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Development of real-time qPCR assays for detecting and quantifying common bacterial pathogens in fish from Mediterranean aquacultures

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Abstract

The aquaculture industry is a rapidly developing and economically important sector for many countries, thus it requires effective solutions to overcome the key challenges that could impede its growth and sustainability. Among the most serious concerns are fish diseases caused by bacterial infections. This study introduces the development of novel rapid molecular methods employing Real-Time Polymerase Chain Reaction (qPCR) for the specific detection and quantification of five major fish pathogenic bacteria: *Vibrio harveyi, V. alginolyticus, V. anguillarum, Photobacterium damselae, and Tenacibaculum maritimum*. These bacteria are responsible for diseases such as vibriosis, photobacteriosis, and tenacibaculosis. The qPCR assays developed in this study are highly specific and extremely sensitive, making them suitable for early detection of these pathogens, thus aiding in the prevention of disease outbreaks in aquaculture farms.

Keywords: qPCR; fish pathogenic bacteria; aquaculture; Vibrionaceae; *Tenacibaculum maritimum.*

Introduction

Mediterranean aquaculture has undergone considerable development in recent decades, and as such, it is of significant economic importance. Greece accounts for 60% of the European sea bass (*Dicentrarchus labrax*) and gilthead sea bream (*Sparus aurata*) farmed in the EU and 25% worldwide, making it the leading producer of Mediterranean fin fish aquaculture in Europe ("FGM; Annual Report; Federation of Greek Mariculture, 2022"). Together with Egypt, Turkey, Italy, and Spain, they account for more than 90% of the aquaculture production in the Mediterranean ("FEAP: European Aquaculture Production Report 2014-2019").

The aquaculture sector faces a major challenge from disease outbreaks brought on by bacterial infections in fish (Irshath *et al.*, 2023). According to Rohani *et al.* (2022), the high density at which fish are raised in aquaculture settings promotes the transfer and spread of pathogenic bacteria, which can result in significant economic losses from morbidity and mortality.

According to a number of recent studies, vibriosis, photobacteriosis, and tenacibaculosis are the three main bacterial infections that affect Mediterranean fish farms, particularly those that involve the European sea bass and the gilthead sea bream (Pujalte *et al.*, 2003; Toranzo *et al.*, 2005; Muniesa *et al.*, 2020; Cascarano *et al.*, 2021). Vibriosis and photobacteriosis are caused by Gram-negative bacteria of the Vibrionaceae family and exhibit a variety of pathologies. More specifically, *Vibrio anguillarum* is responsible for causing highly fatal hemorrhagic septicemia in a wide range of aquacultured fish, with mortality rates in the hyperacute disease stage reaching as high as 80% (Frans *et al.*, 2011). However, vibriosis can also be induced by other bacteria of the *Vibrio* genus, such as *V. alginolyticus* and *V. harveyi* (Toranzo *et al.*, 1997). *V. harveyi* can lead to eye lesions in fish resulting in blindness, gastroenteritis, muscle necrosis, skin ulcers, and caudal fin rot (Zhang *et al.*, 2020). However, even though *V. alginolyticus* is thought to be an opportunistic pathogen in fish, it can also infect humans through the alimentary canal and cause peritonitis and gastroenteritis (Mustapha *et al.*, 2013).

Photobacterium damselae (subsp *piscicida* & *damselae*) causes photobacteriosis or pasteurellosis in fish. The severity of the disease depends on environmental conditions, especially temperature, leading to either low or high mortality rates (Andreoni & Magnani, 2014).

More specifically, *P. d.* subsp. *piscicida* causes a disease characterized by the presence of numerous white nodules (pseudotubercules) in the internal organs, especially the kidney and spleen, and the formation of granulomas (Daly & Aoki, 2011). On the other hand, *P. d.* subsp. *damselae* acts as an opportunistic pathogen, leading to symptoms such as lethargy, hemorrhages at the base of fins and tails, and distended abdomens. This subspecies has a broad host range, encompassing humans among other species (Rivas *et al.*, 2013).

