Detection of virulence Hemolysin gene (HlyA) in Photobacterium damselae subspecies damselae isolates

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Abstract
Polymerase chain reaction (PCR) technique was used to assay for the detection of virulence gene in the genomes of the Photobacterium damselae subsp. damselae isolated from cage culture and wild source fishes, particularly hlyA gene responsible of hemolysin toxins production in this genus. Sixty one strains of P. damselae subsp. damselae were isolated from cage and wild caught fishes. A pair of primers was designed to detect the hemolysin gene (HlyA) of P. damselae subsp. damselae by PCR method. The expected product size (417bp) was obtained from P. damselae subsp. damselae isolates. Photobacterium damselae subsp. damselae grew on TCBS agar plate producing green colonies whereas P. damselae subsp. piscicida did not grow. The PCR methods used are cost and labour effective when compared with the other molecular methods and commercially available kits.

Keywords: HlyA gene, PCR, Photobacterium damselae subsp. damselae, cage culture, wild fish, primer

Introduction
Aquaculture production of aquatic organisms including fish, crustaceans, and mollusks is the fastest growing aquaculture industry globally; while wild capture fisheries have become relatively stable in recent years, aquaculture production has increased by about 9% per year since 1985[9]. Water pollution is one of the most important global problems in aquaculture field. Aquaculture and brackish water fishes can be infected when urban wastes mix with farm water resulting in contamination with bacterial and other microorganism’s infection[2].

Photobacterium damselae subsp. damselae which was formerly classified as Vibrio damselae is a halophilic bacterium causing skin ulcers in warm and cold water fishes[3, 4, 5]. Photobacterium damselae subsp. damselae has been reported to cause wound infections and fatal necrotizing fasciitis in humans[6, 7, 8]. Outbreaks of photobacteriosis may occur in the temperature range of 14-29 °C and at salinities of 3-21ppt however the optimum range for acute disease is 18-25 °C and 5-15ppt. Photobacterium damselae subsp. piscicida is considered an obligate pathogen and its survival is short lived outside the host even in saltwater conditions[9]. Hemolysins contribute to the virulence of P. damselae subsp. damselae for mice and fish[10]. Strains of particular virulence produce plasmid-encoded dam selysin (Dly) and phobalysin P (PhlyP), a phospholipase D, and a small pore-forming toxin (PFT), respectively. All hemolytic strains express chromosomally encoded phobalysin C (PhlyC), which is closely related to PhlyP[11, 12]. Extracellular products (ECPs) from P. damselae subsp. damselae have been shown to display cytotoxic activity for different fish and mammalian cell lines. Only virulent strains produce toxic ECPs and the cytotoxic components are thermo labile[13]. Stephens et al.,[14] investigated the fish infected with P. damselae subsp. piscicida suffer from an acute septicemia, lethargic, swim slowly near the surface and ultimately sink and rest on the bottom prior to death. Infected fish show haemorrhagic septicaemia and the development of whitish areas or granulomas in the spleen, kidney and liver[15].

Molecular diagnosis of disease outbreaks is important for proper management practice and effective control. Recent microbiological methods of culture and biochemical characterization are time consuming and labour intensive. Diagnosis methods using immunological techniques such as agglutination or the ELISA are comparatively quicker[9, 16, 17]. PCR is not only more sensitive, but also faster[18], cost and labour effective when compared with biochemical analysis. The aim of the present study was to develop a convinced PCR-based test for P. damselae subsp. damselae.
The two subspecies have an identical 16S rRNA gene sequences [19] but they differ in their biochemical and physiological characteristics [20, 21]. A pair of primers was designed to detect *P. damselae* subsp. *damselae* by PCR. Also, thiosulphate citrate bile salts–sucrose agar (TCBS) was used to differentiate *P. damselae* subsp. *damselae* from *P. damselae* subsp. *piscicida.*

### Materials and Methods

#### Bacterial strains

The sixty one strains were obtained from cage culture and wild fishes in south east coast of India. All strains of *P. damselae* subsp. *damselae* were cultured in TCBS agar with 2% NaCl.

