J. Ocean Univ. China (Oceanic and Coastal Sea Research)
https://doi.org/10.1007/s11802-021-4544-3
ISSN 1672-5182, 2021 20 (2): 445-453
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Development of a Microfluidics-Based Quantitative Real-Time PCR to Rapidly Identify *Photobacterium damselae* subsp. *damselae* with Different Pathogenicity by Detecting the Presence of *mcp* or *dly* Gene

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(Received March 27, 2020; revised May 8, 2020; accepted August 25, 2020)

Abstract As a marine bacterial pathogen, *Photobacterium damselae* subsp. *damselae* (PDD) is distributed in seawater worldwide. It can infect different animals as well as humans, even cause deaths. The highly conserved regions of PDD *mcp* gene on chromosome and *dly* gene on plasmid were selected as the target fragments to design the specific primers. Recombinant plasmid standard was prepared based on the primers. With GENECHECKER UF-150 qRT-PCR instrument as the platform, a fluorescence-based quantitative real-time PCR (qRT-PCR) method was established for the detection of PDD. This method can specifically detect PDD and distinguish the highly virulent strains. Additionally, the test results can be qualitatively judged by visualization, while the quantitative detection can be achieved through the standard curve calculation. The minimum limit of detection was 1.0×10^1 copies μL^{-1}, and the detection time was less than 20 min. In summary, this new method has outstanding advantages, such as strong specificity, high sensitivity, and low site requirements. It is a rapid on-site detection technology for highly virulent PDD strains.

Key words mariculture; *Photobacterium damselae*; microfluidics; pathogenicity; rapid detection

1 Introduction

Photobacterium damselae subsp. *damselae* (PDD) is a subspecies of *P. damselae* belonging to *Vibrionaceae* family. As an important pathogen of marine animals, it is widely distributed in the seawater worldwide. The bacterium is firstly reported in 1981, which was isolated from skin ulcers of damselfish (*Chromis punctipinnis*), and originally was named as *Vibrio damsel* (Love *et al*., 1981). Subsequently, there are successive reports on PDD infection in different animals around the world, including fish (Ketterer *et al*., 1992; Labella *et al*., 2006), crustaceans (Song *et al*., 1993; Vaseeharan *et al*., 2007), mollusks (Hanlon *et al*., 1984; Lozano-León *et al*., 2003), sea turtles (Obendorf *et al*., 1987), mammals (Fujioka *et al*., 1988; Lee *et al*., 2018). In 1982, the first case of PDD infection in human was reported (Morris *et al*., 1982). Symptoms of human PDD infection usually appear as severe fasciitis (Yuen *et al*., 1993) and septicemia (Perez-Tirse *et al*., 1993). In 2004, Japanese scientists reported two cases of human PDD infection, causing severe fasciitis to acute death (Yamane *et al*., 2004). In 2015, a child was infected with PDD and died in Saudi Arabia (Alhemairi *et al*., 2015). These cases have confirmed that the bacteria are highly pathogenic to humans.

Usually PDD is studied with heterogeneously isolated strains according to their phenotypic characteristics and virulence (Pedersen *et al*., 1997; Terceti *et al*., 2016; Terceti *et al*., 2018). Some of them show strong hemolytic activity and high pathogenicity, while others exhibit weak hemolytic activity and lower pathogenicity, or even no pathogenicity. Early studies have confirmed that the pathogenic strains of PDD can produce a large amount of cytolytic toxin, which is named as damselysin (Dly) (Kothary *et al*., 1985; Kreger *et al*., 1987; Cutter *et al*., 1990). Subsequently, more researches revealed that a pore-forming toxin with hemolytic activity, named as phobalysin P (PhlyP), is another important virulence factor of the strain (Rivas *et al*., 2011, 2013a, 2015; Vences *et al*., 2017). These two toxins are encoded by *dly* gene and $hlyA_{pl}$ gene, respectively, which are located on the virulence plasmid

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named as pPHDD1 (Rivas *et al*., 2011). In 2013, a study reported the third virulence gene on chromosome encoding another hemolysin (HlyA) (Rivas *et al*., 2013a). To date, adequate studies have supported that the conjunction of Dly, PhlyP and HlyA cytotoxins constitutes the virulence system of highly virulent lineages of PDD (Rivas *et al*., 2015; Rivas *et al*., 2013b). In fact, different strains of the same bacteria with significant differences in pathogenicity are common in nature (Saroj *et al*., 2008; Cheng *et al*., 2019).

Until now, infections of mariculture animals by PDD have been rarely reported, and PDD is considered as a newly discovered pathogen in China. Nevertheless, according to available studies, it can infect different marine fishes living in different environments, such as *Epinephelus lanceolatus* (Zhang *et al*., 2009), *Cynoglossus semilaevis* (Yan *et al*., 2018), *Sebastes schlegeli* (Zhang *et al*., 2019b) and so on. There seems to be no obvious regional specificity of this bacterium though the seawater temperatures in different environments are quite different. In 2016, we isolated a PDD strain from black rockfish *S. schlegeli* with the symptom of skin ulceration, which were cultured in net-cage of Changdao County, Shandong Province. The experiment has confirmed its pathogenicity to black rockfish (Zhang *et al*., 2019b). Its whole-genome sequencing has also been completed (Yu *et al*., 2019). Together with other 24 strains isolated previously, there are totally 25 PDD strains in our laboratory. These strains have significantly different physiological and biochemical characteristics as well as hemolytic activity and drug resistance (Shi *et al*., 2019). When 10^8 cfu mL^{-1} live bacterial suspension was injected into the fish, it can cause the death of experimental fish in 2 days, while no death was observed in the fish injected with non-pathogenic strain. In addition, our previous research has confirmed that PDD is widely distributed along China offshore seawater, posing a potential public health risk to fishermen, offshore workers and tourists. Therefore, in order to control PDD infection in animals and humans, it is necessary to establish a rapid detection method to distinguish these strains with different levels of virulence.