Tenacibaculosis has important consequences to the global aquaculture sector, including the Mediterranean (Kolygas *et al.*, 2012; Gourzioti *et al.*, 2016; Muniesa *et al.*, 2020). The causative agent of the disease is *Tenacibaculum maritimum,* a gram-negative bacterium of the *Flavobacteriaceae* family. The symptoms of the disease are distinctive gross lesions on the external body of fish including ulcers, skin necrosis, mouth erosion, frayed fins, and tail rots (Mabrok *et al.*, 2023).

The detection and identification of the aforementioned bacteria by classical microbiological methods are time-consuming as they rely on their cultivation in selective media along with the analysis of their biochemical characteristics (Frans *et al.*, 2011; Mabrok *et al.*, 2023). Although molecular (Polymerase Chain Reaction - PCR) tests have been developed for their specific detection (Osorio *et al.*, 2000; Conejero & Hedreyda, 2003; Pang *et al.*, 2006; Fukui & Sawabe 2007; Zhou *et al.*, 2007; Hong *et al.*, 2007; Jing-jing *et al.*, 2011; Fernández-Álvarez *et al.*, 2019; Mabrok *et al.*, 2023), they have limited or no capacity in quantifying the pathogenic bacterial load. The latter provides crucial prognostic information regarding fish health and environmental contamination. Thus, by focusing on novel genes or improving upon previously employed protocols, our goal in this work was to develop novel, fast molecular tools utilizing Real-Time PCR (qPCR) for the precise identification and quantification of the five most prevalent pathogenic bacteria that infect fish.

Materials and Methods

Sample collection

European sea bass, gilthead sea bream and red porgy, *Pagrus major* were collected from three distinct aquafarms in Northwestern Greece (Ionian Sea). The samplings were performed monthly in 2020 and 2021. According to the history of the farm, samples were taken from cages that exhibited clinical signs and/or mortalities. Ten fish were collected from every cage with a preference to diseased fish. Most of the clinical signs were common like anorexia, darkening of the skin, lethargy while others had signs which were more consistent to specific infections, like vibriosis, photobacteriosis, and tenacibaculosis. Those fish exhibited different levels of ulcers, hemorrhages, and fin necrosis. During the fish necropsy, spleen and kidney tissue samples were aseptically collected with a bacteriological loop, and subsequently inoculated onto two different culture media, i.e. Trypton Soy Agar (TSA) (Oxoid Ltd, Cheshire, England), with the addition of 2% NaCl, and Blood Agar (Columbia blood agar with 5% sheep blood, Oxoid Ltd, Cheshire, England) for 1 to 3 days at 25°C (Austin & Austin, 2016). Colonies from TSA were selected and subcultured to Thiosulfate Citrate Bile Salts Sucrose (TCBS) (Oxoid Ltd, Cheshire, England), a selective media for *Vibrio* species. Pure colonies of the bacterial isolates during that period were re-inoculated into Trypton Soy Broth (TSB) and genomic DNA was extracted using the PureLink Genomic DNA kit (Invitrogen, ThermoFisher Scientific) according to the manufacturer's protocol.

