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Isolation, Molecular Characterization and Virulence Study (Pathogenesis) of *Photobacterium damselae* subsp. *damselae* Isolated from Sea-cage and Wild Fishes

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ABSTRACT

Background: *Photobacterium damselae* subsp. *damselae* is one of the most devastating zoonotic bacterial pathogen affects both fish and human health worldwide. The study aims to isolate and characterize *P. damselae* subsp. *damselae* from both wild caught and cage culture fishes. The virulence potential of *P. damselae* subsp. *damselae* on damselfish also been revealed.

Methods: A total of 212 finfishes from cage culture and wild environment were collected from the south east coast of India and identified for the presence of zoonotic *P. damselae* subsp. *damselae*. Standard biochemical and molecular methods (using specific gene) were employed to identify the *P. damselae* subsp. *damselae* isolates. A total of 61 isolates were identified as *P. damselae* subsp. *damselae*. Dendogram analysis was done for selected 30 strains based on band thickness of PCR product after using IS and ERIC PCR. The data were statistically analysed by unweighted pair group method using mathematic averages (UPGMA) for numerical analysis of banding patterns. The thirty out of sixty one isolates (20% or 50%) showed their presence in both wild caught and cage cultured fishes. The isolates from the wild caught fish were tested for their virulence on damsel fish and histopathological effect also studied.

Result: High prevalence of *P. damselae* subsp. *damselae* in wild caught parrot fish was noticed when compared to the cage culture fishes. The dendrogram obtained after numerical analysis with the Dice coefficient and UPGMA method shows that all patterns shared more than 50% similarity. Experimental challenge revealed that *P. damselae* subsp. *damselae* isolates from wild caught will cause severe tissue damage, abnormal behavior, clinical signs and also leads to fish mortality.

Key words: Bacteria, Cage culture, Marine fish, *Photobacterium*.

INTRODUCTION

Marine capture fish production in India during 2019 showed marginal increase of about 2.1% when compared to the previous year. The South East coast of India bears rich marine fish biodiversity and zone-wise it is the top most producers of marine fish (7.75 lakh tonnes) in India (CMFRI, 2019). Cage culture is also fetching importance due to the presence of confined environment in the south east coast of India and is in emerging phase of development. However, few challenges were needed to addressed in cage culture like disease, environment impacts, *etc*. (Austin and Austin, 2012). Among all, bacterial diseases found to be detrimental for the successful mariculture practices. *Vibrios* come on the top list of bacterial pathogens with direct jeopardy to mariculture development due to high mortalities associated with their invasion to fishes (Austin and Austin, 2012).

P. damselae is a marine bacterium of the family *Vibrionaceae* that is recognized as a pathogen for a wide variety of aquatic animals, including fish, molluscs and crustaceans. It is a halophilic bacterium associated with marine environments that was initially isolated in 1981 as the causative agent of skin ulcers in damselfish. It is a facultative anaerobic, gram negative rod, weakly motile (Love *et al*.1981). In addition, it is a pathogen of concern for humans, as it is capable of causing fatal infections. Most of the reported infections in humans originated from wounds inflicted during the handling of fish and fishing tools or from exposure to marine animals or seawater (Rivas *et al*. 2013). In India, *P. damselae* subsp. *damselae* was isolated from the fishes cultured in cages like cobia (Sharma *et al*. 2016). Bacterial disease transmission between domestic and wild fish populations is a known environmental risk, among that cage farms are generally regarded as having an enhanced

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potential for pathogen and parasite exchange, largely due to greater probability of contact with wild fish aggregated around cage sites. Moreover the wild marine fishes found to be the natural hosts for *P. damselae* subsp. *damselae* isolates (Weston, 2000). Hence it is necessary to investigate the transmission of *P. damselae* subsp. *damselae* from wild caught to cage culture fishes in the southeast coast of India. The pathogenicity of *P. damselae* subsp. *damselae* isolates from wild caught fishes on the cultured fishes also needs to be studied for better understanding of their effect on fishes and proper mitigation measures.

The present study investigated the presence of *P. damselae* subsp. *damselae* isolates from the wild caught marine fishes and cage cultured fishes by employing both conventional and molecular methods. Dendogram analysis has been done for analyzing the genotypic distance of the bacterial isolates to verify the bacterial transmission from wild to cage cultured fishes. Then the pathogenicity of *P. damselae* subsp. *damselae* isolates from wild caught fishes was tested with damsel fish, their behavior and histological changes were recorded and examined.

MATERIALS AND METHODS

Sampling method and study area

Fish samples were collected from 5 cage culture units of 2 farms located at Mandapam, Tamilnadu, India. The species cultured under cages were seabass (n=37) (*Lates calcarifer*) and grouper (n=43) (*Epinephelus* spp.,) with a stocking density of 1000 fishes/m^3. The water temperature and salinity of the cage culture farms were found to be 29±1°C and 32±1.5ppt, respectively during the time of sampling. Moribund/ dead fishes and fishes showed lesions were collected using sterile polythene bags and the live fish samples were brought in oxygen filled polythene bags. All the samples were brought to the laboratory within 24 hr under iced condition. In addition, 3 species of wild caught marine fishes (*Scarus globiceps;* n=45, *Lutjanus sebae;* n=37, *Brama brama;* n=40) from 4 important landing centres and marine ornamental fish (n=10; *Chaetodon octofasciatus*) from one ornamental fish farm, southeast coast of India such as Tharuvaikulam, Theraspuram, Mandapam and Pamban were collected and transported to the bacteriology laboratory under iced condition.

