

## First time isolation of *Photobacterium damsela* subsp. *damsela* from *Caranx sexfasciatus* in Persian Gulf, Iran

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Received: July 2014, Accepted: February 2015

### ABSTRACT

**Background and Objective:** *Photobacterium damsela* subsp. *damsela* is a marine pathogenic bacterium which causes disease in marine animals and human. This bacterium mostly found in coastal shallow seawater. So, the aim of this study was isolation and characterization of *Photobacterium damsela* subsp. *damsela* from edible fish of Persian Gulf, Bandar Abbas.

**Material and Methods:** Totally 100 fish from different species were evaluated and out of that 5 different types of fish with external symptoms including: *Caranx sexfasciatus*, *Lethrinus olivaceus*, *Scomberoid tol*, *Auxis thazard* and *Liza macrolepis*, were collected from Bandar Abbas local fish market in September 2013. The samples were cultured on Marin Agar 2216 and Thiosulfate Citrate Bile salts Sucrose Agar media and incubated at 25°C for 48 hrs. Then the isolates were characterized using biochemical (API 20 NE system) and molecular techniques. In addition, antibiotic susceptibility, presence of poly  $\beta$  hydroxy butyrate and hemolysis activity of isolates were evaluated.

**Results and Conclusion:** Entirely, 30 Gram negative bacterial colonies were isolated from the selected fish. Among the isolates, two suspected colonies were identified as *Photobacterium damsela* from *Caranx sexfasciatus* with API 20NE biochemical test. This results confirmed by 16s rRNA sequencing method. Both isolates showed  $\alpha$  hemolytic with existence of  $\beta$  hydroxyl butyrate. Furthermore, the isolates were susceptible to ciprofloxacin, chloramphenicol and nalidixic acid.

**Conclusion:** Overall, the study indicated first time isolation of this bacterium from one type of fish caught from Persian Gulf, which warns us to pay more attention to fishery in this geographical area.

**Keywords:** Polymerase Chain Reaction, *Photobacterium damsela*, Persian Gulf, *Caranx sexfasciatus*.

### INTRODUCTION

*Photobacterium* is a Gram negative, facultative anaerobic, motile bacterium which is commonly associated with marine animals. Members of this genus are widely distributed in marine environments and usually found in coastal, open oceans and deep-seas (1). The bacterium belongs to the family vibriaceae (2). Although some species of *photobacterium* is lumi-

nous, both luminous and non-luminous species are pathogenic for marine animals and human (3, 4). *Photobacterium damsela* subsp. *damsela* is one of pathogenic members of this family which is originally found in damselfish in 1981. This species normally cause ulcers and septicemia in different type of marine animals such as wild and cultured fish (5). Also unusual case of infections were reported after digestion of raw seafood and urinary tract infection after being exposed to seawater which is mostly occurred in coastal areas of the United States, Australia and Japan (6-8). Many scientists reported that amputation or debridement is effective treatment for patients' survival in early stage of *Photobacterium* infection (9). Moreover *Photobacterium damsela* can cause

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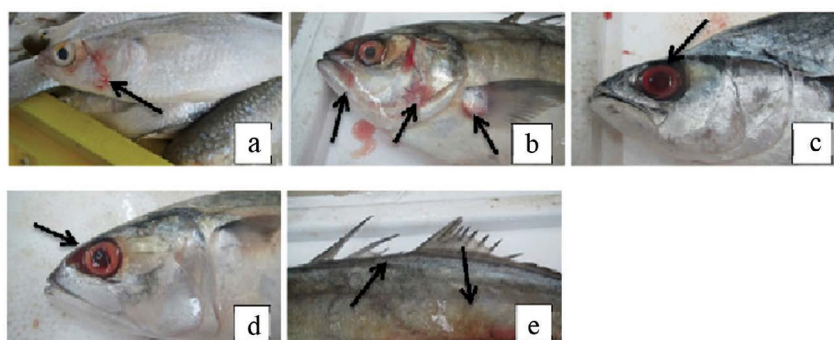
severe fetal infection in humans. These infections related to wound which is caused by fishing tools, during fish handling or being exposed to marine animal and seawater (6, 9, 10). These bacteria interact as commensally, parasites and saprophytes with marine animals and could be found in free living forms (11). In pathogenic form they can be isolated from skin, intestinal tract contents, decaying animal tissues and amphipods hemolymph (12, 14). Various study indicate that powerful cytolysin of *Photobacterium* is one of virulence factor of this bacterium for marine animals. The main external symptoms of this bacterium in fish are ulcerative lesions in skin and hemorrhage in mouth, eyes and muscles (15). All around the world, *Photobacterium damsela* subsp. *damsela* were isolated and reported from different type of fish include rainbow trout (*Oncorhynchus mykiss*), ovate pompano (*Trachinotus ovatus*), sea bass (*Dicentrarchus labrax*), yellowtail (*Seriola quinqueradiata*), redbanded seabream (*Pagrus auriga*) (12, 16-18). Furthermore, in Asia, first case of *Photobacterium damsela* subsp. *damsela* from Asian sea bass (*Lates calcarifer*) was reported in 2008 (19). This bacterium can spread through seawater between marine animals. Salinity and temperate water are two main factors for spreading of *Photobacterium* (20). Because of lack of information about *Photobacterium* in Persian Gulf and importance of this area for its various fish's reservoirs and peoples' contact with seawater we conduct this research to be aware of its spread rate among edible Persian Gulf fish.

