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Simultaneous detection of *Photobacteriumdamselae*, *Vibrio alginolyticus*, *Vibrio harveyi* and *Vibrio parahaemolyticus* using multiplex PCR amplification method

Ransangan J.* **and Lal M.M.T.**
Microbiology and Fish Disease Laboratory, Borneo Marine Research Institute, Universiti Malaysia Sabah, Jalan UMS, 88400 Kota Kinabalu, Sabah, MALAYSIA

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Abstract

The aim of this study was to develop a multiplex PCR amplification method that simultaneously detects the presence of four bacterial pathogens (Photobacteriumdamselae, V. alginolyticus, V. harveyi and V. parahaemolyticus), which are often synergistically caused disease to culture fish throughout the tropical waters, and occasionally cause food poisoning and wound infection to human. Specific multiplex PCR primers targeting conserve regions of virulence genes of the pathogens were designed and tested against different concentrations of $MgCl_2$ *and annealing temperatures. In addition to specificity against different bacterial species, the multiplex PCR was also tested against tissue and environmental samples known to harbor the pathogens. The result showed that the multiplex PCR was highly specific to the target pathogens. The optimum*$MgCl_2$ *concentration and annealing temperature for successful multiplex PCR amplification of the pathogens were at5.0 mM and 56 °C, respectively. The detection limit of the multiplex PCR was at 10 pg of DNA template. Although the concentration of the pathogens in the environment is often lower, enrichment with tryptic soy broth supplemented with 2% NaCl (w/v) has shown to enhance the growth of the bacterial pathogens and hence improved detection. The rapidity, simplicity and cost-effectiveness of the multiplex PCR amplification method described in this paper provide a useful bio-security tool for the determination of the pathogens in aquaculture farms and seafood processing industries throughout the tropical countries.*

Keywords: Multiplex PCR, fish bacterial pathogens, simultaneous detection.

Introduction

Bacterial diseases are one of major problems causing fish mortality and economic loss in aquaculture. Studies showed that *Photobacteriumdamselae*, *Vibrio alginolyticus*, *V. harveyi* and *V. parahaemolyticus* are highly virulent and may responsible for many disease outbreaks occurring in marine aquaculture farms throughout tropical waters[1-4]. These bacteria have also been reported to affect human health especially the *P. damselae*[5],*V. alginolyticus*[6] and *V. parahaemolyticus*[7]. Nevertheless, the current detection methodsfor these pathogens are either time consuming, expensive or only allow detection of single pathogen at a time. Co-existence of these bacteria in a given sample[8] may cause detection difficulty. Moreover, the ability of one bacterium to grow faster may also cause the detection other bacteria difficult. For example, *V. alginolyticus* has been reported to easily out number other *Vibrio* species in environmental samples[9]. Differentiation of these bacteria using phenotypic characterization and 16S rRNA sequencing is also difficult because of high genome homology among*Vibrio*species[10]. Although *Vibrio* species can be accurately identified using other markers such as the *atpA* gene[11], it is a time consuming and expensive process. Considering the damages that these bacteria can bring about to fish and human, rapid, simple, simultaneous and low cost detection method is necessary.

Multiplex PCR amplification method has been widely applied in the detection of fish bacterial pathogens[12] and food-borne pathogens[13-15]. However, no multiplex PCR method has been so far reported that simultaneously determine the presence of the above-mentioned bacterial pathogens. The ability to determine bacterial pathogens using multiplex PCR method was reported dependent to the target genes. Fortunately, both housekeeping and virulent genes can equally serve as good targets for multiplex PCR amplification. Fadaeifard et al.[16] and Mata et al.[12]have used ribosomal genes as the target for multiplex PCR amplification of streptococcal infections. However, Bauer and Rørvik[15] have used virulent genes successfully in the multiplex PCR amplification for food-borne bacteria. According to Panickeret al.[14], different genes could be used as a target for PCR amplification of all subtypes of *V. parahaemolyticus*(*tlh*, *tdh*, *trh* and ORF8), *V. vulnificus* (*vvh* and *viuB*) and *V. cholera* (*ompU*, *toxR*, *tcpI* and *hlyA*). However, Bauer and Rørvik[15] also showed that single gene (*ToxR*) can be used as the target PCR amplification of similar bacterial species. Nevertheless, we strongly believe that virulent genes could serve a better target for multiplex PCR amplification because of their divergence and