In addition, DNA extracts (similarly isolated) originating from the *Tenacibaculum maritimum* DCM 17995 strain (Leibniz Institute DSMZ - German Collection of Microorganisms and Cell Cultures) as well as from *Vibrio harveyi*, *Vibrio anguillarum, Vibrio alginolyticus*, and *Photobacterium damselae* isolates previously identified by Whole Genome Sequencing were used as positive controls. The fastq sequences are accessible at BioProject ID PRJNA1154470, http://www.ncbi.nlm.nih.gov/bioproject/1154470. These bacterial strains were also quantified by the standard plate count method (tenfold serial dilutions of the initial TSB bacterial culture). Each of the quantified initial bacterial culture was subjected to DNA extraction (PureLink Genomic DNA kit, Invitrogen, ThermoFisher Scientific). The estimated final concentrations (copies μl^{-1}) of the DNA extracts were $4X10^6$ for *V. harveyi*, $2X10^5$ for *V. anguillarum*, $2X10^8$ for *V. alginolyticus*, $2X10^7$ for *P. damselae* and $1X10^7$ for *T. maritimum*. Serial dilutions of the above-mentioned quantified DNA extracts were used as matrices to derive the standard curves for the qPCRs. DNA extracts of other common fish pathogenic and nonpathogenic bacterial strains (*Pseudomonas aeruginosa* ATCC 27853, *Staphylococcus aureus* ATCC 25923, *Escherichia coli* ATCC 25923 and *Lactococcus garvieae* ATCC 25922) were used for evaluation of the analytical specificity of the qPCR.

Oligonucleotides and PCR methods

Sequences of the newly designed, modified, or literature-derived primers along with the optimized protocols are presented in Table 1. Analyses for the melting temperature (Tm), dimer formation, and possible hairpin secondary structures were performed using the "OligoAnalyzer 3.1" software (http://eu.idtdna.com/calc/analyzer). All oligonucleotides were synthesized by Integrated DNA Technologies (Leuven, Belgium) and all assays were performed using a CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad Laboratories, USA). Non-template controls (NTCs) were included in duplicate in each run. The fluorescence levels were measured at the end of each cycle, and analysis was performed using CFX Manager™ software (version 3.1; BioRad Laboratories, USA).

For the detection and quantification of *V. harveyi*, a qPCR method was developed for the amplification of a 382 base pair (bp) fragment of the *toxR* gene using the

Table 1. Primer sequences for the detection and quantification of pathogenic bacteria. Modifications of the reference primers are shown in bold.

Target/ Method	Primers	Sequence 5' - 3'	Gene	Product size	Reference
V.harveyi qPCR	VhtoxF	**CT**GAAGCAGCACTCACCGAT	*toxR*	382	Pang *et al.* (2006)
	VhtoxR	**GACT**GGTGAAGACTCATCAGCA			
V.anguillarum qPCR	VangF	TATCACTGTTGAAGAAGGTCAAGCACTG	*groESL*	195	Kim *et al.* (2010)
	VangR	CGCTTCAAGTGCAGGAAGCAG			
V.alginolyticus qPCR	RpoXF	ACGCTGCTCAGGGGTGAAAGC	*rpoX*	330	Jing-jing *et al.*(2011)
	RpoXRnew	**CCGCCTCTATCTGCATCTGACG**			
P.damselae qPCR	Phd16sF2	**GCGAAAGCGTGGGGAGCAAAC**	*16s rRNA*	550	Osorio *et al.* (2000)
	Phdam16S R	CACCTCGCGGTCTTGCTG			
*P.damselae*subsp. *piscicida*&*damselae* multiplex-PCR	Phdam 16S F	GCTTGAAGAGATTCGAGT	*16s rRNA*	267	
	Phdam 16SR	CACCTCGCGGTCTTGCTG			
	Phdamurec F	TCCGGAATAGGTAAAGCGGG	*ureC*	448	
	Phdamurec R	CTTGAATATCCATCTCATCTGC			
T.maritimum qPCR	TenmarF	**AGAGAG GCGAAGCAAATA GCAT**	*IF-2 of infB*	161	This study
	TenmarR	**ACCGATCCATCAACATCTCCTT**			
	TenmarProbe FAM/ ZEN	**TCTCAATTGCAACGTGAGCA**			

