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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 [1]. 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 formely 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 damselysin (*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'- CGCTTTCGGACCATCTTTAC-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.

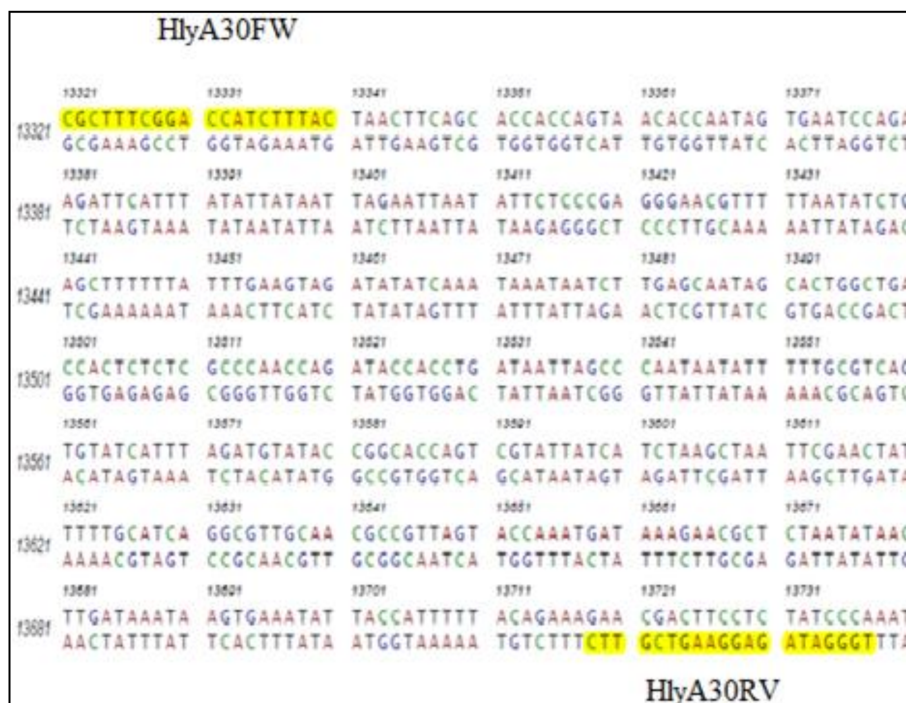

Fig 1: Nucleotide sequence of Photobacterium damselae subsp. damselae hemolysin gene (*HlyA*). Underlined nucleotides (HlyA30FW, HlyA30RV) correspond to the pair of primers used to amplify DNA in PCR Assays

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 and the supernatant was collected in fresh sterile tube. To the supernatant, equal volume of absolute alcohol was added and 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, 1min at 95°C, 1 min at 56°C, and 1min 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 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. 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	CGCTTTCGGACCATCTTTAC	417bp
HIYA30 RV	TGGGATAGAGGAAGTCGTTC	

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. damselae* subsp. *damselae* isolates. Out of the 61 strains, 36/ 61 of *P. damselae* subsp. *damselae* (Fig. 2) isolates were PCR positive for the *hly*A 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. damselae* ssp. *piscicida* and *P. damselae* 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. damselae* subsp. *piscicida* from *P. damselae* subsp. *damselae*. Toranzo *et al.* [24] and Zorrilla *et al.* [25] reported that *P. damselae* subsp. *piscicida* did not grow on TCBS agar plate. Conversely, it was reported that *P. damselae* subsp. *damselae* could grow on TCBS agar plate [3, 26]. The *Photobacterium damselae* 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. damselae* 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. damselae* 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 damselae* subsp. *piscicida* could not grow on TCBS whereas *P. damselae* subsp. *damselae*. The sensitivity of the PCR was evaluated by serial dilution of *P. damselae* subsp. *Piscicida* cell suspension as detailed elsewhere [27]. Amplification resulted in detectable levels of PCR product when a minimum of 13CFU of *P. damselae* 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. damselae* 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. damselae* species in the wild and cage culture fishes.