2 Material and Methods

2.1 Experimental Strains

A total of 20 strains belonging to different bacterial species and 25 different strains of PDD were selected in this study. The 20 different bacterial species included three *V. rotiferianus*, three *V. harveyi*, two *V. anguillarum*, two *V. splendidus*, two *V. parahaemolyticus*, two *V. alginolyticus*, one *Aliivibrio fischeri*, one *V. scophthalmi*, one *V. cyclitrophicus*, one *P. damselae* subsp. *piscicida* (PDP) and two *Escherichia coli*. Among them, seven strains of *V. rotiferi-*

Table 1 The 23 bacteria strains for primers specific detection of PDD *mcp* gene fragment

Serial number	Strain	Strain number	Source of the strain
1	*Photobacterium damselae* subsp. *damselae*	Pdd0905	Isolation of YSFRI
2	*Photobacterium damselae* subsp. *damselae*	Pdd1601	Isolation of YSFRI (SRA accession: PRJNA490082)
3	*Photobacterium damselae* subsp. *damselae*	Pdd1605	Isolation of YSFRI
4	*Vibrio rotiferianus*	VR1601	Isolation of YSFRI (SRA accession: PRJNA498661)
5	*Vibrio rotiferianus*	VR1602	Isolation of YSFRI
6	*Vibrio rotiferianus*	VRST-01	Purchased from CGMCC (CGMCC number:1.8701)
7	*Vibrio harveyi*	VHST-01	Purchased from CGMCC (CGMCC number: 1.8773)
8	*Vibrio harveyi*	VH0207	Isolation of YSFRI
9	*Vibrio harveyi*	VH1809	Isolation of YSFRI
10	*Vibrio anguillarum*	VA1012	Isolation of YSFRI
11	*Vibrio anguillarum*	VA0531	Isolation of YSFRI
12	*Vibrio splendidus*	VSST-01	Purchased from CGMCC (CGMCC number: 1.6382)
13	*Vibrio splendidus*	VS1805	Isolation of YSFRI
14	*Vibrio parahaemolyticus*	VPST-01	Purchased from CGMCC (CGMCC number: 1.1997)
15	*Vibrio parahaemolyticus*	VP0531	Isolation of YSFRI
16	*Vibrio alginolyticus*	VAlST-01	Purchased from CGMCC (CGMCC number: 1.8757)
17	*Vibrio alginolyticus*	VAl1811	Isolation of YSFRI
18	*Aliivibrio fischeri*	AFST-01	Purchased from CGMCC (CGMCC number: 1.3842)
19	*Vibrio scophthalmi*	VSc0531	Isolation of YSFRI
20	*Vibrio cyclitrophicus*	VC0406	Isolation of YSFRI
21	*Photobacterium damselae* subsp. *piscicida*	Pdp1810	Isolation of YSFRI
22	*Escherichia Coli*	EC0701	Isolation of YSFRI
23	*Escherichia Coli*	ECST-01	Purchased from CGMCC (CGMCC number: 1.12883)

Notes: CGMCC, China General Microbiological Culture Collection Center. YSFRI, Yellow Sea Fishery Research Institute.

anus, *V. harveyi*, *V. splendidus*, *V. parahaemolyticus*, *V. alginolyticus*, *A. fischeri* and *E. coli* were purchased from China General Microbiological Culture Collection Center (CGMCC) as standard strains. The remained 13 strains were respectively isolated from different diseased animals in the laboratory of Yellow Sea Fishery Research Institute (YSFRI). More detailed information of these 20 strains was listed in Table 1. The genomic sequences of two strains were detected and submitted to NCBI (Yu *et al*., 2019; Zhang *et al*., 2019a).

All the 25 PDD strains (Fig.4) were isolated from different diseased marine animals along China coastline. They were all stored in YSFRI. These strains showed significant differences in terms of hemolytic phenotype and pathogenicity to black rockfish (Shi *et al*., 2019). Two of these strains, Pdd1601 (α-hemolysis) and Pdd1605 (β-hemolysis), had completed whole-genome sequencing data. One of them has been submitted to NCBI (Yu *et al*., 2019).

2.2 Specific Primer Design

Based on the whole-genome sequencing data of two strains, Pdd1601 and Pdd1605, a highly conserved sequence in *mcp* gene with a length of 348 bp and a highly conserved sequence in *dly* gene with a length of 695 bp were respectively selected as the targets to design specific primers. The *mcp* gene was used to distinguish PDD from other different bacteria, and the *dly* gene was used to distinguish PDD strains with different pathogenicity. Specific primers were designed through Primer Premier 5.0 software and synthesized in Sangon Biotech (Shanghai) Co., Ltd. (Shanghai, China). The *mcp* gene primer sequences were as follows: (5'–3') TGAAATTGCCCAACTGTCCC (forward) and TCACTTACTTGGGCCACATC (reverse). The *dly* gene primer sequences were as follow: (5'–3') TTTGGACGAGCGGTCCATTT (forward) and GGAG CCCAATCTTGACCAGG (reverse).