Isolation, characterization and culture condition of bacterial strain

The detection of *P. damselae* and distinguishing the isolates at the subspecies level were achieved using the standard procedures of morphological and biochemical plate and tube tests as described in Finfish and shellfish diseases Practical Manual (Rijijohn and Rosalind George, 2004). All the dissection utensils and inoculation loop was sterilized using 70% alcohol and flame before isolating the bacteria. The surface of the animal was disinfected by wiping with sterile cotton swab soaked in 70% alcohol. Samples from the gill tissue was collected by inserting the sterile loop into it and immediately streaked on to the pre-dried TCBS agar plates by streaking method. Tissues like kidney, spleen and liver were dissected out aseptically and were scratched with the sterile loops and immediately streaked onto the pre-dried TCBS agar plates. The agar plates were kept under incubation at 27°C for 24 h at incubator (Kemi, India). *P. damselae* subsp. *damselae* grows on TCBS produces green colonies. Therefore, green colonies were selected randomly and streaked on to trypticase soya agar (TSA) slants with 2% NaCl for 24 hrs. All colonies having similar visual appearances were sub-cultured from a single colony for purity. Pure presumptive colonies isolated on plating media were used for biochemical analysis.

Biochemical characterization of *P. damselae* subsp. *damselae* isolates

For biochemical confirmation, colonies showed green colonies in TCBS + 2% NaCl were restreaked in the same medium. Then the reisolated single green colony was inoculated to 5ml of TSB broth. After 24 hrs of incubation, "the TSB broth contains bacteria was used for biochemical analysis". The tests include: motility (hanging drop method), urease production (Urea hydrolysis test), oxidation/ fermentation (O/F test), amino acid decarboxylase (Arginine, lysine and ornithine) and salt tolerance (Rijijohn and Rosalind George, 2004).

Molecular characterization of *P. damselae* subsp. *damselae*

Bacterial DNA extraction

The identities of the isolated bacteria were confirmed using PCR assay. Genomic DNA was extracted according to the protocols of Osorio *et al*. (2000). Briefly, individual green colour colonies grown in 5 ml of TSB broth were incubated at 27°C for 24hrs. After incubation, one ml of sample was transferred to a microcentrifuge tube with a capacity of 1.5ml. The cell suspension was centrifuged for 10 min at 10,000 x g in a refrigerated centrifuge (Sigma, Germany). The supernatant was discarded carefully and the pellet was air dried. Then the pellet was resuspended in 300 µl of DNA – Xpress™ reagent (GeNei™ HIMEDIA) in a dry bath at 60°C for 45 min. After that, the tube was centrifuged for 10 min at 10,000 x g. Then supernatant was carefully transferred to a new microcentrifuge tube and washed with 400 µl of 100% ethanol for 5 min at 10,000 x g. The supernatant was discarded carefully and then the pellet was washed twice with 400 µl of 95% ethanol as said above. After centrifugation, the supernatant was discarded and the tube was inverted for complete drying of pellet. After that, 100µl nuclease free water was added to dissolve the pellet. DNA purity and concentration were assessed using a Nano Drop™ by measuring absorptions at 260 and 280 nm. The DNA concentration of each sample was adjusted to 40ng/µl.

Multiplex PCR assay

A multiplex PCR assay using *P. damselae* specific 16S rRNA gene directed primers and primers designed from the partial urease C (*ureC*) gene sequence are employed to discriminate

between the two subspecies of *P. damselae* (Osorio *et al*., 2000) are shown in Table 1. The PCR condition includes 25 µL reaction included 2 µL forward and reverse primers, 6.5 µL ultrapure water, 12.5 µL master mix and 2 µL template DNA. The thermal cycle (Biorad, USA) program comprised 30 cycles of 4 min at 95°C, 1min at 95°C, 1 min at 60°C and 40 s at 72°C and a final 5 min extension at 72°C. 10 µl of amplified products were separated by electrophoresis on a 1% (w/v) agarose at 100V 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 (Biorad, USA).

Dendrogram analysis

The fingerprints obtained for selected 30 isolates based on band patterns of PCR product after using IS and ERIC PCR were statistically analyzed by unweighted pair group method using mathematic averages (UPGMA) and coefficient cluster analysis (Nei and Li, 1979) by UVI bandmap software in the UVI Doc gel documentation system.