## MATERIALS AND METHODS

**Sample collection.** Persian Gulf is located in

South of Iran (N24°-30°30', E48°-56°25') between United Arab Emirates, Saudi Arabia, Kuwait, Iraq and Iran. Dry and subtropical climate with mean temperature of 26-27°C in surface and also high salinity are its main characteristic (21,22). Hence, for isolation of *Photobacterium* totally 100 fish from different species were evaluated and out of that 5 different types of fish with fracture fins, mucus in gills and hemorrhage in eyes, skin and gills including: *Caranx sexfasciatus*, *Lethrinus olivaceus*, *Scomberoid tol*, *Auxis thazard* and *Liza macrolepis*, were collected from Bandar Abbas local fish market ( Fig. 1). The samples were collected from 9 am up to 1 pm in September 2013. The fish were kept in cold box with approximately 4°C temperature and immediately transferred to the microbiology laboratory.

**Isolation and characterization of *Photobacterium* from fish.** The collected fish with different symptoms were evaluated for isolation of *Photobacterium*. For this purpose the samples were collected from spleen, intestine, eyes, gills, skin, liver and kidney of the fish and seeded on Marine agar 2216 and Thiosulfate Citrate Bile salts Sucrose Agar (TCBS) supplemented with 1.5% NaCl. The plates were incubated at 25°C for 48 hours. The bacterial isolates were examined for morphological and biochemical identification. The preliminary selected tests were: Gram staining, citrate utilization, oxidase and catalase activity. Then the biochemical tests were performed using API 20NE (bioMérieux, France) according to the manufacturer's instructions (23). On the other hand, hemolytic activity and presence of poly beta hydroxyl butyrate granules were assessed by culturing the suspected bacteria on Blood Agar and Sudan Black staining respectively (5, 24).



**Fig. 1.** Symptoms in fish samples. a and b show hemorrhages around the mouth, operculum and pectoral fin, c and d show hemorrhages in eye, e shows fracture fins and hemorrhage in skin.

**Molecular confirmation of the *Photobacterium damsela*.** Molecular identification of the selected isolates was completed using 16s rRNA polymerase chain reaction. For DNA extraction, the isolates were cultured on Luria Bertani Broth medium and incubated at 25 °C for 24h. Then extraction of DNA was done according to Cinna Gen extraction kit instruction (Cinna Gene, Tehran, Iran). PCR process was done with two sets of forward and reverse primers (16s-27F: 5' – TTGGAGAGTTTGATCCTGGCTC-3' and 16s-1492R: 5'- AGGAGGTGATCCAACCGCA – 3') which amplify fragment between position 27 to 1492 bp and PCR complex was consisted of Taq DNA polymerase, 10X PCR buffer, MgCl<sub>2</sub>, dNTPs, primers, purified extracted DNA and distilled water with final volume of 50µl (25, 26). Electrophoresis was used for qualitative analysis of the PCR products and product's bands were observed under UV trans-illuminator. Afterward PCR products were sent to Iranian Biological Resource Center (IBRC) for sequencing. Finally, samples were analyzed by Chromas software and blast in NCBI site.

**Antibiotic Susceptibility.** The presence of β-lactamase genes in members of the family Vibrionaceae has been reported recently (27). Therefore, antibiotic susceptibility of the retrieved bacterial isolates was determined using the Kirby Bauer disk diffusion method (28,29). The following antimicrobial discs (Oxoid) were used: ciprofloxacin 5µg (CIP 5), chloramphenicol 30µg (C 30), nalidixic acid 30µg (NA 30), ampicillin 10µg (AP 10), amoxicillin 25µg (A 25), gentamicin 10µg (GM 10), streptomycin 10µg (S 10), erythromycin 15µg (E15), clindamycin 2µg (CC 2), novobiocin 5µg (NO 5), cotrimaxazole 25µg (CO 25) and rifampin 5µg (RP 5). Since the genus *Photobacterium* is a member of the family *Vibrionaceae*, interpretation of the inhibition zones was done using the CLSI guidelines for *Vibrio* sp (30).