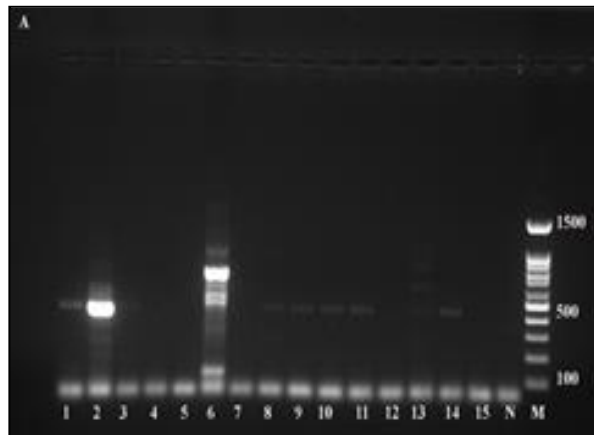

Fig 2: Agarose gel electrophoretic analysis of the PCR amplified product using HlyA30FW and HlyA30RV primers (417bp) for detection of virulence hemolysin gene. A: Lane 1-MP66 (HlyA), Lane 2-MP76 (HlyA), Lane 3-MP140, Lane 4-MP147, Lane 5-MP148, Lane 6-MP157, Lane 7-MP160, Lane 8- MP162 (HlyA), Lane 9-MP189 (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.

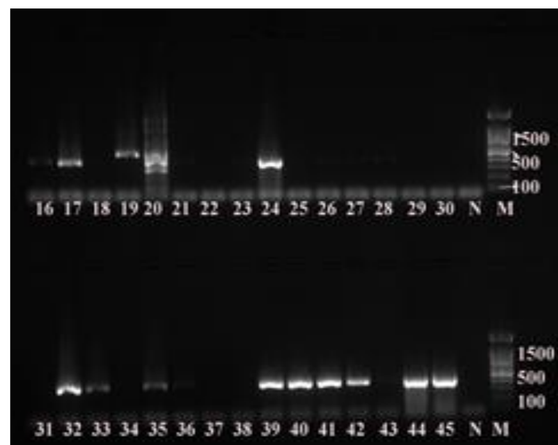

Fig 3: Agarose gel electrophoretic analysis of the PCR amplified product using HlyA30FW and HlyA30RV primers (417bp) for detection of virulence hemolysin gene. B: Lane 16-MP196 (HlyA), Lane 17-MP197 (HlyA), Lane 18-MP198, Lane 19-MP199, Lane 20-MP224, Lane 21-MP233, Lane 22-MP234, Lane 23- MP235, Lane 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.

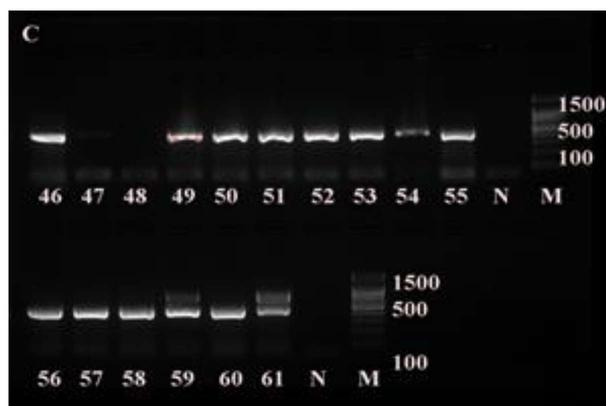

Fig 4: Agarose gel electrophoretic analysis of the PCR amplified product using HlyA30FW and HlyA30RV primers (417bp) for detection of virulence hemolysin gene. C: Lane 46-MP273 (HlyA), Lane 47-MP274 (HlyA), Lane 48-MP276, Lane 49-MP277 (HlyA), Lane 50-MP278 (HlyA), Lane 51-MP279 (HlyA), Lane 52-MP280 (HlyA), Lane 53- MP281 (HlyA), Lane 54-MP282 (HlyA), Lane 55-MP283 (HlyA), Lane 56-MP284 (HlyA), Lane 57-MP285 (HlyA), Lane 58-MP286 (HlyA), Lane 59-MP287, Lane 60-MP288 (HlyA), Lane 61-289MP, Lane 62-Negative control, Lane 63-100bp DNA marker.

Table 2: Details of haemolysin gene(A) detection in *Photobacterium damselae* subsp. *damselae* isolates