Pathogenesis of *P. damselae* subsp. *damselae* isolates in damselfish

For challenge study, healthy and uniform sized damsel fish (*Pomacentrus similis*) fingerlings (80nos) (average size as 6 ± 2 cm and 5 ± 2 g) were obtained from local aquarium at Thoothukudi, India. The fishes were acclimatized before experimentation. The study was conducted in the wet laboratory of Department of Fish Pathology, Tamilnadu Dr. J. Jayalalitha Fisheries University, India. The study was conducted period of 2018-2019. Experiment was conducted in a glass aquarium tanks of 40L capacity with the aerated sea water temperature of 29 ± 1°C, salinity 30 ± 2ppt and pH 8.4 ± 0.2 and the fishes were fed with pellet feed twice daily. Water was replaced daily at a rate of 20% in each tank. All the fishes were acclimated for 3 days prior to the initiation of the experiment. The fish health and water quality were monitored by observing swimming behavior of fishes and appearance of water, respectively. The third day of acclimatization, 8 fingerlings (one from each tank) was randomly collected and their internal organs were checked using microbiological tests. The fourth day, tanks were grouped to 8 Groups [6 test groups and 2 control groups; All treatments in duplicate] were formed and 10 number of fishes were introduced in each groups. One strain of *P. damselae* subsp. *damselae* isolates from natural outbreak was cultured on TCBS agar plate with 2% NaCl and incubated at 27°C for 24hrs. Then after 24 hrs, the culture was centrifuged at 10,000 x g for 10min, supernatant discarded carefully. The pellet was taken and suspended with sterile saline solution. The fishes were intraperitoneally injected with 50 µl of bacterial suspension per fish for test groups and 50 µl of saline for control fishes. For damsel fish challenge study, inoculum strength of bacterial suspension was $3.0x10^4$, $3.0x10^3$ and $3.0x10^2$ CFU $fish^{-1}$ (tenfold dilution from LD_{50} value). The mortality of the fishes was recorded daily up to 7 days post challenge and fish behaviour also recorded. The dead fishes were collected from the tank and tissues such as kidney, spleen and liver were dissected out and bacteria were reisolated for confirmation purpose. The reisolation of bacteria has been done to confirm the mortality due to bacteria. The LD_{50} value was calculated by the Reed and Muench, (1938).

Histopathology of experimentally infected fish

To study the histopathological changes provoked by *P. damselae* subsp. *damselae* in fish tissues, parts of gills and internal organs (liver, kidney and spleen) of dead fishes were dissected out and fixed overnight in 10% buffered formalin. Then the samples were dehydrated in 70 to 100% alcohol, placed in two changes of xylene (10 min each) and then embedded in paraffin (56°C melting point). The parts were sliced transversely into 4-mm-thick sections with the help of microtome (Thermo) and stained with hematoxylin and eosin (Roberts, 2001). Then the stained slides were analyzed and photographed by Nikon microscope (Nikon, Japan) installed with digital camera.

The fish study including animal experimentation, tissue collection and sacrifice was carried out as per the ethical guidelines and adheres to the legal requirements of the study country.

RESULTS AND DISCUSSION

P. damselae subsp. *damselae* is an autochthonous member of aquatic ecosystems. It is considered as primary pathogen of several species of wild fish (damselfish, catfish, shark, stingray, *etc*.) as well as commercially important fish species in mariculture, causing wound infections and hemorrhagic septicemia (Fouz *et al*. 1992). The present study hypothesized that there exist potential for the bacteria to transmit from wild caught fishes to cage cultured fishes. The pathogenic potential of transmitted bacteria on cage culture fishes also been revealed.

Bacterial identification

The bacterial colonies isolated from spleen, kidneys and liver of wild caught and cage culture fishes was inoculated

Table 1: Standard Primer used for multiplex PCR.

Gene	Primer name	Sequence (5′-3′)	Amplicon size	Reference
ureC	ureCpd - F	TCCGGAATAGGTAAAGCGGG	448bp	Osorio *et al*., 2000
	ureCpd - R	CTTGAATATCCATCTCATCTGC		
16S rRNA	Car1-F	GCTTGAAGAGATTCGAGT	267bp	
	Car2-R	CACCTCGCGGTCTTGCTG		

on TCBS agar at 24°C for 1-2 days. In total, 289 green colonies were isolated on TCBS agar. Out of which sixty one isolates were found gram negative, rod-cocci, motile organisms, oxidation/Fermentation test positive and arginine and lysine decarboxylase production, growing in 3% and 6% Nacl and urease production (Table 2). The previous researcher also confirmed that the presence of *P. damselae* subsp. *damselae* when three or more positive results were obtained in the following tests: LDC, motility, TCBS growth and urease production (Holt *et al*. 1994; Zorrilla *et al*. 1999; Rajan *et al*. 2003; Thyssen *et al*.1998).

Molecular identification

The multiplex PCR assay was carried out with 16S rRNA gene and *ureC* gene binding primers. A total of 289 isolates were selected from infected fish, representing different organs and different locations. The expected amplicon sizes were 267bp and 448bp (corresponding to an internal fragment of the 16S rRNA gene and one of the *ureC* genes respectively). A total of 61 isolates were positive for both 16S rRNA gene and *ureC* gene. No isolates were positive for the 16S rRNA gene (267bp) alone indicating the absence of *P. damselae* subsp. *piscicida* (Fig 1). Osorio *et al*. (2000) reported the multiplex-PCR using two primer pairs directed to internal regions of the 16S rRNA and *ureC* genes, was employed to differentiate between the subspecies of *P. damselae*. Labella *et al*. (2006) observed two amplification bands of 267bp and 448bp in the multiplex- PCR assay demonstrated that the isolates from redbanded seabream are members of the *P. damselae* subsp. *damselae*. The 30 PCR product selected based on species wise thickness of PCR generated by the 16S rRNA (1500bp) gene primers was sequenced by outsourcing to MWG, Eurofins Genomics India Pvt Lt Bangalore, India.