primers VhtoxF and VhtoxR, which resulted from the 5'-end modification of previously reported primers for this gene (Pang *et al.*, 2006). The PCR was carried in a final volume of 20 µl containing 1X SYBR™ Select Master Mix (Applied Biosystems™, ThermoFisher Scientific), 400 nM of each primer and approximately 20 ng of bacterial DNA. Thermocycling conditions included Uracil-DNA glycosylase (UDG) activation at 50°C for 2', initial denaturation at 95°C for 2', and 40 cycles consisting of a 15'' denaturation step at 95°C and hybridization/extension step at 63°C for 1'. For the detection and quantification of *V. alginolyticus*, 400nM of the forward primer RpoXF that has been previously described (Jing-jing *et al.*, 2011) and an equal concentration of the newly designed reverse primer RpoXRnew were used for the amplification of a 330bp segment of the *rpoX* gene, under the same thermocycling conditions described above, except for the hybridization/elongation step that was performed at 65°C. For *V. anguillarum* the primer pair VangF-VangR, that has been previously described (Kim *et al.*, 2010) in a conventional PCR, was used for the amplification of a 195 bp segment of the *groESL* gene, with the same thermocycling conditions described above, except that 300nM of each primer were used, and the hybridization/elongation step was performed at 64°C. For the amplification of a 550 bp segment of the *16S rRNA* gene of *P. damselae,* 300nM of the previously described reverse primer Phdam16SR (Osorio *et al.*, 2000) and of the newly designed forward primer Phd16sF2 were used. The hybridization/elongation was performed at 65°C. For the detection and quantification of *T. maritimum*, a novel TaqMan probe-based method was developed for the amplification of a 161 bp fragment of the *infB* gene. The primers used, i.e., TenmarF and TenmarR as well as the TaqMan probe TenmarProbe FAM/ZEN, were designed using the online available Primer3 plus software (https://www.primer3plus.com). The reaction was performed in a final volume of 20µl including 2X KAPA PROBE FAST qPCR mix (KAPA Biosystems®), 300nM of each primer, 300nM probe and approximately 20ng of bacterial DNA. Initial denaturation was performed at 95°C for 3', followed by 40 cycles consisting of denaturation at 95°C for 3'', and hybridization/extension at 56°C for 30''.

Serial tenfold dilutions of DNA extracts from the quantified reference bacterial strains were prepared and each dilution was tested in triplicates to determine the analytical characteristics of each qPCR assay, i.e., amplification efficiency and dynamic range of quantification. Standard curves were generated by plotting the cycle threshold values (Ct) of the amplified genes against the $\log_{10}$ of the bacterial copies. The standard curves were used for the estimation of the detection limit (copies μl^{-1} of the extracted DNA) of each standard bacterial strain. For each bacterial species, standard curves were generated at least three times. Furthermore, the coefficient of variation (CV = standard deviation divided by mean) of the standard curve parameters; efficiency (efficiency CVs) and slope (slope CVs) were calculated (Table 2).

For the discrimination of *P. damselae* in subspecies *piscicida* and *damselae*, an additional multiplex-PCR

Table 2. The percentage of coefficient of variation (CV = standard deviation divided by mean) was calculated for the standard curves' parameters; efficiency (efficiency CVs) and slope (slope CVs) generated for each assay.

qPCR method	Efficiency CV (%)	slope CV (%)
V. harveyi	10	7
V. anguillarum	10	7
V. alginolyticus	10	7
P. damselae	8	6
T. maritimum	5	2

protocol was used with two pairs of primers according to Osorio *et al.* (2000). With this method the strains of *P. d.* subsp. *damselae* yield two amplification products, one 267 bp and the other 448 bp, corresponding to internal segments of the *16s rRNA* and *ureC* genes, respectively. In contrast, in *P. d.* subsp. *piscicida* only the 267 bp product is amplified, as the *ureC* gene is absent from its genome. The multiplex PCR reaction was performed with modifications of the protocol described by Osorio *et al.* (2000) at a final volume of 25 μl, containing 1X KAPA Multiplex Fast PCR (KAPA Biosystems®), 300 nM of each primer and approximately 20 ng of bacterial DNA. The duration of the thermocycler steps was also modified with initial denaturation performed at 95°C for 3', 35 cycles consisting of denaturation at 95°C for 15'', primer annealing at 60°C for 30'', extension at 72°C for 45'' and final elongation at 72°C for 5'. The PCR products were analyzed by agarose gel electrophoresis using a 1.5% agarose gel with the addition of 0.04 μl/ml ethidium bromide.

