when compared using the National Centre for Biotechnology Information (NCBI) website Basic local alignment tool (BLAST) analysis program. Partial theoretical cross reactivity with *Vibrio halioticoli* and other vibrio species was only observed with BLAST analysis, when comparisons were analysed with more dissimilar sequence search settings. However, differences between these sequences occur within the 3′ end terminals of both the forward primer and TaqMan® hydrolysis probe, thereby reducing the likelihood of polymerase initiation. Furthermore, *dnaJ* gene sequences of those species with the highest percentage BLAST similarity scores: *V. halioticoli* (AB263038), *V. anguillarum* (LK021130), *V. brasiliensis* (AB263022), and *V. coralliilyticus* (CP 009617) showed at least 10 nucleotide differences when compared with the TaqMan® PCR amplicon and the *V. aestuarianus* (AB263016) sequence using Lasergene 12 (DNASTAR) sequence analysis software. Empirical selectivity did not yield any amplification fluorescence signal from available vibrios, and aquatic bacterial species isolated at the Marine Institute, Ireland, thus proving agreement between empirical and theoretical results generated using NCBI.

Quantitative real-time TaqMan® PCR reproducibility

Inter- and intra-assay variance was calculated using standard deviation of the *V. aestuarianus* real-time TaqMan® PCR Ct values. The mean intra-assay

Table 3. Reproducibility of TaqMan® PCR for *Vibrio aestuarianus dnaJ* from triplicates of 6 individual *Crassostrea gigas* samples evaluated from intra- and inter-assay variances of 3 PCR assays completed on 3 consecutive days. Sample replicate Ct values, Ct mean ± SD, and relative SD (RSD) are shown

Sample	PCR Assay 1			PCR Assay 2			PCR Assay 3			All assays	
	dnaJ Ct Replicates	Mean ± SD	RSD %	*dnaJ* Ct Replicates	Mean ± SD	RSD %	*dnaJ* Ct Replicates	Mean ± SD	RSD %	Mean Ct ± SD	RSD %
1	28.55 28.49 28.58	28.54 ± 0.05	0.16	28.55 28.48 28.58	28.54 ± 0.05	0.16	28.46 28.46 28.53	28.48 ± 0.04	0.15	28.52 ± 0.05	0.18
2	26.84 26.78 26.81	26.81 ± 0.12	0.12	26.81 26.81 26.76	26.79 ± 0.11	0.11	26.78 26.81 26.75	26.78 ± 0.13	0.11	26.79 ± 0.03	0.11
3	28.32 28.32 28.34	28.33 ± 0.01	0.05	28.37 28.32 28.19	28.29 ± 0.09	0.32	28.30 28.30 28.26	28.30 ± 0.04	0.14	28.31 ± 0.05	0.19
4	27.85 27.77 27.79	27.80 ± 0.04	0.14	27.8 27.84 27.73	27.79 ± 0.05	0.19	27.86 27.83 27.76	27.82 ± 0.05	0.19	27.8 ± 0.04	0.16
5	28.04 27.97 28.00	28.00 ± 0.04	0.13	28.07 28.00 27.97	28.01 ± 0.05	0.17	28.06 27.97 28.12	28.05 ± 0.05	0.17	28.02 ± 0.05	0.17
6	28.97 28.83 28.87	28.89 ± 0.07	0.25	28.94 28.83 28.87	28.88 ± 0.06	0.2	28.83 29.06 28.99	28.96 ± 0.12	0.42	28.91 ± 0.08	0.29

variances ranged from 0.05–0.42% from the 6 samples tested in triplicate (Table 3). Inter-assay variance ranged from 0.11–0.29% following 3 consecutive days of repeat PCR assay testing.

Quantitative real-time TaqMan® PCR sensitivity and matrix test

Ten-fold serial dilutions of plasmid DNA standards were tested using the real-time TaqMan® PCR for *V. aestuarianus*. Fig. 1 shows amplification and standard curve plots from an empirical set of standards typical for the *V. aestuarianus* real-time TaqMan® PCR. The ABI software regression analysis data from 3 PCR assays produced a mean amplification efficiency range between 97 and 99%, with corresponding slope values between −3.2 and −3.3. The assay reliably shows linearity across 7 orders of magnitude and with an accurate LOQ estimated between ≤5 and 10 plasmid copies according to ISO 16140 guidelines. Further dilutions below 5 copies produced less consistent results that were not ISO 16140 satisfactory, but often yielded Ct values across triplicate standard wells and with a typical Ct value standard deviation of ≤0.3. Similarly, 10-fold spiked oyster DNA samples with *V. aestuarianus* DNA gave amplification efficiency averaging 94% with slope values of −3.6.

