Food Microbiology 30 (2012) 17–23

Contents lists available at SciVerse ScienceDirect

Food Microbiology

journal homepage: www.elsevier.com/locate/fm

pilF polymorphism-based real-time PCR to distinguish *Vibrio vulnificus* strains of human health relevance

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ARTICLE INFO

Article history:
Received 1 March 2011
Received in revised form
29 July 2011
Accepted 6 September 2011
Available online 16 September 2011

Keywords:
Pathogen
V. vulnificus
pilF
Real-time PCR
Detection
Oyster

ABSTRACT

The Gram-negative bacterium *Vibrio vulnificus* is a common inhabitant of estuarine environments. Globally, *V. vulnificus* is a significant foodborne pathogen capable of causing necrotizing wound infections and primary septicemia, and is a leading cause of seafood-related mortality. Unfortunately, molecular methods for the detection and enumeration of pathogenic *V. vulnificus* are hampered by the genetically diverse nature of this pathogen, the range of different biotypes capable of infecting humans and aquatic animals, and the fact that *V. vulnificus* contains pathogenic as well as non-pathogenic variants. Here we report an alternative approach utilizing the development of a real-time PCR assay for the detection of pathogenic *V. vulnificus* strains based on a polymorphism in *pilF*, a gene previously indicated to be associated with human pathogenicity. Compared to human serum reactivity, the real-time PCR assay successfully detected pathogenic strains in 46 out of 47 analysed *V. vulnificus* isolates (97.9%). The method is also rapid, sensitive, and more importantly can be reliably utilised on biotype 2 and 3 strains, unlike other current methods for *V. vulnificus* virulence differentiation.

1. Introduction

The Gram-negative halophilic bacterium *Vibrio vulnificus* is a natural inhabitant of tropical as well as temperate estuarine environments. The species is present in high numbers in filtering organisms, such as oysters, especially in warmer months (Oliver, 2006). *V. vulnificus* is a potent human pathogen, and is responsible for more than 95% of all seafood-related deaths (Jones and Oliver, 2009). Isolated incidents of V. vulnificus infections occur globally, with cases frequently reported in the U.S.A., Europe and the Far East (Dalsgaard et al., 1996; Chuang et al., 1992; Hlady and Klontz, 1996; Baker-Austin et al., 2010a). Human infections typically occur after ingestion of raw or undercooked shellfish, particularly oysters, or through entry via a flesh wound (Oliver, 2005; Jones and Oliver, 2009). Significantly, *V. vulnificus*-associated primary septicemia carries the highest fatality rate of any foodborne pathogen (Rippey, 1994). A review of 459 U.S. cases reported by the Food and Drug Administration between 1992 and 2007 revealed that 51.6% of the patients infected with *V. vulnificus* died (Jones and Oliver, 2009). Most cases of infection (~95%) occur in males, who are immunocompromised or who have underlying diseases/syndromes which result in elevated serum iron levels, primarily liver cirrhosis secondary to alcohol abuse/alcoholism (Oliver and Kaper, 2001).

V. vulnificus is currently subdivided into three biotypes based on genetic, biochemical and serological features, as well as host range (Bisharat et al., 1999; Tison et al., 1982). Biotype 1 strains are human pathogens and are responsible for the vast majority of *V. vulnificus* infections reported worldwide. Biotype 2 contains strains which cause disease and infections in aquatic animals such as eels and occasionally in humans (Amaro and Biosca, 1996). A further biotype (biotype 3) was discovered in 1996 after an outbreak of *V. vulnificus* infections in an Israeli fish market, and was later found to be a hybrid of biotypes 1 and 2 (Bisharat et al., 2005). Not all strains of *V. vulnificus* appear to be able to cause disease in humans and aquatic animals, and there is a need for the development of accurate and rapid methods to distinguish virulent and avirulent isolates. *V. vulnificus* is phenotypically and serologically heterogeneous, has a wide host range encompassing different biotypes capable of infecting humans and aquatic animals, and this complexity has hampered efforts to provide a reliable assay to