Sequencing of qPCR products

The specificity of the qPCR methods was confirmed by Sanger dideoxy sequencing of the amplified products using the BigDye™ Terminator v3.1 Cycle Sequencing Kit, an ABI3500 Genetic Analyzer (Applied Biosystems, Darmstadt, Germany) and the qPCR primers. Sequence comparisons were made by BLAST against GenBank (NCBI).

Results

The analytical sensitivity of the developed qPCR assays for the specific detection of *V. harveyi*, *V. anguillarum*, *V. alginolyticus*, *P. damselae*, and *T. maritimum* was tested using tenfold serial dilutions of the quantified DNA extracts. It is possible to estimate the method's detection limit (copies μl^{-1}) and gene copy rate by the indirect method that has been used in the current study, even at a low gene copy number. The detection limit (copies μl^{-1}) of each standard bacterial strain used for the generation of standard curves in Real-Time PCR, and the coefficient of determination (R^2) are shown in Figure 1. More specifically, the sensitivity was found to be one gene copy, i.e., one bacterial cell for *V. harveyi* and *T. maritimum*, and two copies for the other three bacterial species examined. Moreover, the developed methods showed 93.6% efficiency for *V. harveyi*, 100% for *V. anguillarum*, 93.6% for *V. alginolyticus*, 93.1% for *P. damselae*, and 98.3% for *T. maritimum* (Fig. 1A, B, C, D and E). Furthermore, the calculated coefficient of variation of the standard curve parameters, the efficiency and the slope support the low variability and high reproducibility of the assays (Table 2).

The specificity of the newly developed qPCR assays was confirmed by the absence of amplification products when they were applied on DNA extracts from other common fish pathogenic and nonpathogenic bacterial strains (*Pseudomonas aeruginosa* ATCC 27853, *Staphylococcus aureus* ATCC 25923, *Escherichia coli* ATCC 25923 and *Lactococcus garvieae* ATCC 25922), or in non-template reactions.

In total, 116 bacterial isolates were collected from the bacterial cultures of the infected fish tissues. The application of the specific qPCR assays on these 116 bacterial isolates identified 70 of them as *V. harveyi*, 42 as *P.damselae*, two as *V.anguillarum,* and two as *V.alginolyticus*. The *T. maritimum* was not detected in this collection of bacterial isolates. Furthermore, the 42 bacterial isolates identified as *P. damselae* were further differentiated into *piscicida* (N=31) and *damselae* (N=11) subspecies by the multiplex-PCR assay of Osorio *et al.* (2000) (Fig. 2).

The sequencing of the qPCR amplified products confirmed the specificity of the developed qPCR assays. All sequence data have been submitted to the DDBJ/EMBL/GenBank databases under accession numbers LC804752 (*P. damselae*), LC805642 (*V. harveyi*), LC805643 (*V. alginolyticus*), LC805644 (*P. damselae* subsp. *damselae*), LC805645 (*T. maritimum*) and LC805152 (*V. anguillarum*).