Quantitative detection of *V. aestuarianus* in Irish shellfish aquaculture sites

All real-time PCR sample assays achieved acceptable results for amplification and extraction controls, and no PCR or process contamination was experienced from any of the considered *Crassostrea gigas* life stages assessed in this pilot study. Ten sample sites, either with a history of *V. aestuarianus* or a freedom from the pathogen were purposely tested. These were comprised of 12 sample batches and were used to assess the suitability for screening *C. gigas* for *V. aestuarianus* DNA using this TaqMan® PCR assay. Broadly considered, positive and negative results were recorded at each life stage, showing typical amplification equivalent to 10^1 copies per 20 mg of oyster tissue. However, certain sites that were sampled gave levels as high as 10^5 copies, from

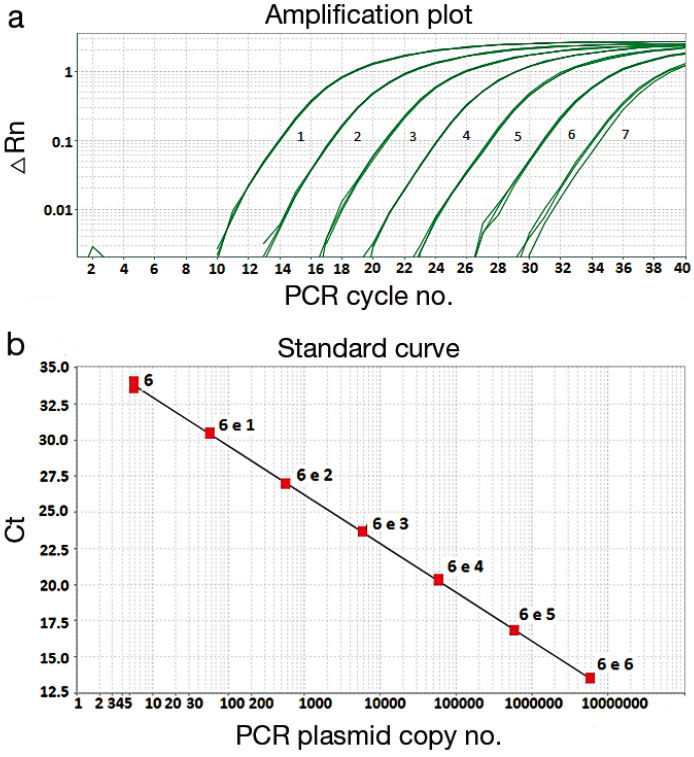

Fig. 1. (a) Real-time TaqMan® PCR amplification plot of *Vibrio aestuarianus dnaJ* gene standards (10-fold dilutions), showing change in reporter signal (ΔRn) relative to PCR cycle number. Curves 1–7 show a typical amplification plot completed in triplicate and inclusive of 6×10^6 – 6 copies per µl range. (b) Real-time TaqMan® PCR standard curve for *Vibrio aestuarianus dnaJ* gene standards (10-fold dilutions) used in (a)