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0740-0020/$ – see front matter
doi:10.1016/j.fm.2011.09.002

identify pathogenic and non-pathogenic variants. Several studies have utilised different molecular markers in biotype 1 *V. vulnificus* as a proxy for potential human virulence, with varying degrees of success. Differences in the sequence of the small subunit 16S rRNA gene, as correlating with either clinical (pathogenic) and environmental (non-pathogenic) origin have been utilised previously (Aznar et al., 1994; Nilsson et al., 2003; Vickery et al., 2007). *V. vulnificus* biotype 1 strains have also been classified into two genotypes based on a virulence-correlated gene, *vcg* (Rosche et al., 2005). This genotype has been correlated with human infection for 90% of isolates from human cases having the *vcgC* sequence type and 87% of environmental strains having the *vcgE* variant (Rosche et al., 2005). Both 16S and *vcg* polymorphism can be used as a predictive assay to distinguish pathogenic potential in biotype 1 *V. vulnificus* strains, but have failed to adequately identify biotype 2 and biotype 3 strains, which are also potentially pathogenic to humans (Sanjuan et al., 2009; Roig et al., 2010).

A recent report by Roig et al. (2010) demonstrated that a polymorphism in the pilus-type IV assembly protein of *V. vulnificus* can be utilised as a potential marker to distinguish pathogenic and non-pathogenic *V. vulnificus* strains, irrespective of biotype. The authors found that *V. vulnificus* strains appeared to be divided into two groups in terms of sequence variability in the *pilF* gene, which correlated strongly with potential pathogenicity (as ascertained by human serum sensitivity). PilF is a protein required for pilus-type IV assembly whose mutation in some bacterial pathogens results in attenuated virulence in mice models (Chakraborty et al., 2008).

The high mortality rates coupled to the rapidity of *V. vulnificus* infections underlie the need for additional assays to detect and enumerate these pathogens in a timely manner. The goal of the current study was the development and evaluation of a real-time PCR assay that would provide a rapid, sensitive and quantitative method for the detection of *V. vulnificus* strains encompassing the entire genetic diversity of human pathogenic isolates. A further objective of this work was the development of an assay that could be used successfully on extracted DNA, boiled cells, as well as directly in artificially spiked shellfish matrices.

2. Materials and methods

2.1. Bacterial strains and growth conditions

V. vulnificus strains, encompassing biotype 1, 2 and 3 isolates, were grown at 28 °C for 24 h in tryptone soy broth or on solid agar media, supplemented with 5 g/liter NaCl. Strains were cryogenically stored at −80 °C prior to use, supplemented with 20% (vol/vol) glyerol. A total of 69 bacterial strains, including *V. vulnificus* ($n = 47$), other *Vibrio* species ($n = 13$), and distantly related reference strains ($n = 9$) were used to assess the specificity of the oligonucleotide probe and primer sets used.

2.2. DNA isolation from individual strains

Late-logarithmically grown bacterial suspensions were pelleted and DNA extracted using a Mini-prep protocol (Ausubel et al., 2007). The quality and quantity of DNA was subsequently ascertained spectrophotometrically using a NanoDrop ND1000 (NanoDrop Technologies, Wilmington, DE). Extracted DNA samples were coded to conceal their identity, and a separate laboratory scientist conducting the real-time PCR testing per strain was “blind” to the original identifier to minimize response measurement bias.

2.3. Sequence analysis and TaqMan probe design

The complete nucleotide sequences (open reading frame regions only) for all full length *pilF* nucleotide sequences, deposited by Roig et al. (2010), and encompassing GenBank accession numbers FJ756476–FJ756489 and FJ899603–FJ899608, were aligned using clustalW (Thompson et al., 1994). A region of the *pilF* genes (approximate position 481–601) was analysed using primer express software from DNASTAR (Madison, WI, USA). Initial sequence alignment comparisons identified a variable region, at approximate positions 534–571 in the *pilF* gene that demonstrated significant polymorphism for the design of an appropriate TaqMan probe (Fig. 1), targeting a sequence specific to human serum-resistant strains. The probe and primers were subsequently assessed for species as well as strain specificity using a BLAST search against publically available databases (Table 1).