Discussion

Disease outbreaks within the aquaculture sector have serious impacts to the industry's production with significant economic losses (Fernández Sánchez *et al.*, 2022). Furthermore, there are reports that *V. alginolyticus*, *V. harveyi* and *P. damselae* subsp. *damselae* are capable of causing food-born infections or zoonoses (Shin *et*

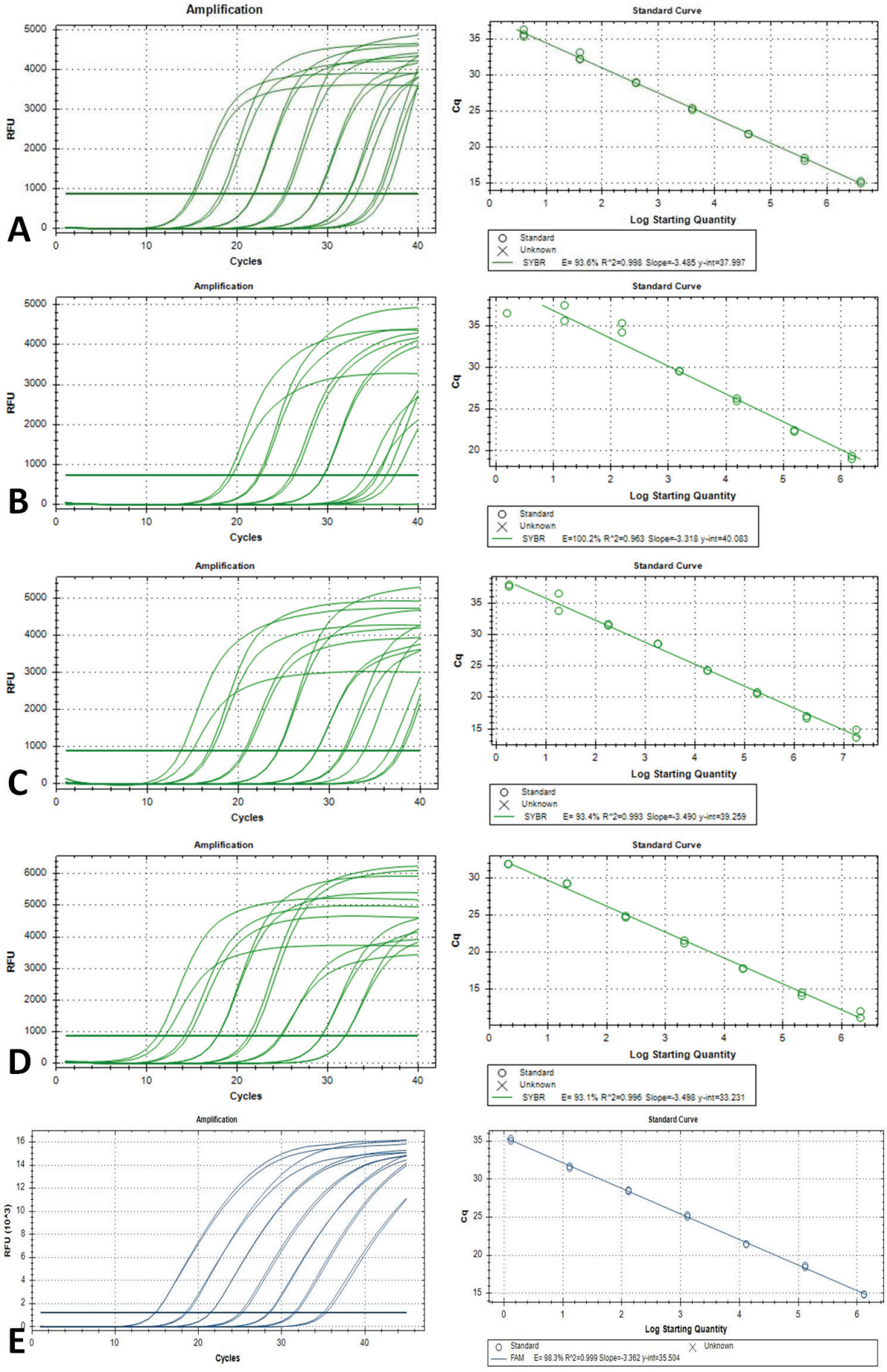

Fig. 1: Amplification plots and corresponding standard curves of quantified bacterial strains. A) *V. harveyi* (tenfold serial dilutions $4X10^{6}$- 4), B) *V. anguillarum* (tenfold serial dilutions $2X10^{5}$-2), C) *V. alginolyticus* (tenfold serial dilutions $2X10^{8}$-2), D) *P. damselae* (tenfold serial dilutions $2X10^{7}$-2), E) *T. maritimum* (tenfold serial dilutions $1X10^{7}$- 1).