Sl. No	Isolate No	Source	Fish Name	Hly A gene
1.	MP66	Marine ornamental fish farm	*Chaetodon octofasciatus*	P
2.	MP76	Landing center (Theraspuram)	*Scarus globiceps*	P
3.	MP162	Cage culture system (Thangachimadam)	*Lates calcarifer*	P
4.	MP189	Cage culture system (Thangachimadam)	*Lates calcarifer*	P
5.	MP190	Cage culture system (Thangachimadam)	*Lates calcarifer*	P
6.	MP191	Cage culture system (Thangachimadam)	*Lates calcarifer*	P
7.	MP194	Cage culture system (Thangachimadam)	*Lates calcarifer*	P
8.	MP196	Cage culture system (Thangachimadam)	*Lates calcarifer*	P
9.	MP197	Cage culture system (Thangachimadam)	*Lates calcarifer*	P
10.	MP236	Cage culture system (Thangachimadam)	*Lates calcarifer*	P
11.	MP238	Landing centers (Tharuvaikulam)	*Scarus globiceps*	P
12.	MP239	Landing centers (Tharuvaikulam)	*Scarus globiceps*	P
13.	MP240	Landing centers (Tharuvaikulam)	*Scarus globiceps*	P
14.	MP245	Landing centers (Tharuvaikulam)	*Scarus globiceps*	P
15.	MP246	Landing centers (Tharuvaikulam)	*Scarus globiceps*	P
16.	MP248	Landing centers (Tharuvaikulam)	*Scarus globiceps*	P
17.	MP249	Landing centers (Theraspuram)	*Brama brama*	P
18.	MP252	Landing centers (Theraspuram)	*Brama brama*	P
19.	MP253	Landing centers (Theraspuram)	*Brama brama*	P
20.	MP254	Landing centers (Theraspuram)	*Brama brama*	P
21.	MP255	Landing centers (Theraspuram)	*Brama brama*	P
22.	MP271	Landing centers (Theraspuram)	*Brama brama*	P
23.	MP272	Landing centers (Theraspuram)	*Brama brama*	P
24.	MP273	Landing centers (Theraspuram)	*Brama brama*	P
25.	MP274	Landing centers (Pamban)	*Lutjanus sebae*	P
26.	MP277	Landing centers (Pamban)	*Lutjanus sebae*	P
27.	MP278	Landing centers (Pamban)	*Lutjanus sebae*	P
28.	MP279	Landing centers (Pamban)	*Lutjanus sebae*	P
29.	MP280	Landing centers (Pamban)	*Lutjanus sebae*	P
30.	MP281	Landing centers (Pamban)	*Lutjanus sebae*	P
31.	MP282	Landing centers (Pamban)	*Lutjanus sebae*	P
32.	MP283	Landing centers (Pamban)	*Lutjanus sebae*	P
33.	MP284	Landing centers (Pamban)	*Lutjanus sebae*	P
34.	MP285	Landing centers (Pamban)	*Lutjanus sebae*	P
35.	MP286	Landing centers (Pamban)	*Lutjanus sebae*	P
36.	MP288	Landing centers (Pamban)	*Lutjanus sebae*	P

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References

1. FAO. Food and Agriculture Organization of the United Nations. Yearbook of Fishery Statistics Rome. ISBN. 2005;92-5-105087-2.
2. Goldburg R, Triplett T. Murky waters: environmental effects of aquaculture in the United States. The Environmental Defense Fund. Washington D.C: https://www.researchgate.net/publication/270582938 1997.
3. Love M, Fisher DT, Hose JE, Farmer JJ, Hickman FW,