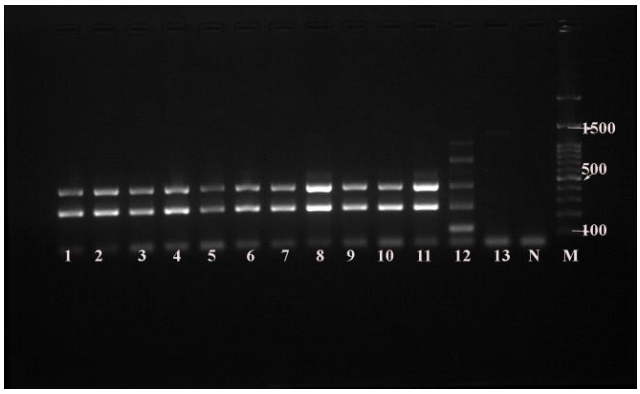

Fig 1: Agarose gel electrophoresis analysis of the multiplex PCR products obtained from bacterial DNA with primers targeting 16S rRNA (448bp) and *ureC* gene (267bp).

Lane 1-MP189 (Pdd), Lane 2-MP190 (Pdd), Lane 3-MP191 (Pdd), Lane 4-MP192 (Pdd), Lane 5-MP193 (Pdd), Lane 6-MP194 (Pdd), Lane 7-MP195 (Pdd), Lane 8-MP196 (Pdd), Lane 9-MP197 (Pdd), Lane 10-MP198 (Pdd), Lane 11-MP199 (Pdd), Lane 12-MP200, Lane 13-MP201, Lane 14-Negative control, Lane 15-100bp DNA marker.

Prevalence of *P. damselae* subsp. *damselae* isolates from wild and cage culture fishes

The prevalence of *P. damselae* subsp. *damselae* isolates are presented in Table 3. A total of 61 *P. damselae* subsp. *damselae* were identified from 289 isolates of bacteria collected from different fish samples. Out of all *P. damselae* subsp. *damselae* 86.91% isolates were found in wild caught fishes and 13.09% in cage cultured fishes collected from South east coast of India. Highest number of *P. damselae* subsp. *damselae* were obtained from wild caught parrot fish samples (55.73%) followed by wild *Lutjanus* sp. (22.95%). Of the 61 isolates obtained, 9.83% were from seabass cultured in cages. The lowest number of *P. damselae* subsp. *damselae* (0.35%) was isolated from cage grouper and eightband butterflyfish. The more amounts of *P. damselae* subsp. *damselae* isolates present in parrot fish when compared to cage culture fishes was noticed. Similarly, studies noticed high bacterial loads in parrot fish tissues when compared to other marine fish species (Tarnecki *et al*. 2016). This suggests that their feeding behavior found correlated with the abundance of *P. damselae* subsp. *damselae* in parrot fish. It has been noticed that gut microbiota of coral reef associated fishes contains more amount of *Photobacterium.* The parrot fish feeding on coral reef, seagrass and algae (Smriga *et al*., 2010). Eissa *et al*. (2018) also reported total prevalence of *P. damselae* subsp. *damselae* among naturally infected marine fishes was 45.04%. Essam *et al*. (2016) reported that total prevalence of *P. damselae* subsp. *damselae* in naturally infected fishes was 20.76%. The low bacterial presence in cage culture fishes might be due to the reason that hatchery produced seeds may devoid of *P. damselae* subsp. *damselae*. Once the fishes were introduced into the cage environment, it becomes more vulnerable to *P. damselae* subsp. *damselae* infection (Essam *et al*., 2016). The reason for Cross-contagion between cage and wild fish species with shared pathogens may occur either through movement of individual fish or through species-specific migrations. Unlike parasitic pathogens, bacteria seem to exhibit higher potential to spread between wild and cage fish. Infected wild fish might also transfer pathogens to the cage fish. This co-infection process leads to a large variety of shared pathogens among wild and cage fish, while the various pathways of pathogen transmission increase the potential for infection and render epidemiological risk management difficult (Diamant *et al*., 2007) Therefore, the present study suggests that proper site selection and screening of *P. damselae* subsp. *damselae* in the cage culture environment before incorporating cages in the natural environment is very much necessary to avoid the *P. damselae* subsp. *damselae* infection and associated fish mortality.

Dendrogram analysis of *P. damselae* subsp. *damselae* isolates from wild caught and cage culture fishes

A cluster analysis of the pairwise distance matrix among patterns was performed using the unweighted pair group

Table 2: Biochemical characteristics of the *Photobacterium damselae* subsp. *damselae* isolates obtained in the study.