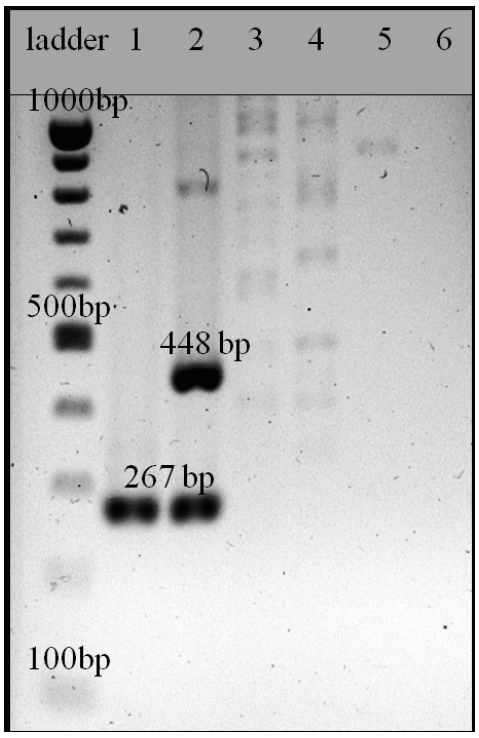

Fig. 2: Discrimination of *P. damselae* subsp. *damselae* &*piscicida* by multiplex-PCR and agarose gel electrophoresis. Ladder: 100 base pair (New England Biolabs, UK), 1: *P. damselae* subsp. *piscicida* 2: *P. damselae* subsp. *damselae,* 3: *V. alginolyticus*, 4: *V. harveyi*, 5:*L. garviae*, 6: non template.

al., 1996; Yamane *et al.*, 2004; Kim *et al.*, 2009; Austin, 2010; Mustapha *et al.*, 2013). For the control of the bacterial fish diseases, aquafarmers rely mostly on the use of antibiotics (Rigos *et al.*, 2021), a practice that poses serious concerns for the potential presence of antibiotic residues in fish products and the development of antibiotic resistance. Climate change is expected to further accentuate disease outbreaks, with important consequences on both global and Mediterranean aquaculture (Cascarano *et al.*, 2021). Therefore, the need for highly sensitive diagnostic tools for the early detection of diseases, along with management strategies to minimize the use of antibiotics, has become of primary importance (Rosa *et al.*, 2012; Baker-Austin *et al.*, 2017; Montánchez & Kaberdin, 2020; Cascarano *et al.*, 2021).