2.3 Preparation of Standards

The 348-bp target fragment in *mcp* gene was amplified using the designed primers, and the PCR product was ligated to PMD18-T vector, and transformed into DH-5α competent cells. The positive clones were enriched to extract plasmids, which were then digested and identified through sequencing. The correctly recombined plasmid was named Pl_{mcp}-Pdd and used as the positive standard in the present study. The copy number of recombinant plasmids was calculated using a previously established method (Raymond *et al*., 2004). Briefly, the OD_{260nm} was firstly determined to calculate the plasmid concentration. Then the copy number was calculated according to the formula as follows:

$$\text{Copies} = \frac{\text{Plasmid concentration (g } \mu\text{L}^{-1}) \times 6.02 \times 10^{23}}{660 \times \text{Total length of plasmid}}.$$

2.4 Establishment of qRT-PCR System and Standard Curve

The genomic DNA of bacterial strains was extracted using TIANamp Bacterial DNA kit (TIANGEN Biotech Co., Ltd., Beijing, China). The qRT-PCR was conducted using Realplex model quantitative real-time PCR instrument (Eppendorf Co., Ltd., Hamburg, Germany) in a 20-μL reaction system consisting of 10 μL of 2×SYBR Green Pro Taq HS PREMIX (Takara Biomedical Technology Co., Ltd., Beijing, China), 0.4 μL of 10 μmol L^{-1} *mcp* gene forward primer, 0.4 μL of 10 μmol L^{-1} *mcp* gene reverse primer, 0.2 μL of 20 μmol L^{-1} ROX Reference Dye, 2 μL of DNA template and 7 μL of RNase-free water. Briefly, after an initial denaturation step at 95℃ for 30 s, the amplifications were carried out with 40 cycles at a melting temperature of 95℃ for 10 s, an annealing temperature 60℃ for 20 s and an extension temperature of 72℃ for 20 s, followed by a melting curve analysis (95℃ for 15 s, 60℃ for 15 s, 60℃ to 95℃ for 20 min, 95℃ for 15 s).

The prepared standard plasmid Pl_{mcp}-Pdd was diluted into different gradient concentrations using 10-fold serial dilution, and a qRT-PCR was performed according to the optimized reaction conditions to draw a standard curve.

2.5 The Primer Specificity and Universality

The specificity of designed primers of *mcp* gene fragment was verified by qRT-PCR under the optimized conditions using the genomic DNA of 23 bacterial strains (Table 1) as the template, and RNase-free water was used as a negative control.

The universality of *mcp* gene primers was verified using the genomic DNA of 25 PPD strains as the template, and RNase-free water was used as a negative control.

The specificity of designed primers of *dly* gene fragment was verified by qRT-PCR using the genomic DNA of 25 PPD strains as template, and RNase-free water as a negative control.

2.6 The Sensitivity of *mcp* Gene Primers

The standard plasmid Pl_{mcp}-Pdd was serially diluted into a concentration gradient consisting of 1.0×10^{1}–1.0×10^{7} copies μL^{-1} using 10-fold serial dilution, which was used as templates. The primer sensitivity was verified by qRT-PCR under the reaction conditions in Section 2.4. Meanwhile, ordinary PCR was carried out to compare the sensitivity for designed primers of *mcp* gene.

2.7 Establishment of a Microfluidics-Based Real-Time PCR Method for PDD Detection

GENECHECKER UF-150 Ultra-Fast qRT-PCR instrument (CHK Biotech Co., Ltd., Shanghai, China) was used to establish a microfluidics-based qRT-PCR method for PDD detection. The PCR was conducted in a 10-μL reaction system consisting of 5 μL of 2 × ChamQ Universal SYBR qPCR Master Mix (Vazyme Biotech Co., Ltd., Nanjing, China), 1 μL of 10 μmol L^{-1} forward primer, 1 μL of 10 μmol L^{-1} reverse primer, 2 μL of bacterial DNA template and 1 μL of RNase-free water. Briefly, after an initial denaturation step at 95℃ for 30 s, the amplifications were carried out for 30 cycles with a melting temperature of 95℃ for 5 s, an annealing temperature of 60℃ for 20 s, and

an extension temperature of 72℃ for 5 s, followed by melting curve analysis (95℃ for 5 s, 60℃ for 40 s, and 95℃ for 5 s). Fluorescence detection was performed after the qRT-PCR was finished. The amplified products using specific primers of *mcp* and *dly* genes in different channels of the chip were required by synchronous detection.

3 Results

3.1 The Recombinant Plasmid Pl$_{mcp}$-Pdd

In the present study, we successfully amplified the target sequence with 348 bp in length using the Pdd1601 strain DNA as template and *mcp*-F/R primer pairs. The PCR product was recovered and ligated, and the constructed recombinant standard plasmid Pl$_{mcp}$-Pdd (Fig.1) was transformed into DH-5α competent cells. The recombinant plasmid was used as the positive standard, and its concentration was detected as 67.70 ng μL^{-1}, which was converted into 2.03×10^{10} copies μL^{-1}.