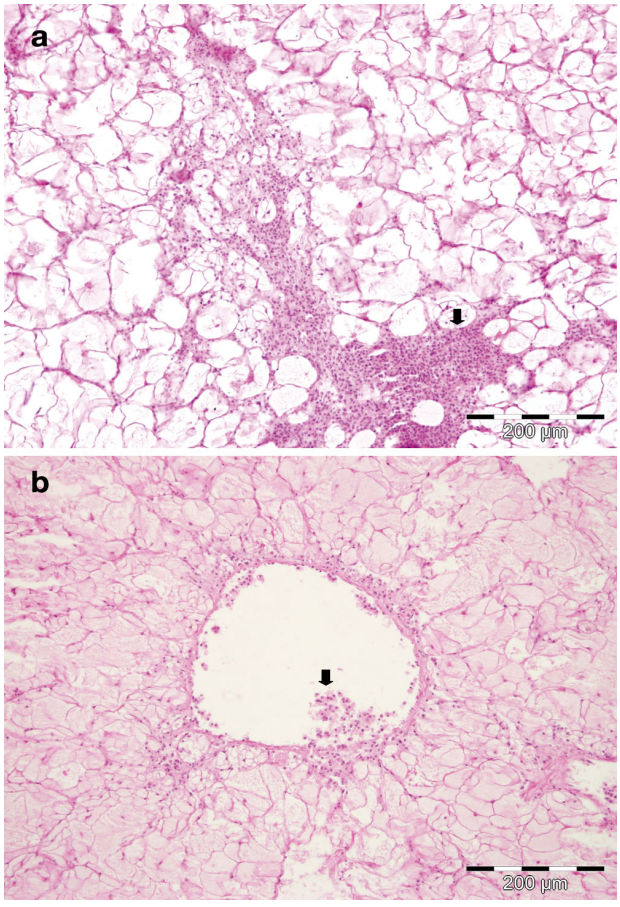

Fig. 2. Pathology caused by *Vibrio aestuarianus* in *Crassostrea gigas* oysters stained with haemotoxylin and eosin. Arrows show regions of (a) haemocyte accumulation in the connective tissues and (b) haemocytes entering the sinus

which pathology was observed when tissue sections were examined by histology (Fig. 2). Some samples also generated Ct values above the established LOQ cut off value, and as a result were considered negative. PCR results are shown in Table 4. A number of real-time PCR sample positives were confirmed for *V. aestuarianus* DNA by sequencing a PCR amplicon specific for the 16s rRNA gene, which is unrelated to the *dnaJ* gene used in the TaqMan® PCR.

DISCUSSION

Real-time PCR is becoming increasingly used as a method of choice by diagnostic laboratories and industry

Table 4. Real-time TaqMan® qPCR results from various *Crassostrea gigas* production life stages sampled in batches of approx. 20 animals from a selection of Irish aquaculture sites, and tested for amplification of *Vibrio aestuarianus dnaJ* gene. S20/10 from Woodstown and S33/13 from Castlemaine were confirmed with PCR amplification of ribosomal 16s genetic sequence comparison

Aquaculture site	MI sample batch no.	*C. gigas* life stage	PCR copies (per 20 mg) of *C. gigas* tissue
Dungloe	S37/09	Spat	Negative
Carlingford	S9/09/a	Spat	Negative
Castlemaine	S59/10	Spat	10^1 – 10^3
Woodstown	S20/10	Juvenile <70 mm	10^1 – 10^3
Kenmare	S62/12	Juvenile <70 mm	Negative
Dungarven	S103/12	Juvenile <70 mm	$\geq 10^1$
Waterford	S13/09	≥1yr old	10^2
Donegal	S18/09	≥1yr old	10^1
Castlemaine	S28/13	Adult 2 yr+	10^1
Mannin Bay	S78/10	Adult 2 yr+	Negative
Castlemaine	S33/13	Adult 2 yr+	10^1 – 10^5
Killala	S71/11	Adult 2 yr+	Negative

that require rapid and targeted results (Monis & Giglio 2006). This is particularly evident, since more recent revisions to the World Organisation for Animal Health (OIE 2014a,b) diagnostic manual(s) for terrestrial and aquatic diseases show continued uptake of real-time PCR technologies. The significant benefits of using real-time PCR for diagnostic purposes have been widely discussed, and a multitude of these benefits apply for detection of shellfish pathogens affecting aquaculture. There is a breadth of current literature detailing the use of real-time PCR for detection of shellfish diseases, inclusive of vibrios that impact the shellfish aquaculture industry (Pepin et al. 2008, Saulnier et al. 2009, Corbeil et al. 2010, Martenot et al. 2011, Jiang et al. 2012). The detection of *Vibrio aestuarianus* as a shellfish pathogen has newly emerged in the last few years with increasing severity (Azandégbé et al. 2010, Wendling et al. 2014), and aquaculture management strategies to safeguard *C. gigas* culture stand to benefit from the rapid and sensitive detection capabilities that real-time PCR can offer.