2.4. Production of positive control material

To produce appropriate positive control material for the *pilF* real-time PCR, the generation of cloned positive PCR was adopted. Briefly, for conventional PCR 50 μl per reaction mixes consisted of 30.65 μl molecular grade water, 10 μl 5× Green GoTaq® Flexi Buffer (Promega), 0.62 μl dNTP mixture (100 μM each); 1.2 μl of the *pilF* primers (100 μM each, Table 1), 5 μl of $MgCl_2$ (25 mM), 0.25 μl Taq flexi polymerase and 2.5 μl of extracted DNA samples. The temperature profile for this assay consisted of 3 min denaturation at 94 °C, followed by 30 cycles of 95 °C for 45 s, 45 s at 55 °C and

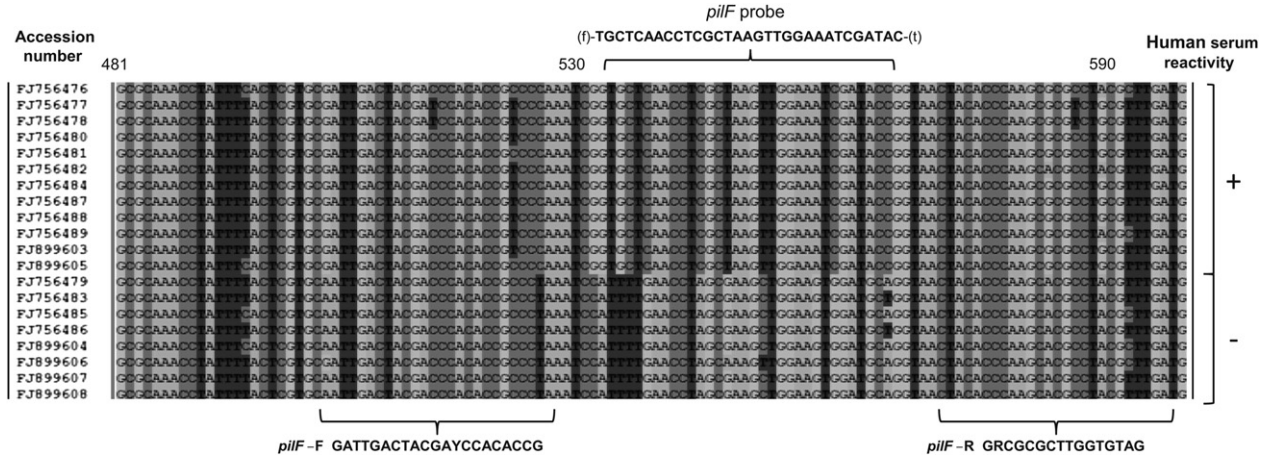

Fig. 1. Alignment of *pilF* gene sequences from a range of clinical and environmentally-derived *V. vulnifius* isolates, indicating primer binding regions used in this study.

Table 1
Primers and probes used in this study.

Name	Sequence (5'–3')	Reference
Pil F-F	GATTGACTACGAYCCACACCG	This study
Pil F-R	GRCGCGCTTGGGTGTAG	This study
PilF Probe	$^{(FAM)}$-TGCTCAACCTCGCTAAGTTGGAAATCGATAC-$^{(TAMRA)}$	This study
vvC-F	MMAAACTCATTGARCAGTAACGAAA	Baker-Austin et al. (2010b)
vvC-R	AGCTGGATCTAAKCCCAATGC	Baker-Austin et al. (2010b)
vcgC Probe	$^{(TAMRA)}$-AATTAAAGCCGTCAAGCCACTTGACTGTAAAGAA-(FAM)	Baker-Austin et al. (2010b)