Isolate No.	Source of the isolates	Salt tolerance					Amino acid decarboxylase			Urease
		0%	3%	6%	8%	11%	A	O	L	
MP66	Ornamental fish farm (Mandapam)	-	+	+	+	-	+	-	+	+
MP76	Landing center (Theraspuram)	-	+	+	+	-	+	-	+	+
MP140	Cage culture (Thangachimadam)	-	+	+	-	-	+	-	+	+
MP147	Cage culture (Thangachimadam)	-	+	+	-	-	+	-	+	+
MP148	Cage culture (Thangachimadam)	-	+	+	-	-	+	-	+	+
MP157	Cage culture (Thangachimadam)	-	+	+	-	-	+	-	+	+
MP160	Cage culture (Thangachimadam)	-	+	+	-	-	+	-	+	+
MP162	Cage culture (Thangachimadam)	-	+	+	-	-	+	-	-	+
MP189	Landing center (Tharuvaikulam)	-	+	+	+	-	+	-	+	+
MP190	Landing center (Tharuvaikulam)	-	+	+	+	-	+	-	+	+
MP191	Landing center (Tharuvaikulam)	-	+	+	+	-	+	-	+	+
MP192	Landing center (Tharuvaikulam)	-	+	+	+	-	+	-	+	+
MP193	Landing center (Tharuvaikulam)	-	+	+	+	-	+	-	+	+
MP194	Landing center (Tharuvaikulam)	-	+	+	+	-	+	-	+	+
MP195	Landing center (Tharuvaikulam)	-	+	+	+	-	+	-	+	+
MP196	Landing center (Tharuvaikulam)	-	+	+	+	-	+	-	+	+
MP197	Landing center (Tharuvaikulam)	-	+	+	+	-	+	-	+	+
MP198	Landing center (Tharuvaikulam)	-	+	+	+	-	+	-	+	+
MP199	Landing center (Tharuvaikulam)	-	+	+	+	-	+	-	+	+
MP224	Landing center (Theraspuram)	-	+	+	+	-	-	-	+	+
MP233	Landing center (Theraspuram)	-	+	+	-	-	+	-	+	+
MP234	Landing center (Theraspuram)	-	+	+	-	-	+	-	+	+
MP235	Landing center (Theraspuram)	-	+	+	-	-	+	-	+	+
MP236	Landing center (Theraspuram)	-	+	+	-	-	+	-	+	+
MP237	Landing center (Theraspuram)	-	+	+	-	-	+	-	+	+
MP238	Landing center (Theraspuram)	-	+	+	-	-	+	-	+	+
MP239	Landing center (Theraspuram)	-	+	+	-	-	+	-	+	+
MP240	Landing center (Theraspuram)	-	+	+	-	-	+	-	+	+
MP241	Landing center (Theraspuram)	-	+	+	-	-	+	-	+	+
MP242	Landing center (Theraspuram)	-	+	+	-	-	+	-	+	+
MP244	Landing center (Theraspuram)	-	+	+	-	-	+	-	+	+
MP245	Landing center (Theraspuram)	-	+	+	-	-	+	-	+	+
MP246	Landing center (Theraspuram)	-	+	+	-	-	+	-	+	+
MP247	Landing center (Theraspuram)	-	+	+	-	-	+	-	+	+
MP248	Landing center (Theraspuram)	-	+	+	-	-	+	-	+	+
MP249	Landing center (Theraspuram)	-	+	+	-	-	+	-	+	+
MP250	Landing center (Theraspuram)	-	+	+	-	-	+	-	+	+
MP251	Landing center (Theraspuram)	-	+	+	-	-	+	-	+	+
MP252	Landing center (Theraspuram)	-	+	+	-	-	+	-	+	+
MP253	Landing center (Theraspuram)	-	+	+	-	-	+	-	+	+
MP254	Landing center (Theraspuram)	-	+	+	-	-	+	-	+	+
MP255	Landing center (Theraspuram)	-	+	+	-	-	+	-	+	+
MP270	Landing center (Pamban)	-	+	+	+	-	+	-	+	+
MP271	Landing center (Pamban)	-	+	+	+	-	+	-	+	+
MP272	Landing center (Pamban)	-	+	+	+	-	+	-	+	+
MP273	Landing center (Pamban)	-	+	+	+	-	+	-	+	+
MP274	Landing center (Pamban)	-	+	+	+	-	+	-	+	+
MP276	Landing center (Pamban)	-	+	+	+	-	+	-	+	+

Table 2: Continue...

Table 2: Continue...

MP277	Landing center (Pamban)	-	+	+	+	-	+	-	+	+
MP278	Landing center (Pamban)	-	+	+	+	-	+	-	+	+
MP279	Landing center (Pamban)	-	+	+	+	-	+	-	+	+
MP280	Landing center (Pamban)	-	+	+	+	-	+	-	+	+
MP281	Landing center (Pamban)	-	+	+	+	-	+	-	+	+
MP282	Landing center (Pamban)	-	+	+	+	-	+	-	+	+
MP283	Landing center (Pamban)	-	+	+	+	-	+	-	+	+
MP284	Landing center (Pamban)	-	+	+	+	-	+	-	+	+
MP285	Landing center (Pamban)	-	+	+	+	-	+	-	+	+
MP286	Landing center (Pamban)	-	+	+	+	-	+	-	+	+
MP287	Landing center (Pamban)	-	+	+	+	-	+	-	+	+
MP288	Landing center (Pamban)	-	+	+	+	-	+	-	+	+
MP289	Landing center (Pamban)	-	+	+	+	-	+	-	+	+

A - Arginine, O - Ornithine, L - Lysine, 0/F – Oxidation / Fermentation.

Table 3: Prevalence of *Photobacterium damselae* subsp. *damselae* in different fishes isolated from different locations of the south east coast of India.