Fanning GR, *et al*. *Vibrio damsela*, a marine bacterium, causes skin ulcer on the damselfish *Chromis punctipinnis*. Science 1981;214:1139–1140.
4. Sakata T, Matsuura M, Shimkawwa Y, *et al*. Characteristics of *Vibrio damsela* isolated from diseased yellowtail *Seriola quinqueradiata*. Bulletin of the Japanese Society of Scientific. Fisheries 1989;55:135-141.
5. Fouz B, Conchas RF, Magarinos B, Amaro C, Toranzo AE, *et al*. *Vibrio damsela* strain virulence for fish and mammals. FHS/AFS Newsletter 1992a;20:56-59.
6. Clarridge JE, Zighelboimdaum S. Isolation and characterization of 2 hemolytic phenotypes of *Vibrio damsela* associated with a fatal wound infection. Journal of Clinical Microbiology 1985;21:302–306.
7. Tang WM, Wong JWK. Necrotizing fasciitis caused by *Vibrio damsela*. Orthopedics 1999;22:443-444.
8. Barber GR, Swygert JS. Necrotizing fasciitis due to *Photobacterium damsela* in a man lashed by a stingray. New England Journal of Medicine 2000;342(11):824.
9. Janssen WA, Surgalla MJ. Morphology, physiology, and serology of a *Pasteurella* species pathogenic for white perch (*Roccus americanus*). Journal of Bacteriology 1968;96:1606-1610.
10. Rivas AJ, Lemos ML, Osorio, CR, *et al*. *Photobacterium damselae* subsp. *damselae*, a bacterium pathogenic for marine animals and humans. Frontiers in Microbiology 2013a;4:283.
11. Vences A, Rivas AJ, Lemos ML, Husmann M, Osorio CR, *et al*. Chromosome-encoded hemolysin, phospholipase, and collagenase in plasmidless isolates of Photobacterium damselae subsp. damselae contribute to virulence for fish. Applied and Environmental Microbiology 2017;83:e00401-17. doi: 10.1128/AEM. 00401-17.
12. Osorio CR, Vences A, Matanza XM, Terceti MS, *et al*. Photobacterium damselae subsp. damselae, a generalist pathogen with unique virulence factors and high genetic diversity. Journal of Bacteriology 2018, 00002-18. doi: 10.1128/JB.00002-18.
13. Labella A. Aislamientoy Caracterizacion de Bacterfias Potencialmente Patogenas Asociadas a Nuevas Especies de Esparidos Cultivados, PhD Thesis, University of Malaga, Spain 2010.
14. Stephens FJ, Raidal SR, Buller N, Jones B, *et al*. Infection with *Photobacterium damselae* subspecies *damselae* and *Vibrio harveyi* in snapper, *Pagrus auratus* with bloat. Australian Veterinary Journal 2006;84(5):173-177.
15. Kubota S, Kimura M, Egusa S, *et al*. Studies on bacterial tuberculoidosis in cultured yellowtail. I. Symptomatology and histopathology. Fish Pathology 1970;4:111-118.
16. Toranzo AE, Baya AM, Roberson BS, Barja JL, Grimes DJ, Hetrick FM, *et al*. Specificity of slide agglutination test for detecting bacterial fish pathogens. Aquaculture. 1987;61:81-97.
17. Jung TS, Thompson KD, Morris DJ, Adams A, Sneddon K, *et al*. The production and characterization of monoclonal antibodies against *Photobacterium damselae* ssp. *piscicida* and initial observations using immunohistochemistry. Journal of Fish Diseases 2001;24:67-77.
18. Eisenstein BI. The polymerase chain reaction: a new method of using molecular genetics for medical diagnosis. New England Journal of Medicine 1990;322:178-183.
19. Osorio CR, Collins MD, Toranzo AE, Barja JL, Romalde JL, *et al*. 16S rRNA gene sequence analysis of *Photobacterium damselae* and nested PCR method for rapid detection of the causative agent of fish pasteurellosis. Applied and Environmental Microbiology 1999;65:2942-294.
20. Fouz B, Larsen JL, Nielsen B, Barja JL, Toranzo AE, *et al*. Characterization of *Vibrio damsela* strain isolated from turbot *Scophthalmus maximus* in Spain. Diseases of Aquatic Organisms. 1992b; 12: 155–166.
21. Magarinos B, Toranzo AE, Romalde JL, *et al*. Phenotypic and pathobiological characteristics of *Pasteurella piscicida*. Annual Review of Fish Diseases 1996;6:41-64.
22. Gauthier G, Lafay B, Ruimy R, Breittmayer V, Nicolas JL, Gauthier M, *et al*. Small-subunit rRNA sequences and whole DNA relatedness concur for the reassignment of *Pasteurella piscicida* (Sniezko *et al*.) Janssen and Surgalla to the genus *Photobacterium* as *Photobacterium damsela* subsp. *piscicida* comb. nov. International Journal of Systematic Bacteriology 1995;45:139-144.
23. Rajan PR, Lopez C, Lin JH, Yang HL, *et al*. *Vibrio alginolyticus* infection in cobia (*Rachycentron canadum*) cultured in Taiwan. Bulletin of the European Association of Fish Pathologists 2001;21:228-234.
24. Toranzo AE, Barreiro S, Casal JF, Figueras A, Magarinos B, Barja J, *et al*. Pasteurellosis in cultured gilt-head seabream (*Sparus aurata*): first report in Spain. Aquaculture 1991;99:1-15.
25. Zorrilla I, Balebona MC, Morinigo MA, Sarasquete C, Borrego JJ, *et al*. Isolation and characterization of the causative agent of pasteurellosis, *Photobacterium damsela* ssp. *piscicida*, from sole, *Solea senegalensis* (Kaup). Journal of Fish Diseases 1999;22:167-172.
26. Thyssen A, Grisez L, Van Houdt R, Ollevier F, *et al*. Phenotypic characterization of the marine pathogen *Photobacterium damselae* subsp. *Piscicida*. International Journal of Systematic Bacteriology 1998;48:1145-1151.
27. Cascon A, Anguita J, Hernanz C, Sanchez M, Fernandez M, Nanarro G, *et al*. Identification of Aeromonas hydrophila hybridization group 1 by PCR assays. Applied and Environmental Microbiology 1996;62:1167-1170.