#### DNA primers

A pair of primers was selected to amplify a 417bp fragment of hemolysin gene (*HlyA*) derived from *P. damselae* subsp. *damselae*. The forward primer, *HlyA30FW*, was 20 nucleotides long (5'-CGCTTTCGGACCATTTAC-3') corresponding to positions 13321-13340 of the *P. damselae* subsp. *damselae* gene for hemolysin (Fig. 1) and the reverse primer, *HlyA30RV*, was 20 nucleotides long (5'-TGGGATAGAGGAAGTCGTTC-3') corresponding to positions 13718-13730. Primers were designed using Primer3plus software (www.bioinformatics.nl/primer3plus) and then the analysis of the designed primers was done using DNA MAN and manually check was the target gene.

#### Preparation of samples

**DNA extraction**

Pure cultures were inoculated in trypticase soya broth and incubated for 24 hours. After 24hrs the cultures was centrifuged at 6000rpm and the supernatant is discarded. The bacterial pellet was collected aseptically and 1ml of DNA extraction solution (GeNei™ HIMEDIA, Mumbai) was added into the cells. The mixture was homogenized well and incubated at 60°C for 60 min. The mixture was centrifuged at 10,000 x g for 10 min. The supernatant was centrifuged at 10,000 x g for 5 min. The supernatant was discarded and the DNA pellet was washed twice with 1 ml of 95% alcohol and centrifuged at 6000 x g for 5 min. The supernatant was removed and the DNA pellet was allowed to air dry for 5 min and 100 µl of deionized water was added and stored at 4°C for further use.

**PCR reaction**

The PCR condition includes 25 µL reaction included 2 µL forward and reverse primers, respectively, 6.5 µL ultrapure water, 12.5 µL master mix and 2 µL template DNA. The thermal cycle (Biorad) program comprised 35 cycles of 5 min at 95°C, 1 min at 95°C, 1 min at 56°C, and 1 min at 72°C, and a final 10 min extension at 72°C. The cycling conditions and sequence of the primer listed in the Table 1. The 10 µl of amplified products were separated by electrophoresis on a 1% (w/v) agarose gel run for 50-90min in Trisborate – EDTA (TBE) buffer (0.89 M tris, 0.89 M boric acid, 0.02 M EDTA, pH 8.0) and a 100bp DNA ladder was used as a size reference. This gel was then stained with 0.5µg/ml ethidium bromide solution for 20 min and visualized by UV – induced fluorescence. The presence of a clear fragment with the correct amplification size was assessed as a positive signal indicating the presence of the gene.

**Table 1:** Primer designed for target gene of *Photobacterium damselae* subsp. *Damselae*

| Primer Name | Sequence | Product Size |
|-------------|----------|--------------|
| *HlyA30FW*  | CGCTTTCGGACCATTTAC | 417bp |
| *HlyA30 RV* | TGGGATAGAGGAAGTCGTTC | 417bp |

#### Results and discussion

The specific-PCR was performed using the primers *HlyA30FW* and *HlyA30RV* to determine whether a 417bp of *hlyA* gene fragment, respectively, could be detected among...
the 61 *P. damsela* subsp. *damselae* isolates. Out of the 61 strains, 36/ 61 of *P. damsela* subsp. *damselae* (Fig. 2) isolates were PCR positive for the *hlyA* gene. This could be easily explained as the two subspecies differed only by a single nucleotide [22]. Rajan et al. [23] evaluated the pair of primers, CPSF and CPSR, designed from the capsular polysaccharide gene successfully amplified an expected 410-bp DNA fragment of all *P. damsela* ssp. *piscicida* and *P. damsela* strains tested. Osorio et al. [19] reported that the two subspecies have the same 16S rRNA gene sequence and have used the nested PCR to differentiate *P. damsela* subsp. *piscicida* from *P. damsela* subsp. *damselae*. Toranzo et al. [24] and Zorrilla et al. [25] reported that *P. damsela* subsp. *piscicida* did not grow on TCBS agar plate. Conversely, it was reported that *P. damsela* subsp. *damselae* could grow on TCBS agar plate [1, 26]. The *Photobacterium damsela* subsp. *damselae* isolated from different cage culture and wild caught fishes (Table 2) in south east coast of India. Out of the 36 positive *hlyA* genes, 22.2% of the cage culture system isolates, 75% of wild caught isolates and 2.8% of marine ornamental fish isolates present the *hlyA* gene. Cage culture fish’s incident of *P. damsela* subsp. *damselae* isolates during the summer season. The pH, salinity and temperature of the cage water were measured during the sampling period. Temperature of the water during the sampling period was 31±0.5 °C, salinity (36%) and pH (8.0-8.1).