72 °C for 45 s with a final extension step of 72 °C for 2 min. Following PCR, amplicons were visualised by ethidium bromide staining (0.5 mg/l) on 2.0% (wt/vol) agarose gels. Positive PCR amplicons of predicted size were subsequently purified using a Qiagen PCR purification kit (Qiagen, Valencia, CA) following the manufacturer's recommendation and cloned into a TOPO™ TA cloning kit (K4530-20, Invitrogen, Carlsbad, CA). Putative clones were tested by PCR and positive transconjugants were subsequently plasmid extracted using a Qiagen mini-prep kit (Qiagen, Valencia, CA). Plasmid DNA was subsequently serially diluted, and standard weight/volume concentrations were converted to *pilF* copies/microlitre concentrations by using the calculation: Copies per microliter = grams per microliter/FW $\times$ 6.0221415 $\times$ 10^{23}, where FW = Amplicon Length (in Base Pairs) $\times$ 660. Known concentrations of *pilF* copies/microlitre were subsequently used for quantification purposes (Section 2.5).

2.5. TaqMan PCR assay

For real-time PCR experiments, the assay comprised of a total reaction of 25 μl, consisting of 12.5 μl TaqMan Universal PCR Master Mix (Applied Biosystems), 0.45 μl each of forward and reverse primer (as appropriate), *V. vulnificus pilF* and *vcgC* primers (100 nM), 5.6 μl nuclease-free water, and 1 μl of probe (500 nM, Table 1). Five microliters of template (either chromosomal DNA, plasmids, boiled cell lysate) was subsequently added, and each reaction was performed in triplicate. Amplification was performed using an Applied Biosystems SDS 7900 real-time PCR machine with the following cycling parameters: 1 cycle at 95 °C for 10 min followed by 50 cycles at 95 °C for 15 s and 60 °C for 90 s. For each assay, samples generating a positive reaction result (sigmoid-shaped amplification curve rising above the threshold) in any replicate were considered positive. For each TaqMan sample replicate, a PCR unit quantity (genome copies of cloned *pilF* per reaction) was calculated using the slope of a standard curve of target DNA, with a C_p value of 42.75 representing the theoretical limit of detection of the assay equalling 1 genome copy of target per PCR reaction. Standard curves for the determination of *pilF* DNA copy number (per PCR reaction) were constructed using the average of duplicate C_p values encompassing a concentration range of four serially diluted cloned material samples (typically from −4 to −8 dilution of cloned material). Curves with r^2 values of <0.99 were not used for quantification purposes. Analysed TaqMan sample replicates that did not generate positive amplification curves were omitted from final analyses, and the average from the three replicates was calculated to give an overall quantity for that sample. To ascertain potential PCR inhibition, a commercially available amplification control was utilised (TaqMan® Exogenous internal positive control reagents, Applied Biosystems), with minor modifications. Briefly, each sample of extracted shellfish and extracted water (5 μl) were analysed as above in triplicate, with the addition of 2.5 μl of 10× EXO IPC mix, 0.5 1 μl of 50× EXO IPC DNA, 12.5 μl TaqMan Universal PCR Master Mix (Applied Biosystems), and 4.5 μl of nuclease-free water. Inhibition in this context was defined by the absence of target amplification.