highly conserved among *Vibrionaceae*[17]. In this paper, we described a multiplex PCR amplification method which can detect the presence of the four bacterial pathogens simultaneously.

Material and Methods

Bacterial specimens: The four bacterial specimensused in this studyincluded *V. alginolyticus* (ATCC 17749), *V. harveyi* (ATCC 35084), *V. parahaemolyticus* (ATCC 17802) and *Ph.damselae*SA1. The three bacterial specimens were acquired from American Type Culture Collection (ATCC), while *Ph. damselae* SA1 was isolated from diseased fish during fish mortality event in Sabah (Malaysia) in 2011. All the bacterial specimens were authenticated using 16S rRNA gene sequencing (unpublished data). The bacteria were maintained in tryptic soy broth (Merck) supplemented with 2% sodium chloride (Merck) at 28^0C. Bacterial genomic DNA was extracted from overnight bacterial culture using the DTAB-CTA Bextraction method described by Philips and Simon[18].

Primer design and synthesis: Selected virulence genes (AF170886[17], X62635[19], GQ149070[20] and AB300869[21]) used inprimer design are listed in table-1. DNA sequences ofthose selected genes were downloaded from GenBank database at http://www.ncbi.nih.gov. Subsequently, the DNA sequences werealigned using the MegAlign, DNAstarLasergene Version 7. The primers consisted of 17 to 23 base pairs with the percentage of GC content ranged from 40 – 65 %.The forward and reverse primers were carefully selected in order to generate perfect lengthof non-overlapping PCR products.The PCR primers were synthesized at AIT Biotech Ptd. Ltd (Singapore). Finally, the PCR primers (in lyophilized form) were diluted in TE Buffer (pH 8.0) to make the final concentration to 10 µM and stored at-20 °C until used.

Specificity of PCR primers: Each pair of PCR primers was tested against DNA from target and non-target bacteria. Sterile double distilled water was used as negative control. The PCR mixtures consisted of 1 X PCR buffer (Promega), 1.7 mM $MgCl_2$ (Promega), 200 µM dNTPs (Promega), 0.4 µM of each forward and reverse primers, 1 U Taq DNA polymerase (Promega) and 50 ng DNA in 25 µl reaction. The PCR was carried out under the following conditions; 3 min in 95°C, 30 cycles of each 95°C (30 sec), 56°C (30 sec) and 72°C (30 sec) and final extension for 5 min at 72°C in a thermal cycler(Applied Biosystems). The PCR products were separated on 1.5% agarose gel electrophoresis stained withethidium bromide and visualized using a Gel Documentation System (Alpha Innotech).

Optimization of primersfor multiplex PCR amplification: The PCR primers was optimized against different $MgCl_2$ concentrations (1.8, 2.0, 3.0, 4.0, 5.0 and 6.0 mM) and annealing temperatures (56, 58, 60, 62 and 64 °C). The DNA template used for the optimization test was a combination of DNA from the four target bacteria. 2µl (25 ng/µl) of DNA from each target bacteriawas used in every25 µl reaction. PCR amplification was carried out according to the conditions described above except for $MgCl_2$ and the annealing temperatures.