3.2 Amplification Curve and Standard Curve of the Plasmid Pl$_{mcp}$-Pdd

The standard plasmid Pl$_{mcp}$-Pdd was diluted to 2.03×10^{2}–2.03×10^{8} copies μL^{-1}. Using it as a template, the amplification was performed under the optimized qRT-PCR conditions mentioned in section 2.4. The amplification curve (Fig.2I) and standard curve (Fig.2II) were obtained based on the results, indicating that there was a good linear relationship between the amount of Pl$_{mcp}$-Pdd plasmid copies and Ct value. The formula was y=−3.012x+36.70, and the correlation coefficient R^2 was 0.998.

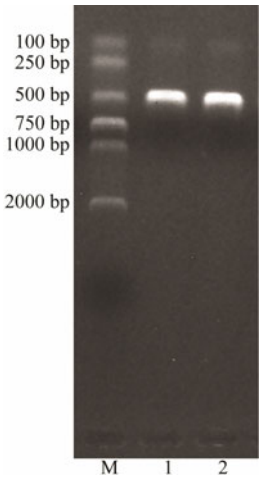

Fig.1 Verification of ligation and transformation of PDD *mcp* gene target fragment with electrophoresis. M, DNA molecular quality standards (DL2000); 1, 2, Enzyme-digested products.

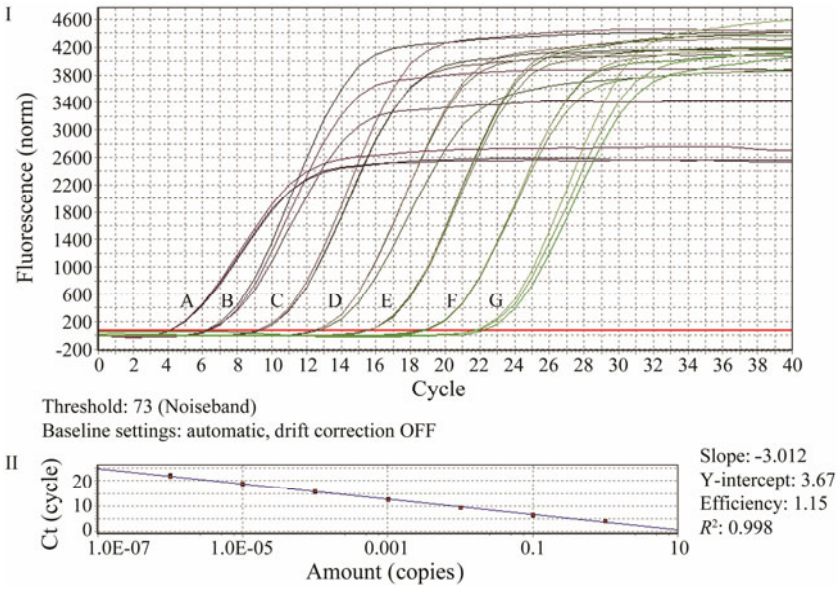

Fig.2 Amplification curve and standard curve of standard plasmid Pl$_{mcp}$-Pdd. I, Amplification curve of different concentrations of plasmid Pl$_{mcp}$-Pdd. A, 2.03×10^{8} copies μL^{-1}; B, 2.03×10^{7} copies μL^{-1}; C, 2.03×10^{6} copies μL^{-1}; D, 2.03×10^{5} copies μL^{-1}; E, 2.03×10^{4} copies μL^{-1}; F, 2.03×10^{3} copies μL^{-1}; G, 2.03×10^{2} copies μL^{-1}. II, Standard curve of plasmid Pl$_{mcp}$-Pdd.

3.3 Specificity and Universality of the Designed Primers

The designed primers for *mcp* gene were used to amplify the DNA fragments of 23 strains listed in Table 1. The results showed that only three PDD strains were successfully amplified. The target sequence was about 348 bp in length (Fig.3), confirming the specificity of the designed primers to PDD strains.

Moreover, qRT-PCR was performed to amplify the plasmid Pl$_{mcp}$-Pdd and the other 10 strains using the designed primers for *mcp* gene. These 10 strains were chosen from Table 1, including *V. rotiferianus* (VRST-01), *V. harveyi* (VHST-01), *V. splendidus* (VSST-01), *V. parahaemolyticus* (VPST-01), *V. alginolyticus* (VAlST-01), *A. fischeri* (AFST-01), *V. anguillarum* (VA0531), *V. cyclitrophicus* (VC0406), PDP (Pdp1810) and *E. coli* (ECST-01). The amplification curve was only observed from the plasmid Pl$_{mcp}$-Pdd, while