2.6. Shellfish bioaccumulation and DNA extraction procedures

For shellfish bioaccumulation experiments, four 50 litre capacity tanks (3 experimental and 1 control) were filled with 22 l (±0. 5 l) sterile seawater and maintained at 15 °C (±1 °C) with constant air sparging. Twenty-five un-depurated live Pacific oysters (*Crassostrea gigas*) were obtained from a local wholesaler (Weymouth, UK), evenly distributed into each tank and left for 24 h to acclimatise, prior to the addition of bacterial amendments. For bioaccumulation experiments, *V. vulnificus* strain E12 (a strain previously identified *pilF* positive by real-time PCR, Table 2) was grown up overnight in alkaline saline peptone water ASPW (Oxoid, Basingstoke, United Kingdom), and added to the test tank. The required quantities of bacterial amendments were assessed by analysing the cell cultures spectrophotometrically, and by adjusting the concentration of strain E12 in appropriate volumes of sterile ASPW prior to use. A spiking volume of strain E12 (optical density of 1) corresponding to approximately 10^9 cells was added to the test tank and mixed. Ten *C. gigas* were removed after 6 h of exposure, and opened. For each sample, the digestive glands (stomach and digestive diverticula) were removed from each animal and weighed. The digestive glands were subsequently pooled together, and then finely chopped using a sterile razor blade. Homogenates were then prepared by treating the chopped digestive glands with 100 μg/ml Proteinase K solution (30 U/mg; Promega) as previously described (Jothikumar et al., 2005; Baker-Austin et al., 2010b). Homogenates were stored at 4 °C prior to testing. For each shellfish sample, 500 μl of homogenate was processed using the NucliSens magnetic extraction kit (BioMerieux, Lyon, France) essentially following the manufacturer's instructions. Extracted samples (final volume 100 μl) were subsequently used directly in real-time PCR experiments.

3. Results and discussion

To date, no single molecular approach has been developed that can determine potential human pathogenicity in *V. vulnificus* strains with 100% accuracy and confidence, although recent studies have demonstrated the utility of combining several molecular virulence testing approaches simultaneously (Han and Ge, 2010). In this regard, we believe that *pilF* is a useful and reliable additional target for epidemiological and diagnostic tests alongside other reported assays for virulence testing in *V. vulnificus*, such as 16S rRNA (Aznar et al., 1994; Nilsson et al., 2003; Vickery et al., 2007), capsular polysaccharide genes (Han et al., 2009), and *vcgC* and *vcgE* analysis (Rosche et al., 2005; Baker-Austin et al., 2010b; Warner and Oliver, 2008).

Initial experiments using a cloned *pilF* fragment from strain E12, a previously identified pathogenic and *vcgC*+ *V. vulnificus* isolate, showed excellent linear agreement (r^2 0.999) between the expected standard curve and detection of the *pilF* target (Fig. 2, inset B). Subsequent analysis of DNA diluted over 10 orders of magnitude was capable of reliably identifying this target using real-time PCR. Repeated analysis of highly diluted cloned material indicated that the observed limits of detection correspond to less than 5 copies of *pilF* per reaction, demonstrating optimum reaction kinetics of the

Table 2
Bacterial strains analysed in this study.