Fishes	Source	Scientific name	Total No. of isolates	No. of. Pdd. total isolates	Percentage of Isolates (%)
Eightband butterfly fish	Marine ornamental fish farm (Mandapam)	*Chaetodon octofasciatus*	57	1 (1.63%)	0.35%
Parrot fish	Landing centers (Theraspuram and Tharuvaikulam)	*Scarus globiceps*	62	34 (55.73%)	11.76%
Sea bass	Cage culture (Mandapam)	*Lates calcarifer*	45	6 (9.83%)	2.08%
Grouper	Cage culture (Mandapam) Landing center (Theraspuram)	*Epinephelus* spp.	59	1 (1.63%)	0.35%
Pomfret	Landing center (pamban)	*Brama brama*	39	5 (8.19%)	1.73%
Lutjanus	Landing center (Mandapam)	*Lutjanus sebae*	27	14 (22.95%)	4.84%
	Total isolates		289	61	21.11%

method with average linkage (UPGMA) (Sneath and Sokal, 1973). The dendrogram obtained after numerical analysis with the Dice coefficient and UPGMA method (Fig 2, 3, 4 and 5) shown that all patterns shared more than 50% similarity. The isolates (MP66, MP147 and MP157) showed 87% similarity, will belong to same genogroup. The isolate no MP66 isolated from eightband butterflyfish collected at marine ornamental fish farm, Mandapam. MP147 and MP 157 isolate in wild caught parrot fish collected from south east coast of India. The dendrogram obtained after numerical analysis with the Dice coefficient and UPGMA method shows that all patterns shared more than 45% similarity (Botella *et al*., 2002). The isolates (MP189, MP190, MP191, MP193 and MP194) are one genogroups showed 85% similarity. All isolates were collected from wild caught parrot fish. The isolate (MP162, MP189 and MP190) belongs to one genogroup and showed 70% similarity. MP162 isolate collected from cage culture seabass in Mandapam. MP189 and MP190 isolate collected from wild caught fishes. So, these isolates showed abundance in both wild caught and cage culture fishes. Regev *et al*. (2020) revealed that all detected Vibrio strains were divided into four different genogroups of *Vibrio* sp., with an overlap in one group between the wild and the cultured species. This may suggest a spontaneous transmission between the wild and the cage fish. The first group showed high similarity to *V. parahaemolyticus* and *V. alginolyticus*. The second group showed high similarity to *V. harveyi*. The third group contained only one strain that belonged to the Lessepsian fish *S. lessepsianus*, which showed similarity to uncultured Vibrionaceae bacterium isolated from pinfish (*Lagodon rhomboids*), with a 95% homology. In addition, the fourth group contained three identical strains all belonging to the cultured *S. aurata* from 2017 without any similar references. The thirty out of sixty isolates (20% or 50%) showed their presence in both wild caught and cage cultured fishes. The presence of similar genogroup in both wild and cage cultured fishes confirmed the horizontal transfer of these bacteria from wild caught fishes to cage cultured fishes. Similarly reports from various countries also showed horizontal transfer (Chiu *et al*., 2013). Therefore, it is suggested that while selecting sites for cage culture, the place which showed abundance in reef associated fishes should be neglected to avoid *P. damselae* subsp. *damselae* infection.

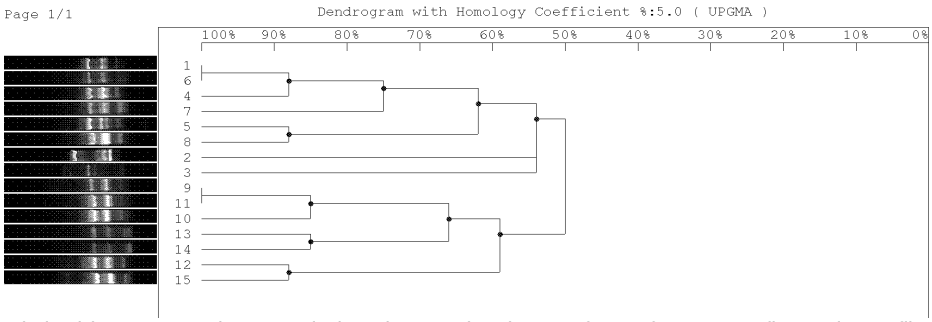

Fig 2: Dendrogram derived by UPGMA cluster analysis using UVI bandmap software for IS-PCR fingerprint profiles of 15 strains of *P. damselae* subsp. *damselae*. Lane 1-MP66, Lane 2-MP76, Lane 3-MP147, Lane 4-MP192, Lane 5-MP193, Lane 6-MP196, Lane 7-MP198, Lane 8-MP224, Lane 9-MP234, Lane 10-MP235, Lane 11-MP237, Lane 12-MP238, Lane 13 MP241, Lane 14-MP246, Lane 15-MP248.

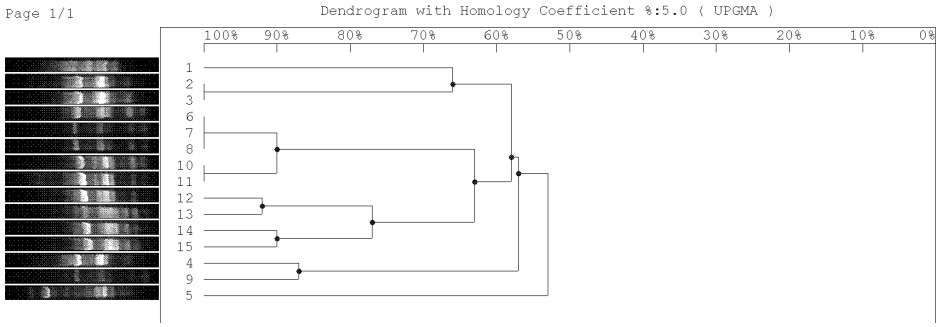

Fig 3: Dendrogram derived by UPGMA cluster analysis using UVI bandmap software for IS-PCR fingerprint profiles of 15 strains of *P. damselae* subsp. *damselae*. Lane 1-MP252, Lane 2-MP253, Lane 3-MP271, Lane 4-MP272, Lane 5-MP273, Lane 6-MP274, Lane 7-MP276, Lane 8-MP277, Lane 9-MP278, Lane 10-MP279, Lane 11-MP280, Lane 12-MP283, Lane 13 MP285, Lane 14-MP287, Lane 15-MP289.