Specificity of the multiplex PCR amplification: The specificity of the multiplex PCRamplification was evaluated against DNA from 24 bacterial species (*Aeromonascaviae* ATCC 15468, *A. hydrophila* ATCC 7965, *A. salmonicida*subsp. *Salmonicida* ATCC 33658, *Edwardsiellatarda* ATCC 15947, *Escherichia coli* ATCC 25922, *E. coli* JM109, *Micrococcuslysodeikticus* ATCC 4698, *Proteus mirabilis* ATCC 29245, *Pseudomonasaeruginosa* ATCC 27853, *Ps. Fluorescens* ATCC 13525, *V. anguillarum* ATCC 19264, *Yersiniaruckeri* ATCC 29473, *Ph. Damselae* PDTG2, *V. harveyi* VHJR7, *V. harveyi* VHJR4 and *V. parahaemolyticus* VPHG1.The multiplex PCR was also evaluated against total bacterial DNA from environmental samples previously enriched with tryptic soy broth (Merck) at 28^0C for overnight incubation.

Table-1
List of primers used in the multiplex PCR amplification of the bacterial pathogens

Target bacteria	Primer name	Primer sequence (5'-3')	Expected size (bp)	Gene Reference (Accession number)
Ph. damselae SA1	MpJRPdF MpJRPdR	CGGTTATCAAATGATCGCAAC CTTGCACCCCTTTAACCG	355	AF170886
*V. alginolyticus*ATCC17749	MpJRValF MpJRValR	CTCTCCCAATTCAGCCCTCTA GACTCTTCACAACAGAACTC	773	X62635
V. harveyi ATCC 35084	MpJRVhF MpJRVhR	ACGCTTGATGGCTACTGGTGGAG CTTCGCACCTGCATCGG	606	GQ149070
V. parahaemolyticus ATCC 17802	MpJRVpF MpJRVpR	CCGTTCCAAAACGAGGCTATC CGAGTGGTTGCTGTCATGA	521	AB300869

Detection limit of the multiplex PCR amplification: The detection limit of the multiplex PCR amplification was tested against different concentrations of genomic DNA (10, 1, 0.1, 0.01 and 0.001ng) at the conditions described above.

Results and Discussion

The primers specifically amplified the genes in the target bacteria (figure-1) with non-overlapping PCR products. The optimum concentration of $MgCl_2$ and annealing temperatures required for the multiplex PCR amplification (figure-2) of the four bacteria simultaneously was at 5.0 mM and 56 – 58°C, respectively.

The multiplex PCR amplification showed high specificity to target bacteria. It did not amplify the non-target bacteria. Interestingly, it has excellently amplified total DNA from environmental samples previously enriched with the target bacterial cells (figure-3). The test against DNA concentration showed that the multiplex PCR amplification method efficiently amplified as low as 0.01 ng or 10 pg of DNA from target bacteria (figure-4).