Strain	Isolation/origin	*vcgC* RT-PCR	*pilF* RT-PCR	Human serum resistance[a]
Vibrio vulnificus biotype 1 strains				
ATCC 33816	Human blood (USA)	+	+	+
E12	Oyster (USA)	+	+	+
CECT 5168	Human blood (USA)	+	+	+
CECT 529	Human blood (USA)	–	–	–
V4	Human blood (Australia)	+	+	+
N87	Human blood (Japan)	+	+	+
KH03	Human blood (Japan)	+	+	+
YJ106	Human blood (Taiwan)	+	+	+
CECT 4867	Diseased eel (Sweden)	–	–	–
PD-1	Eel tank water (Spain)	–	–	–
L49	Brackish water (Japan)	–	–	–
Riu1	Seawater (Spain)	–	+	–
CECT 4608	Eel farm water (Spain)	–	+	+
CG100	Oyster (Taiwan)	+	+	+
CS9133	Human blood (South Korea)	+	+	+
CG106	Oyster (Taiwan)	+	+	+
CECT 4606	Eel (Spain)	–	–	–
Vibrio vulnificus biotype 2 strains				
CECT 5198	Diseased eel (Spain)	–	–	–
A13	Diseased eel (Spain)	–	–	–
CECT 5769	Diseased eel (Spain)	–	–	–
A11	Diseased eel (Spain)	–	–	–
A14	Diseased eel (Spain)	–	–	–
21A	Diseased eel (Denmark)	–	–[b]	–
22	Diseased eel (Denmark)	–	–	–
27	Diseased eel (Denmark)	–	–	–
CECT 4862	Diseased eel (Japan)	–	+	+
CECT 4604	Diseased eel (Spain)	+	+	+
CECT 4999	Diseased eel (Spain)	–	+	+
CECT 5763	Eel tank water (Spain)	–	+	+
CIP8190	Human blood (France)	–	+	+
CECT 4866	Human blood (Australia)	–	+	+
CECT 5762	Healthy eel (Spain)	–	+	+
Riu-2	Seawater (Spain)	–	+	+
CECT 4868	Diseased eel (Norway)	–	+	+
90-2-11	Diseased eel (Denmark)	–	+	+
94-8-112	Human wound (Denmark)	–	+	+
94-9-123	Seawater (Denmark)	–	+	+
CECT 4865	Diseased shrimp (Taiwan)	–	+	+
UE516	Diseased eel (Taiwan)	–	+	+
CECT 897	Diseased eel (Japan)	–	+	+
95-8-162	Diseased eel (Denmark)	–	+	+
95-8-6	Diseased eel (Denmark)	–	–[b]	–
Vibrio vulnificus biotype 3 strains (Serovar O)				
11028	Human disease (Israel)	–	+	+
162	Human disease (Israel)	–	+	+
12	Human disease (Israel)	–	+	+
32	Human disease (Israel)	–	+	+
97	Human disease (Israel)	–	+	+
Other *Vibrio* spp.				
V. fluvialis VF10	Type strain NCTC 11327	–	–	NA
V. cholerae VC9	Type strain NCTC 80442	–	–	NA
V. alginolyticus	05/073	–	–	NA
V. cholerae	V05/086	–	–	NA
V. fluvialis	Type strain NCTC 11327	–	–	NA
V. parahaemolyticus	V05/070	–	–	NA
V. parahaemolyticus	V05/086	–	–	NA
V .parahaemolyticus	Type strain NCTC 10885	–	–	NA
V. parahaemolyticus	V05/062	–	–	NA
V. furnissi	V06/003	–	–	NA
V. cincinnatiensis	V06/001	–	–	NA
V. metschinovii	V06/004	–	–	NA
V. mimicus	V06/300	–	–	NA
Other bacteria				
S. paucimobilis SP21	Type strain NCTC 11030	–	–	NA
S. nottingham	Type strain NCTC 7832	–	–	NA
R. planticola	Type strain NCTC 9528	–	–	NA
P. aeruginosa PA20	Type strain NCTC 10332	–	–	NA
P. mirabilis PM22	Type strain NCTC 10975	–	–	NA
E .coli E012	Type strain NCTC 12241	–	–	NA

Table 2 (*continued*)

Strain	Isolation/origin	*vcgC* RT-PCR	*pilF* RT-PCR	Human serum resistance[a]
E .coli ECL13	Type strain NCTC 13216	–	–	NA
K. aerogenes KA23	Type strain NCTC 9528	–	–	NA
E. faecalis EF9	Type strain NCTC 775	–	–	NA

NA, not assessed.
[a] Previously determined by Roig et al. (2010).
[b] Showed weak non-sigmoidal amplification of *pilF*.