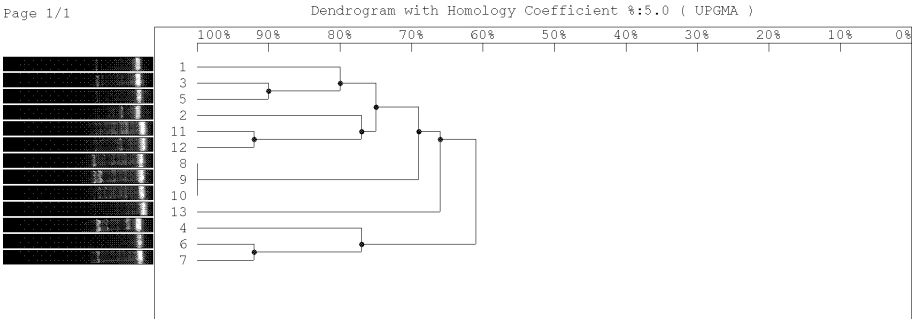

Fig 4: Dendrogram derived by UPGMA cluster analysis using UVI bandmap software for ERIC-PCR fingerprint profiles of 13 strains of *P. damselae* subsp. *damselae*. Lane 1-MP66, Lane 2-MP76, Lane 3-MP147, Lane 4-MP192, Lane 5-MP193, Lane 6-MP196, Lane 7-MP198, Lane 8-MP224, Lane 9-MP234, Lane 10-MP235, Lane 11-MP237, Lane 12-MP238, Lane 13-MP241.

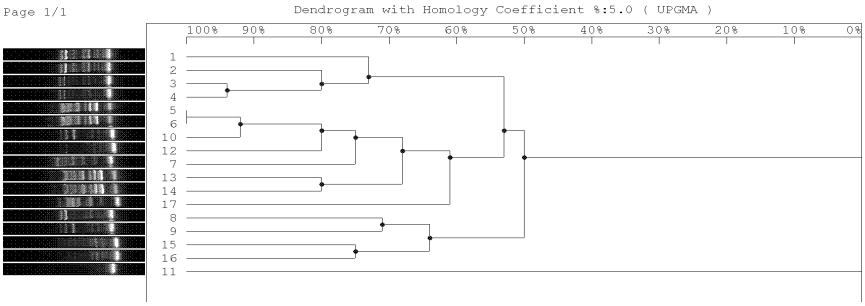

Fig 5: Dendrogram derived by UPGMA cluster analysis using UVI bandmap software for ERIC-PCR fingerprint profiles of 13 strains of *P. damselae* subsp. *damselae*. Lane 14-MP246, Lane 15-MP248, Lane 16-MP252, Lane 17-MP253, Lane 18-MP271, Lane 19-MP272, Lane 20-MP273, Lane 21-MP274, Lane 22-MP276, Lane 23-MP277, Lane 24-MP278, Lane 25-MP279, Lane 26-MP280, Lane 27-MP283, Lane 28-MP285, Lane 29-MP287, Lane 30-MP289.



Fig 6: Reisolated bacteria from dead damselfish.

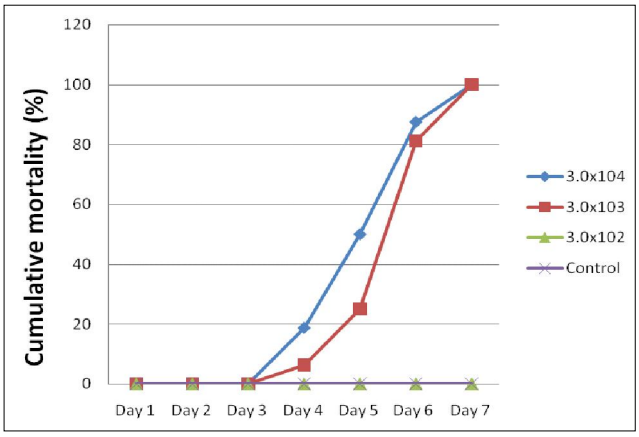

Fig 7: Cumulative mortality percentage observed in damselfish experimental infection.

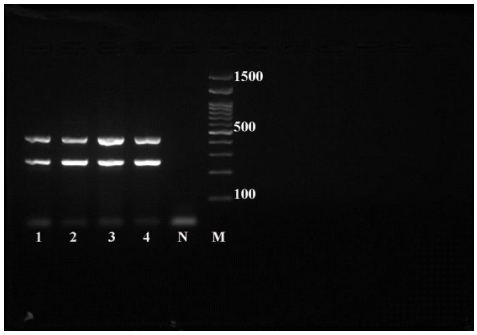

Fig 8: Multiplex-polymerase chain reaction assay for *Photobacterium damselae* subsp. *damselae* from tissue samples in damselfish. Lane: 1-4, *P. damselae* subsp. *damselae*; Lane N: Negative control; Lane M: Molecular weight (100bp ladder).