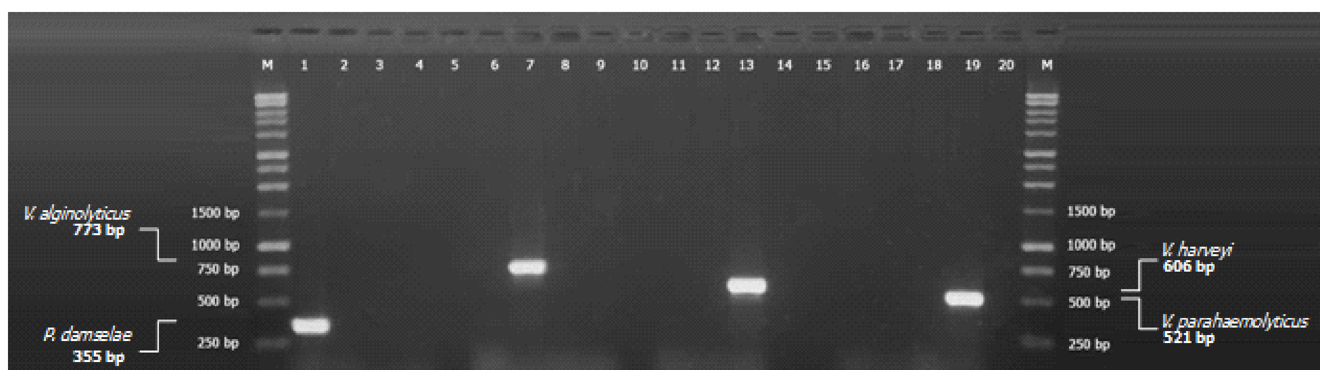

Figure-1

PCR amplification was specific to target bacterial pathogens. Lane 1 to 5 = *Ph. damselae*, *V. alginolyticus*, *V. harveyi*, *V. parahaemolyticus* and sterile distilled water amplified with MpJRPdF-MpJRPdR primers; Lane 6 to 10 = *Ph. damselae*, *V. alginolyticus*, *V. harveyi*, *V. parahaemolyticus* and sterile distilled water amplified with MpJRValF-MpJRValR primers; Lanes 11 to 15 = *Ph. damselae*, *V. alginolyticus*, *V. harveyi*, *V. parahaemolyticus* and sterile distilled water amplified with MpJRVhF-MpJRVhR primers; Lanes 16 to 20 = *Ph. damselae*, *V. alginolyticus*, *V. harveyi*, *V. parahaemolyticus* and sterile distilled water amplified with MpJRVpF-MpJRVpR primers. Lane M = 1 kb DNA Ladder (Promega)

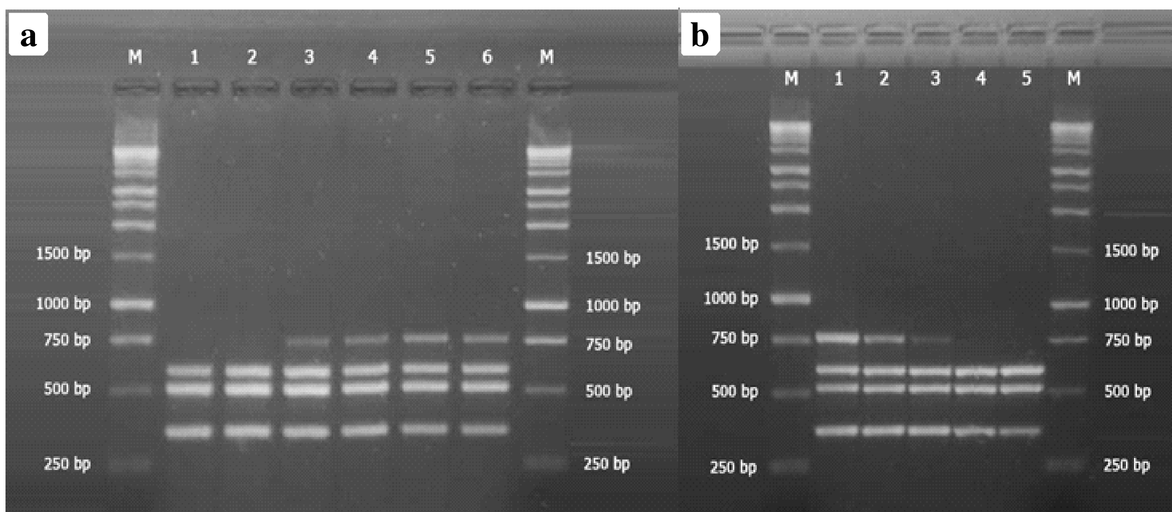

Figure-2

Optimization of Multiplex PCR amplification a: against bacterial pathogens at various concentration of MgCl2. Lane M = 1 kb DNA Ladder (Promega); Lane 1 = 1.8 mM; Lane 2 = 2.0 mM; Lane 3 = 3.0 mM; Lane 4 = 4.0 mM; Lane 5 = 5.0 mM; Lane 6 = 6.0 mM; b: against bacterial pathogens at various annealing temperatures. Lane M = 1 kb DNA Ladder (Promega); Lane 1 = 56°C; Lane 2 = 58°C; Lane 3 = 60°C; Lane 4 = 62°C; Lane 5 = 64°C