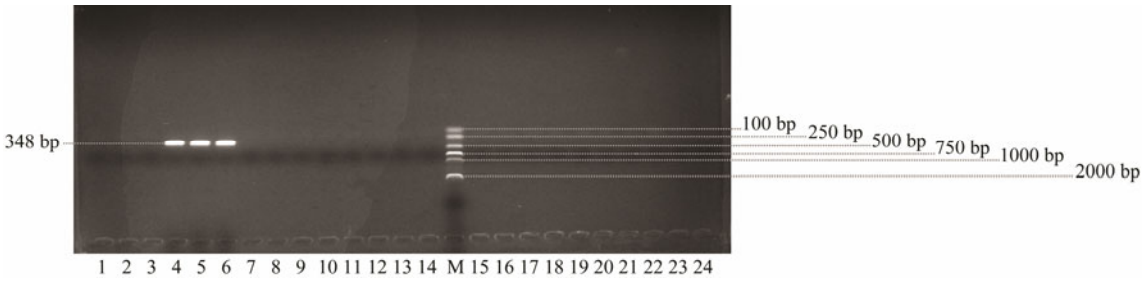

Fig.3 Specific detection of the designed primers for PDD *mcp* gene fragment. M, DNA molecular quality standards (DL2000); 1, *V. rotiferianus* (VR1601); 2, *V. rotiferianus* (VR1602); 3, *V. rotiferianus* (VRST-01); 4, PDD (Pdd0905); 5, PDD (Pdd1601); 6, PDD (Pdd1605); 7, *V. harveyi* (VHST-01); 8, *V. harveyi* (VH0207); 9, *V. harveyi* (VH1809); 10, *V. anguillarum* (VA1012); 11, *V. anguillarum* (VA0531); 12, *V. splendidus* (VSST-01); 13, *V. splendidus* (VS1805); 14, *V. parahaemolyticus* (VPST-01); 15, *V. parahaemolyticus* (VP0531); 16, *V. alginolyticus* (VAlST-01); 17, *V. alginolyticus* (VAl 1811); 18, *A. fischeri* (AFST-01); 19, *V. scophthalmi* (VSc0531); 20, *V. cyclitrophicus* (VC0406); 21, PDP (Pdp1810); 22, *E. coli* (EC0701); 23, *E. coli* (ECST-01); 24, RNase-free H_2O.

negative results were found in other 10 strains. The results reconfirmed the specificity of the designed primers to PDD *mcp* gene.

Amplification results of the 25 PDD strains showed that all these strains harbored the target fragment (Fig.4), demonstrating the good intraspecific universality of the designed *mcp* gene primers in PDD strains.

The 25 PDD strains were amplified with the designed *dly* gene primers. The results showed that the target fragment of 695 bp in length was only detected in two strains with high hemolytic activity (Fig.5), confirming the good specificity of the designed primers to PDD *dly* gene.

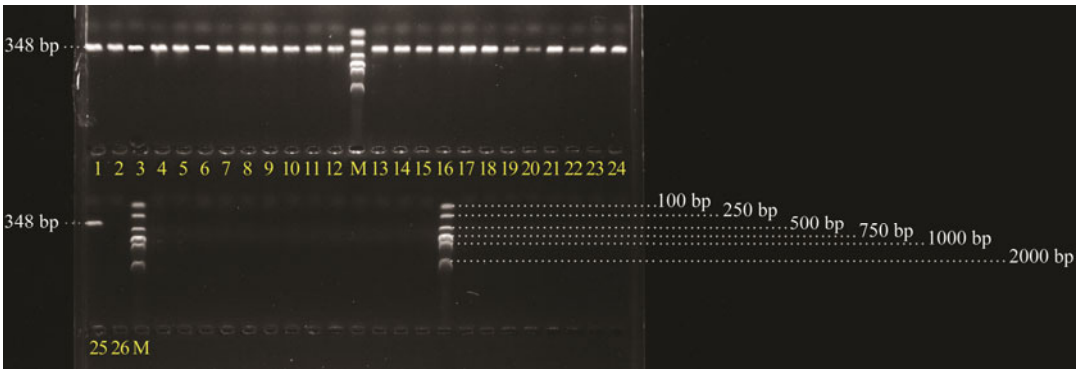

Fig.4 Intra-specific universality detection of the designed primers for PDD *mcp* gene fragment. M, DNA molecular quality standards (DL2000); 1, Pdd0210; 2, Pdd0905; 3, Pdd0906; 4, Pdd0907; 5, Pdd0908; 6, Pdd0909; 7, Pdd1208; 8, Pdd1308; 9, Pdd1605; 10, Pdd1606; 11, Pdd1607; 12, Pdd1608; 13, Pdd1609; 14, Pdd1611; 15, Pdd1612; 16, Pdd1613; 17, Pdd1614; 18, Pdd1615; 19, Pdd1616; 20, Pdd1617; 21, Pdd1701; 22, Pdd1706; 23, Pdd1807; 24, Pdd1809; 25, Pdd1808; 26, RNase-free H_2O.