While qPCRs-based assays have been previously used for the detection of fish pathogens, the present study is the first to provide a comprehensive methodology for the identification of the most important pathogenic bacteria. Specifically, in two different studies the amplification of the *16S rRNA* gene was used for the detection of *T. maritimum* (Fringuelli *et al.*, 2012; Fernández-Álvarez *et al.*, 2019). The reported sensitivities of these methods were reported at 4.8 copies μl^{-1} (Fernández-Álvarez *et al.*, 2019) and at 2.22 copies of the gene (Fringuelli *et al.*, 2012). Our assay for this bacterium, targeting the *infB* gene, provides superior sensitivity as we were able to detect down to 1 copyμl^{-1}. Similarly, two different methods for the detection of *V.anguillarum* have been previously reported, one targeting both the *16S rRNA* and *toxR* genes, with reported sensitivity of 1-10 bacterial cells (Crisafi *et al.*, 2011), and one reported to detect10^4 cfu ml^{-1}, with the *virA/virB* gene as the target sequence (Chapela *et al.*, 2018). Our *V.anguillarum* assay, targeting the g*roESL* gene, showed higher sensitivity, as the level of detection was 2 copies μl^{-1}. Two different assays have also been reported for *V. alginolyticus*. The first, that used as target the *rpoX* gene, had minimum detection limit 10^3 cells from pure culture and 10^2 cells from seawater (Jing-jing *et al.*, 2011). The second, by targeting the *gyrB* gene, was able to detect 100 cells in 1 ml of seawater or seafood tissue homogenates (Zhou *et al.*, 2007). The *V. alginolyticus* assay that we developed, used as target the *rpoX* and achieved detection limit of 2 copies μl^{-1}. For the simultaneous detection and quantification of both subspecies of *P. damselae* (subsp. *piscicida* and *damselae*), a previously described qPCR assay, targeting the *toxR* gene of both subspecies, reported detection limit of about 1×10^3 target molecules per µl (Martins *et al.*, 2015). In contrast, our assay, targeting a region of the *16S rRNA* gene, was able to detect 2 copies μl^{-1}. All our assays showed linear range of quantification that extended over a 6-$\log_{10}$ range, high amplification efficiency (93.1% - 100%) and low detection limit (1-2 copies μl^{-1}). Sequencing of the qPCR amplified products confirmed the specificity of the assays.

The application of the developed assays on the 116 collected bacterial isolates from infected fish tissues showed that the most common fish pathogen was *V. harveyi*, since 70 out of the 116 isolates were identified as this species. The second most abundant bacterial species was *P. d.* subsp. *piscicida* (N=31), followed by *P. d.* subsp. *damselae* (N=11). Only two isolates for each of *V. anguillarum* and *V. alginolyticus* were identified. In contrast, *T. maritimum* was not detected in this collection. This negative result may reflect the absence of this pathogen in the fish samples examined. Alternatively, this could be attributed to the fact that *T. maritimum* isolation from diseased fish is not always successful and definitive diagnosis requires colony isolation on specific media (i.e., Flexibacter Maritimus Medium agar or media supplemented with antibiotics) (Pazos *et al.*, 1996).

The molecular techniques developed in this study offer an advantage over traditional microbiological methods due to their speed and reliability in distinguishing bacterial species that are phenotypically similar. Furthermore, the Real-Time PCR format offers the benefit of quantification, speed, lower cost, minimal risk of laboratory contamination and aids in the prevention of false-positive results when compared to the available gel-based assays. The assays developed in the present study could also be used for the quantification of the pathogenic bacterial load in other matrices such as DNA from infected fish tissues or from environmental samples in contrast to the aforementioned gel-based assays (Bader & Shotts, 1998; Osorio *et al.*, 2000; Conejero & Hedreyda, 2003; Cepeda *et al.*, 2003; Avendaño-Herrera *et al.*, 2004; Avendaño-Herrera *et al.*, 2006; Pang *et al.*, 2006; Fukui & Sawabe, 2007; Hong *et al.*, 2007; Kim *et al.*, 2010) and other available Real-Time PCRs that have limited or no quantification capacity (Zhou *et al.*, 2007; Jing-jing *et al.*,

2011; Fringuelli *et al.*, 2012; Fernández-Álvarez *et al.*, 2019).

In conclusion, the qPCR assays of the present study are sensitive and specific methods for detection and quantification of the five most important bacteria species affecting Mediterranean aquaculture. Future efforts should involve their application and evaluation for the detection and quantification of bacteria load in environmental samples and in affected fish tissues.

In conclusion, the outbreak of fish diseases is considered a major threat for the development of the aquaculture industry, rendering the early detection of common fish pathogens crucial for effective preventive measures in any aquaculture setting. The molecular methods developed and optimized in the present study are fast and reliable in identifying phenotypically similar bacterial species, also providing quantification of the bacterial load. Therefore, they can support fish farming by helping in the early identification of the main pathogenic bacteria in fish tissues and potentially, in environmental samples.

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