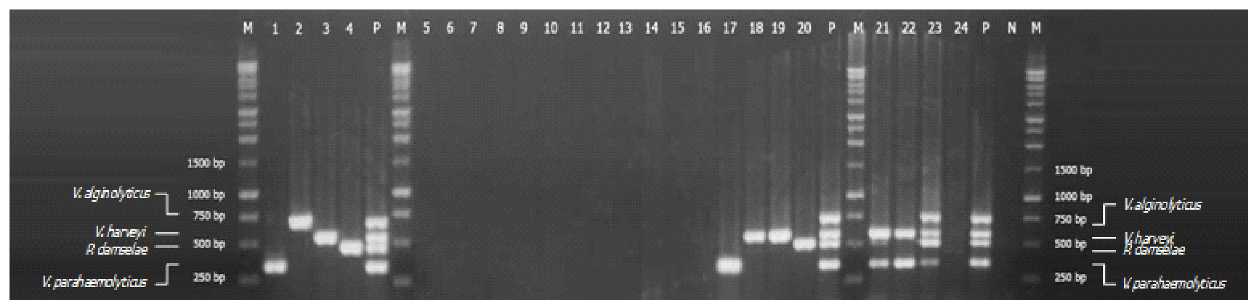

Figure-3

Specificity test against pure bacterial strains and total bacteria from environmental samples. Lane 1 = *Ph. damselae* SA1; Lane 2 = *V. alginolyticus* ATCC 17749; Lane 3 = *V. harveyi* ATCC 35084; Lane 4 = *V. parahaemolyticus* ATCC 17802; Lane 5 = *A. caviae* ATCC 15468; Lane 6 = *A. hydrophila* ATCC 7965; Lane 7 = *A. salmonicida* subsp. *salmonicida* ATCC 33658; Lane 8 = *Ed. tarda* ATCC 15947; Lane 9 = *E. coli* ATCC 25922; Lane 10 = *E. coli* JM109; Lane 11 = *M. lysodeikticus* ATCC 4698; Lane 12 = *P. mirabilis* ATCC 29245; Lane 13 = *Ps. aeruginosa* ATCC 27853; Lane 14 = *Ps. fluorescens* ATCC 13525; Lane 15 = *V. anguillarum* ATCC 19264; Lane 16 = *Y. ruckeri* ATCC 29473; Lane 17 = *Ph. damselae* PDTG2; Lane 18 = *V. harveyi* VHJR7; Lane 19 = *V. harveyi* VHJR4; Lane 20 = *V. parahaemolyticus* VPHG1; Lane 21 = Total bacteria from fish tissue; Lane 22 = Total bacteria from seawater; Lane 23 = Total bacteria from biofilms; Lane 24 = Total bacteria from freshwater; Lane P = Genomic DNA from all target bacteria; Lane N = Sterile distilled water; Lane M = 1 kb DNA Ladder (Promega)