real-time PCR assay. The species specificity of the real-time PCR assay was evaluated by testing pathogenic and non-pathogenic *V. vulnificus* strains (deemed pathogenic to humans based on previous human serum reactivity analysis), as well as a range of closely and distantly related bacteria. To compare *pilF* with established pathogenicity analysis we analysed strains using *vcgC* real-time PCR, an assay previously shown to successfully identify pathogenic biotype 1 *V. vulnificus* isolates (Baker-Austin et al., 2010b). In agreement with previous work (Roig et al., 2010), the results here demonstrated that the *pilF* was capable of correctly identifying the vast majority of human serum-resistant strains, irrespective of biotype (Table 2). Of the 17 biotype 1 *V. vulnificus* strains analysed for *pilF* and *vcgC*, 16 strains were correctly identified by both assays (94.1%). Of the 25 biotype 2 strains analysed, only 9 strains were correctly identified using *vcgC* real-time PCR, compared to 25 using *pilF* (36% and 100% identifications, respectively). All five biotype three strains were correctly identified using *pilF* (100%), whilst no positive results were detected by *vcgC* (0%), Table 2. No amplification of closely related vibrio or non-vibrio strains were observed, indicating the specificity of the *pilF* real-time PCR assay (Table 2). The *pilF* assay thus contrasts with other targets used to identify pathogenic strains of *V. vulnificus*, such as 16S rRNA (Aznar et al., 1994; Nilsson et al., 2003; Vickery et al., 2007) and *vcgC* polymorphisms (Rosche et al., 2005; Warner and Oliver, 2008; Baker-Austin et al., 2010b), which have shown limited usefulness in detecting biotype 2 and biotype 3 *V. vulnificus* strains potentially dangerous to human health (Roig et al., 2010; Sanjuan et al., 2009). Both biotype 2 (serovar E and serovar I) and biotype 3 *V. vulnificus* strains represent important human pathogens (Amaro and Biosca, 1996; Bisharat et al., 1999), and the ability to identify potentially virulent strains rapidly is of paramount importance. We found that a small number of non-pathogenic *V. vulnificus* strains (i.e. isolates possessing no serum resistance) did demonstrate weak amplification when analysed with the *pilF* real-time PCR assay (Table 2). However, these strains did not show true sigmoidal amplification curves, which were evident in all analysed human serum resistant *V. vulnificus* strains (data not shown). The non-specific amplifications are probably caused by the relatively close sequence homology of the two major polymorphisms in the *pilF* gene, whereby some co-amplification of non-pathogenic *pilF* DNA from non-pathogenic strains is difficult to eliminate. We found that reducing the number of thermal cycles, increasing the cycle threshold and iterative analysis of all amplifications during the real-time PCR assay successfully eliminated these false-positive results. Irrespective, several strains producing discordant results are currently subject to in-depth characterization including additional human serum analysis and genomic sequencing to glean additional insights into the pathogenic potential of these isolates.

Real-time PCR demonstrated a strong dynamic range of detection, with samples reliably detected from cloned plasmid template diluted to over 9 orders of magnitude in serial dilution experiments (Fig. 2), corresponding to single number copies of *pilF* per PCR reaction. Real-time PCR assays were performed on DNA extracted from bioaccumulated shellfish samples (Fig. 3). We were able to identify *pilF* directly from shellfish matrices (Pacific oyster, average C_t value 28.99, Fig. 3), whereas no signal was evident from negative control samples (no vibrio amendment), or from extracted water. No matrix (shellfish tissue) inhibition was observed during these experiments. The results from artificially bioaccumulated samples (Fig. 3) correspond to detection of approximately 7600 genome copies of *pilF* per reaction based on cloned *pilF* target DNA (Section