The assay design described here is based on the real-time PCR method by Saulnier et al. (2009) which targets a 266 bp amplicon from the *dnaJ* gene of *V. aestuarianus*. This new assay uses TaqMan® MGB real-time PCR chemistry to recognise a significantly shorter 58 bp target amplicon from the same gene. Short TaqMan® MGB hydrolysis probe real-time PCR assays are a widespread and preferred diagnostic choice. This is supported by the vast majority of real-time PCR methods described in OIE (2014a) that are designed with Taqman® chemistry. This is due in part to the performance benefits afforded from improved primer binding with higher melt temperatures. The latter creates an enhanced discriminatory capacity for short specific sequence hybridisation, resulting in less tolerance to nucleotide mismatches and non-specific amplification results (Kutyavin 2000, Andersen et al. 2006, Yao et al. 2006). No cross reactions were found with this assay when tested empirically against relevant vibrio and bacterial species, or theoretically when tested by BLAST similarity analyses (Altschul et al. 1990). TaqMan® MGB assays are also recognised for their amplification efficiency due to their shortened amplification targets. PCR efficiency ranged between 94 and 99% for this assay, the lower values representing the effect of *C. gigas* sample matrix inhibitors. Furthermore, well designed TaqMan® MGB assays when assayed with TaqMan® PCR reagents containing AmpErase® UNG, offer a useful countermeasure to PCR carry-over contamination within the laboratory (Tetzner et al. 2007). This further limits the opportunity for false results and should therefore be favoured in diagnostic laboratories. No contamination or false amplification was observed with this assay at any stage of the validation and testing of *C. gigas* tissue samples.

Validation of this TaqMan® MGB real-time PCR has shown it to be a sensitive and versatile alternative method for detection of *V. aestuarianus* in *C. gigas* tissue samples, in both quantitative and semi-quantitative formats. Although Saulnier et al. (2009) report that the existing standard real-time PCR assay for *V. aestuarianus* has similar testing performance, in our lab this method lacked robustness proving difficult to adopt in-house, despite attempting various modifications to reagent chemistry and run conditions. Indicative TaqMan® MGB real-time PCR results from *C. gigas* sample testing demonstrated comparable performance using 2 manufacturer platforms (Life Technologies ABI 7500 and Roche LC96 instruments). Similarly, robust assay performance was further established through its compatibility with various reagent chemistries e.g. Taqman®Universal PCR Master Mix, FastStart Universal Probe Master (Rox) and FastStart Essential DNA Probes Master reagents (Roche) (data not included). We also found the new TaqMan® MGB method to be more sensitive when testing a selection of aquaculture samples for *V. aestuarianus*. However, a comprehensive comparison of the 2 methods was not included in this study, since it was not possible to attain consistent and satisfactory PCR amplification profiles with the existing method. This may have been caused by a difference in laboratory instrumentation compared to that originally used by Saulnier et al. (2009); ultimately this prevented an accurate and fair comparison of the 2 methods.

V. aestuarianus was reliably detected for the first time from a number of aquaculture sites around Ireland using real-time TaqMan® MGB PCR. Positive results at sites located in Castlemaine, County Kerry, and Woodstown, County Waterford, were confirmed, both by microbiological and genetic sequencing. Most sites that generated positive detections did not present tissue pathology when examined using histology. Negative results were also found during this study, consistent with previous site history profiles for *V. aestuarianus* detection and isolation. Interestingly, in one sample taken from Castlemaine, *V. aestuarianus* was initially detected at sub clinical levels i.e. with no evidence of histopathology. Yet a follow-up sample tested 2 weeks later from the same oyster stocks showed a marked increase in real-time TaqMan® MGB PCR detection concentration. Certain

oysters displayed levels that were 4 orders of magnitude greater. When these oysters were examined using hematoxylin and eosin staining with histology, there was evidence of haemocytic infiltration present in the sinuses of the oyster, which is symptomatic of *V. aestuarianus* infection. This result represents the first reported symptoms of tissue pathology presented by *V. aestuarianus* in Irish *C. gigas* culture. In practical terms, these samples effectively demonstrate the assay detection sensitivity and also importantly the potential benefit for application of this real time TaqMan® MGB PCR in *V. aestuarianus* disease diagnostics. Early detection and selective screening using this novel and sensitive assay could become part of an effective management strategy in response to limiting the impact of this pathogen on *C. gigas* aquaculture sites. Also, early detection of *V. aestuarianus* in oyster spat may limit the spread of the pathogen through movements of infected stocks. This assay is currently being used in a comprehensive survey for *V. aestuarianus* in Irish shellfish aquaculture sites, sampling from 2003 through to the present day. This will purposely test the link between *C. gigas* pathology and production mortality associated with *V. aestuarianus*.

In conclusion, the results from this assay validation show the development of an alternative and more robust quantitative or semi-quantitative real-time TaqMan® MGB PCR for the rapid, sensitive and specific detection of *V. aestuarianus* from *C. gigas* shellfish tissue. Adaptation of this assay within a laboratory should involve complete validation to determine laboratory-specific performance characteristics and therefore should not rely upon a simple transfer of the results presented herein.

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