Pathogenicity study

Clinical signs and mortality

Pathogenicity of *P. damselae* subsp. *damselae* strain to damsel fish was investigated using standard protocols and the LD_{50} was calculated. After injection, the first three days of post challenge no fishes showed symptoms, but fourth and fifth days of post challenge, reduced feed intake was noticed. Six days of post challenge, infected fish developed clinical signs such as lethargy, discolouration of skin, followed by abnormal swimming before mortality. By day 7, the 100% cumulative mortality was observed in damselfish injected with two higher concentrations of bacteria (Fig 7). As compared to infected fish, no clinical signs and mortality were noticed in control fish injected with saline solution. Mahmoud *et al*. (2018) has done the pathogenicity assay and revealed that *P. damselae* subsp. *damselae*, were pathogenic for seabass at LD50 of (1.5×10^8 CFU/g body weight), causing 66.67% mortality. Infected seabass showed ulcers and hemorrhages all over the body with fin erosions and pale gills (Mahmoud *et al*., 2018). Love *et al*. (1981) discussed with ulcers typically occur in the region of the pectoral fin and caudal peduncle of naturally infected damselfish and may reach 5-20 mm in diameter. LD_{50} value of this injected culture of *P. damselae* subsp. *damselae* strain was calculated to be $2.48x10^3$ CFU/mL. Bacteria were re-isolated from the kidney, spleen and liver of moribund fish (Fig 6) and the tissue extracts were directly analysed for the presence of injected pathogen by means of multiplex PCR and the results are given in Fig 8. It has confirmed that mortality and the clinical sign developed in the experimental fish was due to *P. damselae* subsp. *damselae*.

Histopathological findings

Histopathology was carried out from moribund fish tissues such as kidney, liver, spleen and gill (Fig 9). In kidney, tubular epithelial fusion and hyperplasia of haematopoietic tissue, hyperactivity of melanomacrophage centers and coagulative

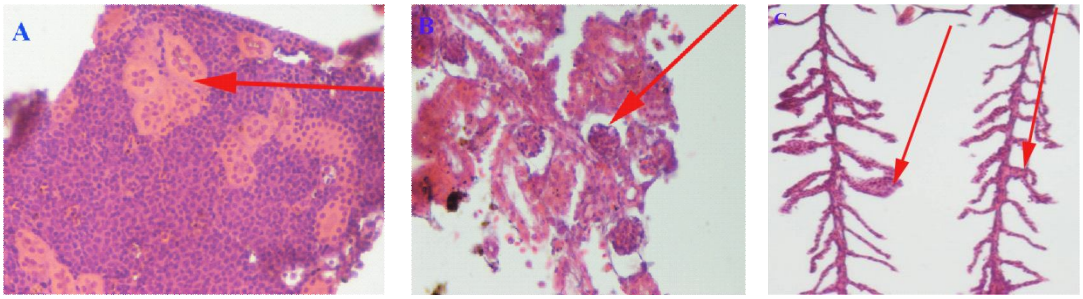

Fig 9: Histopathology section of experimentally infected damselfish with *Photobacterium damselae* subsp. *damselae*.
A and B) kidney, starting is tubular epithelial fusion and hyperplasia of haematopoietic tissue, hyperactivity of melanomacrophage centers and coagulative necrosis of epithelial lining of some renal tubules and Glomerulonephritis in kidney (60X), C). Gills show destructive changes in both primary and secondary lamellae, collapsed and curled secondary lamellae, lamellar fusion and hyperplasia in gill tissue of infected of damselfish (60X).

necrosis of epithelial lining of some renal tubules and glomerulonephritis was noticed (Fig 7). Similarly, *P. damselae* subsp. *damselae* infected fish kidneys showed congestion of blood vessels, necrosis of renal tubules and hyperplasia of melanomacrophage centres (Eissa *et al*., 2018, Mladineo *et al*., 2006). The presence of highly dense clusters of bacteria in the kidney in seabass suggested that kidney should be microbiologically examined in a first attempt (Avci *et al*., 2013). Gills of infected damselfish showed destructive changes in both primary and secondary lamellae, collapsed and curled secondary lamellae, lamellar fusion and hyperplasia. Essam *et al*. (2016) investigated the histological alterations in gills were characterized by changes in both primary and secondary lamellae, collapsed and curled secondary lamellae, edema in filaments, severe lamellar aneurism and hyper activation of goblet cells. These sever lesions in the gills suggests their primary role in the bacterial entrance into the fish body. Hence, the study suggest that *P. damselae* subsp. *damselae* isolates from wild caught fishes found virulent to the culture fishes and cause severe tissue damage and mortality to the individuals.

CONCLUSION

Photobacterium damselae subsp. *damselae* is the most devastating bacterial pathogen and a global health issue. Photobacteriosis found to be one of the major causes for the failure of cage culture industry. The present study revealed higher prevalence of *P. damselae* subsp. *damselae* in wild caught parrot fish when compared to the other wild and cage culture fishes. Among cage culture fishes, seabass found more infected with *P. damselae* subsp. *damselae* when compared to grouper. There exists possibility for transmission of *P. damselae* subsp. *damselae* from wild caught to cage culture fishes and could cause severe tissue damage, lesions and mortality of the individuals. Therefore proper site selection, screening of *P. damselae* subsp. *damselae* presence in cage sites proximity area and good management practices during cage culture is very much necessary for the prevention of *P. damselae* subsp. *damselae* infection in fishes and their associated economic loss.

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