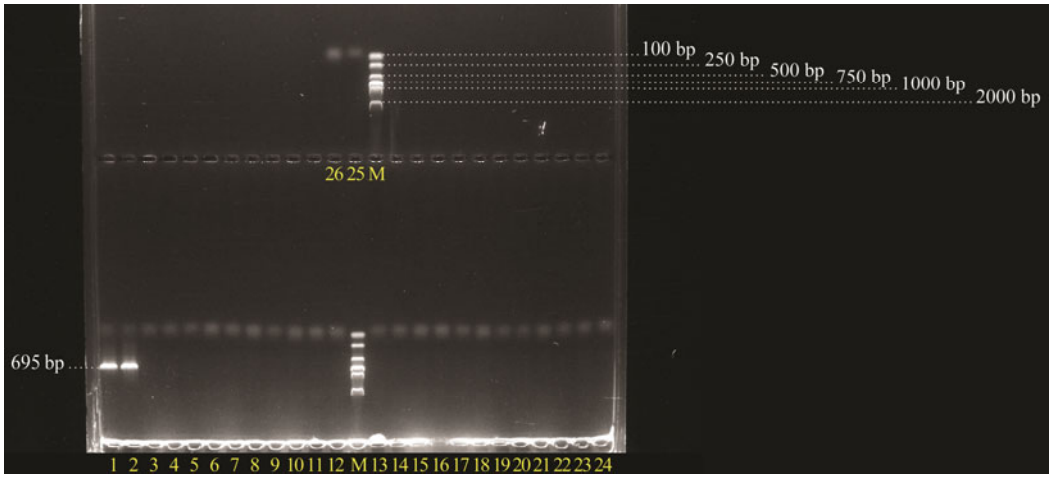

Fig.5 Specific detection of the designed primers for PDD *dly* gene fragment. M, DNA molecular quality standards (DL-2000); 1, Pdd1605; 2, Pdd1608; 3, Pdd0210; 4, Pdd- 0905; 5, Pdd0906; 6, Pdd0907; 7, Pdd0908; 8, Pdd0909; 9, Pdd1208; 10, Pdd1308; 11, Pdd1606; 12, Pdd1607; 13, Pdd1609; 14, Pdd1611; 15, Pdd1612; 16, Pdd1613; 17, Pdd1614; 18, Pdd1615; 19, Pdd1616; 20, Pdd1617; 21, Pdd1701; 22, Pdd1706; 23, Pdd1807; 24, Pdd1809; 25, Pdd1808; 26, RNase-free H_2O.

3.4 The Sensitivity of *mcp* Gene Primers

Comparing the results of qRT-PCR and ordinary PCR amplifications using the designed *mcp* gene primers showed that the detection limit of qRT-PCR was as low as 1.0×10^{1} copies μL^{-1}, while it was 1.0×10^{4} copies μL^{-1} for ordinary PCR (Fig.6). The sensitivity of qRT-PCR was 1000 times higher compared with the ordinary PCR.

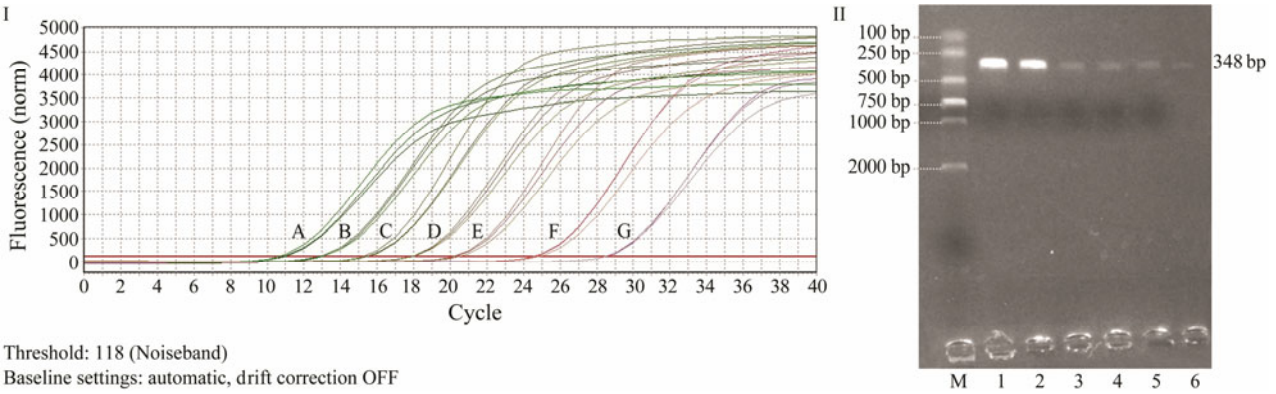

Fig.6 Detection sensitivity comparison of qRT-PCR and ordinary PCR using the primers of *mcp* gene. I, Amplification curve of qRT-PCR; II, Gel detection of ordinary PCR; A, 1.0×10^{7} copies μL^{-1}; B, 1.0×10^{6} copies μL^{-1}; C, 1.0×10^{5} copies μL^{-1}; E, 1.0×10^{4} copies μL^{-1}; E, 1.0×10^{3} copies μL^{-1}; F, 1.0×10^{2} copies μL^{-1}; G, 1.0×10^{1} copies μL^{-1}; M, DNA molecular quality standards (DL2000); 1, 1.0×10^{5} copies μL^{-1}; 2, 1.0×10^{5} copies μL^{-1}; 3, 1.0×10^{3} copies μL^{-1}; 4, 1.0×10^{2} copies μL^{-1}; 5, 1.0×10^{1} copies μL^{-1}; 6, RNase-free H_2O.

3.5 The Microfluidics-Based qRT-PCR Detection

The microfluidics-based ultra-fast PCR method for PDD detection was established using the specific primers of *mcp* gene and *dly* gene under the optimized reaction conditions, and GENECHECKER UF-150 was used as the instrument platform. Into two channels in the chip, *mcp* gene primers and *dly* gene primers added respectively. After the PCR amplification, detection results were visually judged through fluorescence signal. If fluorescence signal simultaneously appeared in both two channels, the test object could be confirmed as the highly pathogenic PDD strain. If fluorescence signal appeared in the channel with *mcp* gene primers and no fluorescence signal was detected in the channel with *dly* gene primers, the test object could be confirmed as the low or non-pathogenic PDD strain. If no fluorescence signal was detected in both two channels, or if fluorescence signal was only detected in the channel with *dly* gene primers, the test object was not a PDD strain (Fig.7). The reaction time of qRT-PCR was reduced to about 17 min. To obtain the Ct value in the amplification curve based on the standard curve, the quantitative detection of PDD strains was performed. The lowest limit detection was 1.0×10^{1} copies μL^{-1}, with extremely high sensitivity. Due to the outstanding portability of the instrument, this method could be used for field testing.