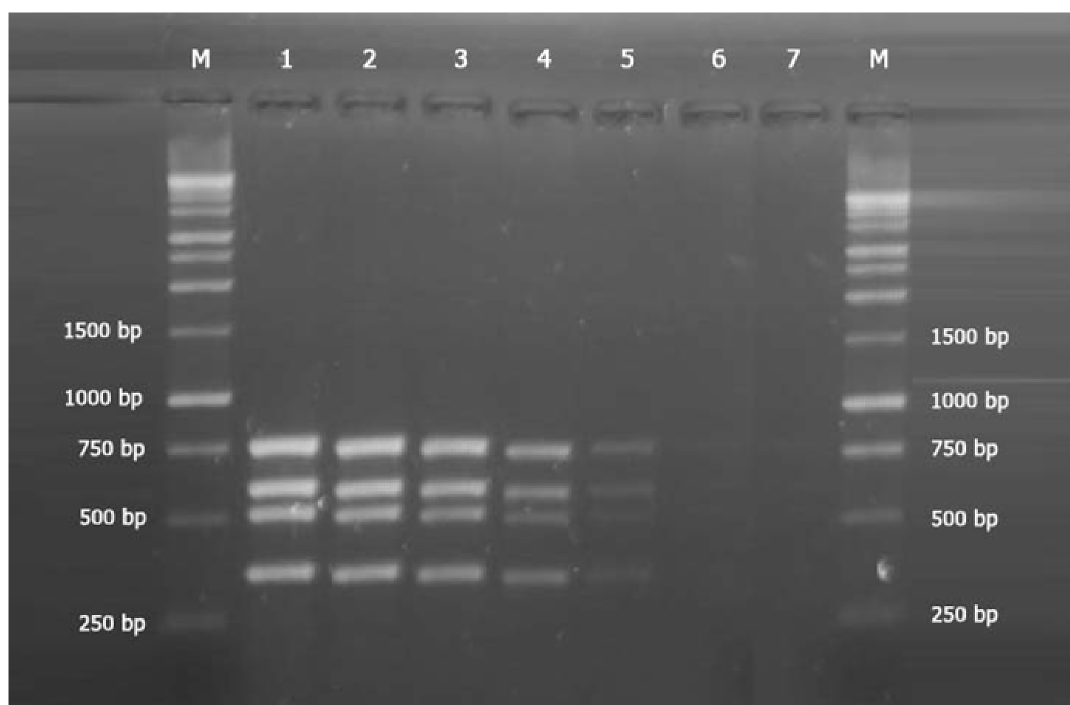

Figure-4

Multiplex PCR amplification against different concentration (nanogram) of genomic DNA from target bacterial pathogens. Lane M = 1 kb DNA Ladder (Promega); Lane 1 = 50 ng; Lane 2 = 10 ng; Lane 3 = 1 ng; Lane 4 = 0.1 ng; Lane 5 = 0.01 ng; Lane 6 = 0.001 ng; Lane 7 = Sterile distilled water

Bacterial species such as the *Ph. damselae*, *V. alginolyticus*, *V. harveyi* and *V. parahaemolyticus*are often reported to cause diseases not only to culture fish[1-4]but also to human[5-7].Hence, a rapid and simultaneous detection method for these bacterial pathogens is necessary.The multiplex PCR amplification method described in this study is very helpful for this purpose. Mata et al.[12] andFadaeifardet al.[16] have also reported that multiplex PCR method was useful for diagnosis of streptococcal

infections in fish. It is preferable because of its simplicity and cost efficiency compared to the conventional single PCR method[22].

The multiplex PCR amplification method we developed in this study has been shown to be highly specific to the target bacteria. It has also successfully amplified the target bacteria seeded in environmental and tissue samples. Although the use of multiple virulent genes (up to 10 genes) was not highly recommended for multiplex PCR[14], the result of our study showed otherwise. However, the drawback of multiplex PCR is often related to its sensitivity. In this study, the lowest concentration of DNA which can be successfully amplified was at 10pg. This is in agreement with the findings of Wei et al.[23]. Generally, the concentration of bacterial pathogens in environment is often low, which causes detection difficulty and can lead to false-negative result. However, it can be overcome by the enrichment of bacterial cells using a general-purpose media such as peptone water[24] and tryptic soy broth[25]. Such situation has also been shown in this study where environmental and tissue samples seeded with the bacterial cells were successfully amplified. Panicker et al.[14]have also suggested that cell enrichment improve detection capacity of multiplex PCR to food-borne pathogens (*V. cholerae*, *V. parahaemolyticus* and *V. vulnificus*) in shellfish tissues.

Conclusion

The multiplex PCR amplification method described in this study was shown to be specific and simultaneously amplified the target bacterial pathogens. The rapidity, simplicity and cost effectivenessof the multiplex PCR amplification method may provide useful biosecurity tool for determination of the pathogens in aquaculture farmsand seafood processing industries throughout tropical countries.

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