In order to identify *P. damsela* subsp. *damselae*, bacterial colonies of the bacteria that gave a PCR product were placed on TCBS plate. In previous reports, bacteria grown on BHIA were scraped off from the plates, resuspended in saline to extract DNA of the mixed cultures and were then used for PCR [12]. *Photobacterium damsela* subsp. *piscicida* could not grow on TCBS whereas *P. damsela* subsp. *damselae*. The sensitivity of the PCR was evaluated by serial dilution of *P. damsela* subsp. *Piscicida* cell suspension as detailed elsewhere [27]. Amplification resulted in detectable levels of PCR product when a minimum of 13CFU of *P. damsela* subsp. *damselae* was used (Fig. 3 and 4). In conclusion, Screening of specific hemolysin gene (*HlyA*) appeared to be the most effective way of detecting and characterizing *P. damsela* virulence factors. The high throughput and cost-effective specific-PCR system used in this study could provide a powerful supplement to the conventional methods for a more accurate risk assessment and monitoring of *P. damsela* species in the wild and cage culture fishes.

**Fig 2:** Agarose gel electrophoretic analysis of the PCR amplified product using HlyA30FW and HlyA30RV primers (417bp) for detection of virulence hemolysin gene. A: Lane 1-MP66 (HlyA), Lane 2-MP76 (HlyA), Lane 3-MP140, Lane 4-MP147, Lane 5-MP148, Lane 6-MP157, Lane 7-MP160, Lane 8- MP162 (HlyA), Lane 9-MP189 (HlyA), Lane 10-MP190 (HlyA), Lane 11-MP191 (HlyA), Lane 12-MP192, Lane 13-MP193, Lane 14-MP194 (HlyA), Lane 15-MP195, Lane 16-Negative control, Lane 17-100bp DNA marker.

**Fig 3:** Agarose gel electrophoretic analysis of the PCR amplified product using HlyA30FW and HlyA30RV primers (417bp) for detection of virulence hemolysin gene. B: Lane 16-MP196 (HlyA), Lane 17-MP197 (HlyA), Lane 18-MP198, Lane 19-MP199, Lane 20-MP224, Lane 21-MP233, Lane 22-MP234, Lane 23- MP235, Lane 24-MP236 (HlyA), Lane 25-MP237, Lane 26-MP238 (HlyA), Lane 27-MP239 (HlyA), Lane 28-MP240 (HlyA), Lane 29-MP241, Lane 30-MP242, Lane 31-MP244, Lane 32-MP245 (HlyA), Lane 33-MP246 (HlyA), Lane 34-MP247, Lane 35-MP248 (HlyA), Lane 36-MP249 (HlyA), Lane 37-MP250, Lane 38-MP251, Lane 39-MP252 (HlyA), Lane 40-MP253 (HlyA), Lane 41-MP254 (HlyA), Lane 42-MP255 (HlyA), Lane 43-MP270, Lane 44-MP271 (HlyA), Lane 45-MP272 (HlyA), Lane 46-Negative control, Lane 47- 100bp DNA marker.

![Image](http://www.entomoljournal.com)
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