4 Discussion

As a pathogenic bacterial strain, which is widely distributed in the marine environment worldwide, PDD is also an important zoonotic pathogen. PDD has no obvious host specificity, and it can infect not only poikilotherm, but also homotherm (Rivas *et al*., 2013), or even cause acute death of humans (Yamane *et al*., 2004). Sufficient attention has to be paid to this pathogen. From the existing studies, there are changeable phenotypes among different PDD strains. They show diversities in pathogenicity, hemolysis, antibiotic resistance, physiological and biochemical characteristics (Takahashi *et al*., 2008). Environmental stress factors, such as temperature and salinity, can also affect the virulence of PDD strains (Vences *et al*., 2017; Matanza *et al*., 2018). Our previous research has also confirmed that the strains isolated from the same mariculture environment have significantly different pathogenicities to black rockfish, *S. schlegeli*. Therefore, it is important to quickly detect the PDD strains with different pathogenicities to prevent and control the infections of such bacteria.

The classical method of bacterial detection usually uses 16S *rDNA* gene (Cole *et al*., 2009) and *gyrB* gene (Sun *et al*., 2019). Many bacterial PCR-based classification and identification methods have been successfully established according to these genes. However, it is difficult to accurately distinguish PDD species due to the extremely high homology of *Vibrionaceae* 16S *rDNA*. For example, the homology between *V.cholerae* and *V. mimicus* is 99.6%, while it is 99.8% between *V. alginolyticus* and *V. parahaemolyticus* (Wen *et al*., 2009). The *gyrB* gene also needs to cooperate with other genes to achieve higher inter-species discrimination (Teh *et al*., 2010). The highly virulent plasmid pPHDD1 encoding damselysin (Dly) and phobalysin P (PhlyP), two key virulence factors of highly virulent PDD strains, has been reported in 2011 (Rivas *et al*., 2011). Moreover, the third hemolysin encoding gene of $hlyA_{ch}$ located on PDD chromosome has been found in 2013 (Rivas *et al*., 2013). From the existing researches, *dly* and $hlyA_{pl}$ on the plasmid pPHDD1 and $hlyA_{ch}$ on the chromosome constitute the virulence system of PDD together. Their synergy and participation of some functional genes create the high pathogenicity and strong hemolytic activity. The absence or silence of key virulence factors