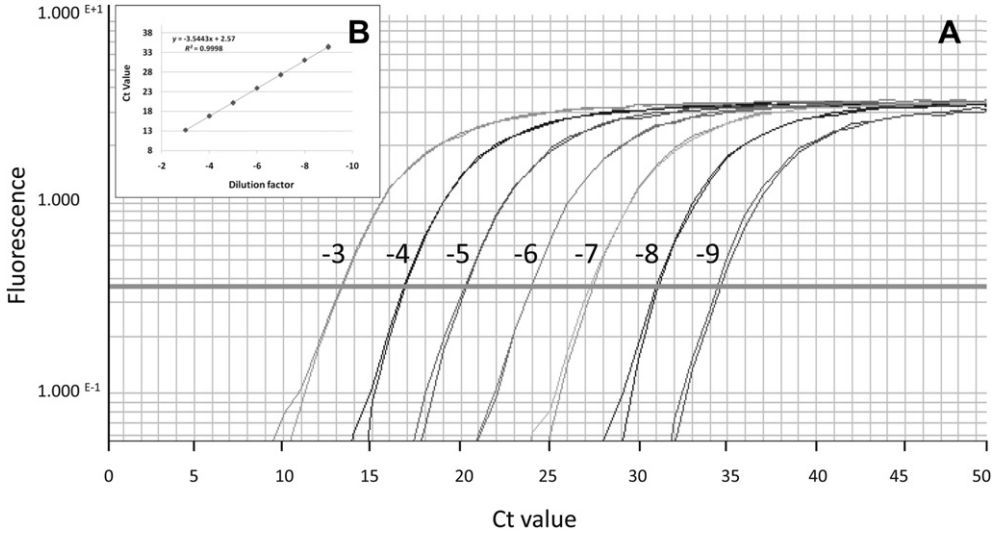

Fig. 2. Example of real-time PCR curves generated by a serial dilution range of cloned material (A) and corresponding standard curves for *V. vulnificus* as detected by real-time PCR (B).

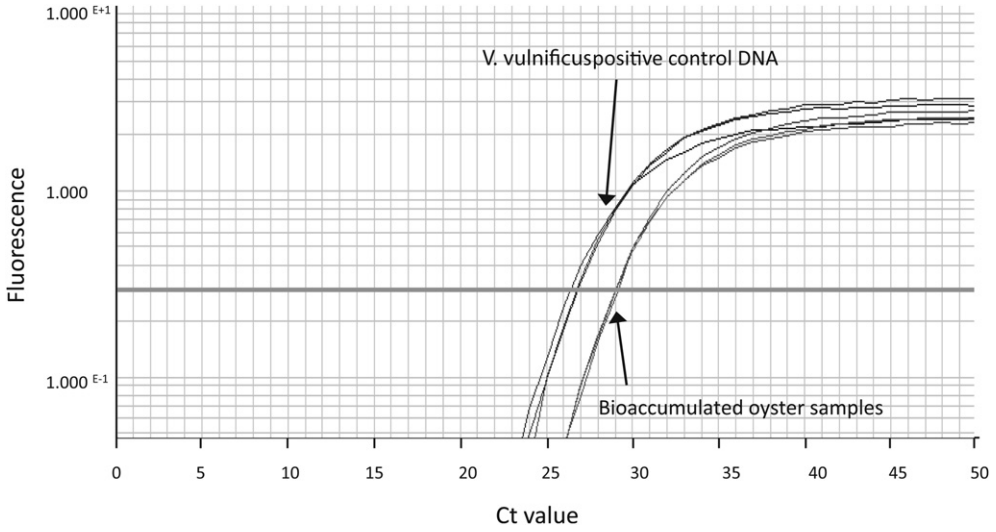

Fig. 3. Detection of *pilF* in artificially amended shellfish matrices.

2.5), highlighting the sensitivity of this approach for detection purposes in shellfish matrices. These results mirror prior findings (Baker-Austin et al., 2010b) which demonstrated that the nucleic acid extraction procedure detailed here can be used to directly extract bacterial nucleic acids from shellfish matrices, without the need for prior enrichment of samples. Given the promising sensitivity of this assay, the ability to identify pathogenic *V. vulnificus* from naturally contaminated rather than artificially spiked samples is an area that will be further investigated in future work.

In conclusion, this real-time PCR assay represents a rapid means of distinguishing *V. vulnificus* strains potentially harmful to human health, irrespective of biotype. The assay was applied to single cultures, extracted DNA and to artificially bioaccumulated shellfish samples, and is an especially useful target to detect potentially pathogenic biotype 2 and 3 strains of human health relevance. This tool will enable early detection capability in a range of different applications, such as food processing, regulatory and clinical settings.

Acknowledgments

We would like to that F. Roig for technical assistance. The European Community Reference laboratory is funded by D.G. Sanco of the European Commission, with matching funding by the Department of Environment, Food and Rural Affairs, and the Food Standards Agency of the United Kingdom. This work has been partially financed by grants AGL2008-03977/ACU and Programa Consolider-Ingenio CSD2009-00006 from MICINN (Spain).

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