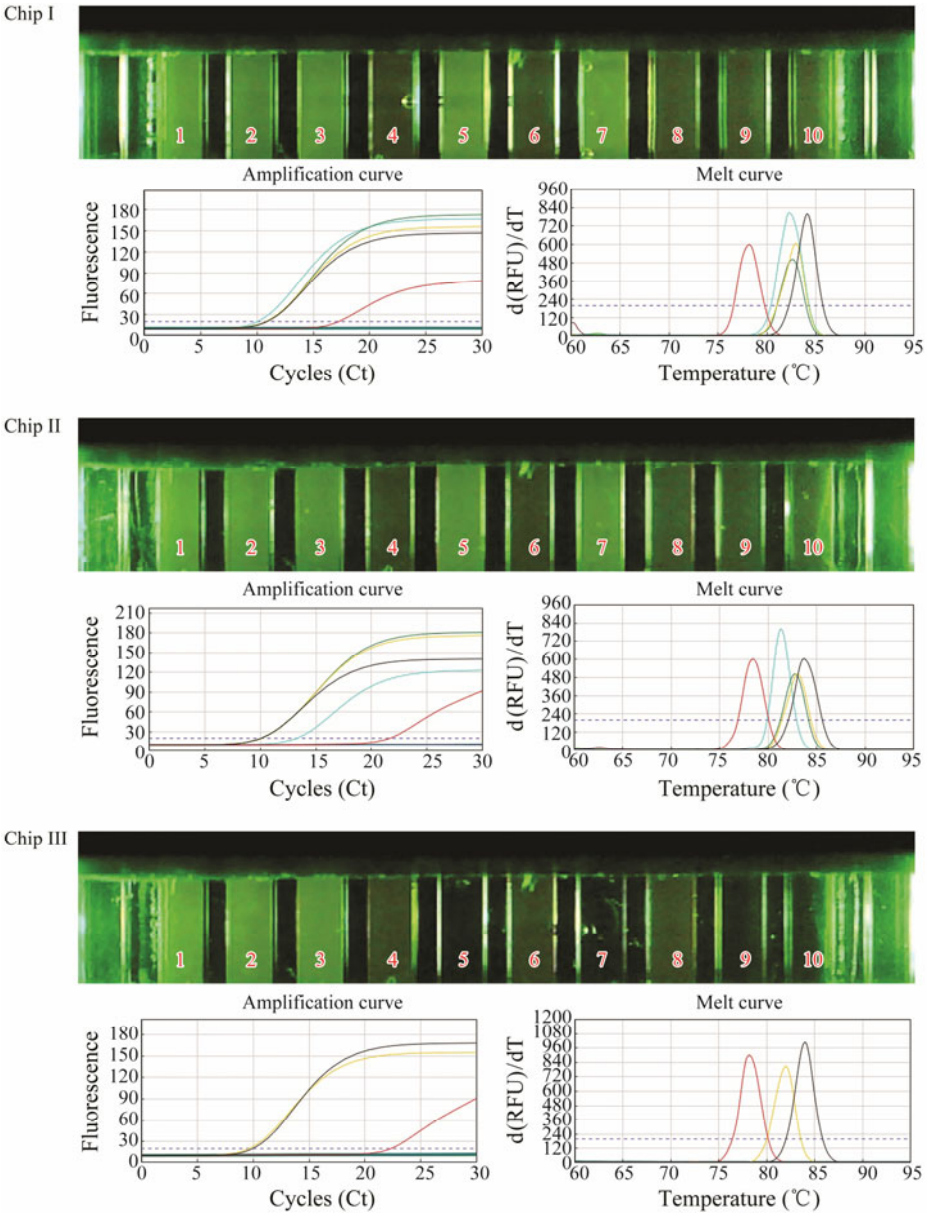

Fig.7 Detection results of different bacterial strains using GENECHECKER UF-150 qRT-PCR instrument. The chip has 10 reaction channels, of which the 1st, 3rd, 5th and 7th channels are added with *mcp* gene primers, the 2nd, 4th, 6th and 8th channels are added with *dly* gene primers, and the 9th and 10th channels are added with RNase-free H_2O. In chip I, the 1st, 2nd, 3rd, 5th and 7th channels appear fluorescence, 1st and 2nd channels are highly pathogenic PDD strain (Pdd1605), 3rd and 4th channels are lowly pathogenic PDD strain (Pdd0906), 5th and 6th channels are non-pathogenic PDD strain (Pdd0909), 7th and 8th channels are lowly pathogenic PDD strain (Pdd0210), 9th and 10th channels are RNase-free H_2O. In chip II, the 1st, 2nd, 3rd, 5th and 7th channel appear fluorescence, 1st and 2nd channels are highly pathogenic PDD strain (Pdd1608), 3th and 4th channels are lowly pathogenic PDD strain (Pdd1208), 5th and 6th channels are lowly pathogenic PDD strain (Pdd1611), 7th and 8th channels are lowly pathogenic PDD strain (Pdd1616), 9th and 10th channels are RNase-free H_2O. In chip III, the 1st, 2nd and 3rd channel appear fluorescence, 1st and 2nd channels are highly pathogenic PDD strain (Pdd1605), 3rd and 4th channels are lowly pathogenic PDD strain (Pdd1809), 5th and 6th channels are PDP (Pdp1810), 7th and 8th channels are *V. parahaemolyticus* (VP^{ST}-01), and 9th and 10th channels are RNase-free H_2O.

can cause a significant decrease of pathogens' virulence (Rivas *et al*., 2013; Rivas *et al*., 2015; Luo *et al*., 2019). It offers a new possible method for distinguishing PDD strains with different virulence through detecting the existence of virulence factors or functional genes.

In the present study, we collected 25 different PDD strains along the coast of China. These strains exhibited significant differences in pathogenicity to black rockfish. We completed the whole-genome sequencing of two strains, which displayed notable difference in hemolytic phenotype and pathogenicity. From the results, both *dly* gene on plasmid and *mcp* gene on chromosome were selected together as target sequences for PDD detection. The *mcp* gene was employed to distinguish PDD from other different bacteria, while the *dly* gene was used to identify PDD strains with high pathogenicity. It has been confirmed that Dly is

one of the most important virulence factors encoded by plasmid pPHDD1, which can vanish with the loss of the plasmid. Methyl-accepting chemotaxis proteins (Mcps) are the most common receptors widely found in bacteria and archaea. They not only participate in various physiological activities of cells, but also play an important role in pathogenicity of many bacteria (Ud-Din *et al*., 2017). Gene *mcp* is usually considered to be a housekeeping gene, while its sequence greatly varies in different species. Through genome-wide analysis, we screened out the *mcp* gene of PDD and found that this gene was ubiquitous in PDD strains with excellent specificity. It can be employed to distinguish PDD from other strains, including subsp. *piscicida*, and can be used as a target gene for PDD detection. Moreover, such gene might also be a potential indicator to identify the two subspecies of *P. damselae*.

Notably, the two strains that were detected to harbor *dly* gene showed strong hemolytic activity on sheep blood plate and high pathogenicity to black rockfish, whereas the other stains without *dly* gene exhibited weak hemolytic ability and low or non-pathogenicity. From these results, we speculated that *dly* gene is directly associated with the strong hemolytic activity and high pathogenicity of PDD. The sequence of *dly* gene in this study was derived from a megaplasmid (374 kb) of a highly virulent PDD strain, which was much larger than the 153-kb virulent plasmid pPHDD1. This finding confirmed the existence of other highly virulent plasmids in addition to pPHDD1 in PDD.

Another aim of this study was to establish a microfluidics-based qRT-PCR technique for the detection of PDD strains. As an emerging technology of molecular biology, microfluidics-based PCR, with the advantages of short-time consumption and low cost, has been widely used in medical application and research areas (De Paz *et al*., 2014; Park *et al*., 2011). We successfully established a microfluidics-based qRT-PCR method for PDD detection through specific primers. The test results can also be qualitatively judged by visualization. Quantitative detection would be achieved through the standard curve calculation. The minimum detection limit was as low as 1.0×10^{1} copies μL^{-1}, and the detection time was shortened to less than 20 min. This method has the feasibility of on-site detection, which is suitable to develop fast and accurate PDD detection for field research.

Acknowledgements

This work was supported by the National Key Research and Development Program of China (No. 2019YFD0900 104), and the Key Projects of Science and Technology Innovation of Shandong Province (No. 2018YFJH0703).

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(Edited by Qiu Yantao)