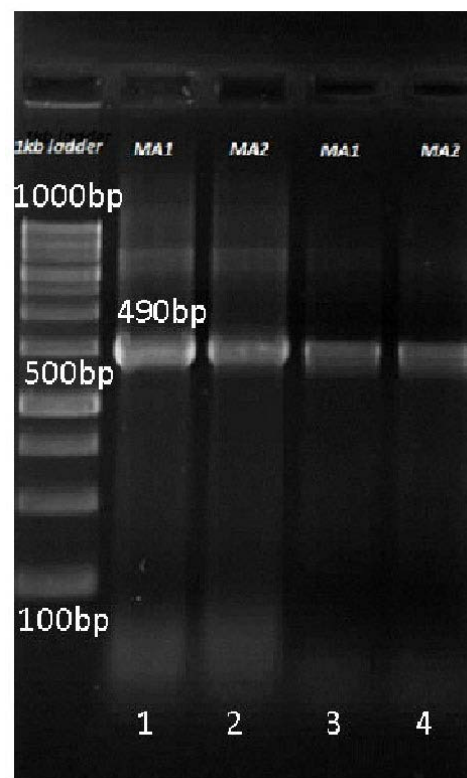
**Presence of β hydroxybutyrate butyrate and hemolysis test.** Presence of β hydroxyl butyrate and hemolysis were checked using Sudan black staining and growth on Blood agar medium respectively.

## RESULTS

**Isolation, characterization and molecular confirmation of isolates.** Totally, 30 Gram negative

bacterial colonies were isolated from the selected fish. Out of that, 20 isolates were oxidase positive and 10 were negative. Among the 20 isolates, 7 isolates were catalase positive and rests of them were negative with citrate negative reaction. The suspected colonies were evaluated using API 20 NE system and 2 isolates were identified as *Photobacterium damsela* with excellent identification rate (99.9%) (Table1). Both of the strains were isolated from *Caranx sexfasciatus* fish. Colonies of the isolates were small, convex, circular and white. Furthermore, it's morphology under light microscope were bipolar coccobacilli.

The results obtained from PCR and gel electrophoresis of two isolates showed two DNA fragments with 490 bp length which sequencing of 16s rRNA gene identification showed that both isolates belonged to the genus *Photobacterium damsela* subspecies *damsela* (Fig. 2) ( Table 2).



**Fig. 2.** Gel electrophoresis of PCR products. Lane 1 and 2 show DNA fragments of two *Photobacterium damsela* isolates which result from PCR process with high density. Lane 3 and 4 show DNA fragments of same *Photobacterium* with lower density.

**Table1.** Characterization of *Photobacterium damsela* isolated from *Caranx sexfasciatus*

Tests	Isolated <i>Caranx sexfasciatus</i> strains	
	Intestine sample	Skin sample
Gram stain	-	-
Oxidase	+	+
Catalase	±	±
Citrate utilization	-	-
Haemolysis	α	α
Growth on TCBS	G+	G+
Nitrates reduction	+	+
Indole production	-	-
Glucose fermentation	+	+
Arginine dihydrolase	+	+
Urease	+	+
β-glucosidase	-	-
Gelatinase	-	-
β-galactosidase(PNPG)	-	-
Assimilation:		
D-glucose	-	-
L-arabinose	-	-
D-mannose	-	-
D-mannitol	-	-
N-acetyl-glucosamine	-	-
D-maltose	-	+
Potassium gluconate	-	+
Capric acid	-	-
Adipic acid	-	-
Malic acid	-	+
Trisodium citrate	-	-
Phenylacetic acid	-	+
Poly beta hydroxybutyrategranul	+	+

**Table 2.** molecular identification of the isolates after Blasting in NCBI

Sample name	Sequence Length	Max. Coverage	Max. Identity (%)	Max. Identity with
Bacterial intestine sample	1393bp	100%	100%	<i>Photobacterium damsela</i> subsp. <i>damsela</i> strain ATCC33539
Bacterial skin sample	1400bp	100%	99%	<i>Photobacterium damsela</i> subsp. <i>damsela</i> strain ATCC33539

*Photobacterium* cultures on Blood Agar shows  $\alpha$  hemolytic activity with dark greenish color around colonies. Furthermore, these isolates had Poly  $\beta$  hydroxy butyrate granules which were seen dark blue pattern in pink bacterial background.

**Antibacterial susceptibility test.** According to the results obtained from the antibacterial susceptibility testing it could be concluded that *Photobacterium* isolates were sensitive to ciprofloxacin, chloramphenicol and nalidixic acid, while resistance to ampicillin, amoxicillin, gentamicin, streptomycin, erythromycin, clindamycin and novobiocin. Furthermore, intermediate sensitive was found to cotrimaxazole.

## DISCUSSION

Persian Gulf in terms of nutrition and economy is an important geographical area for Iranian people. Therefore, special attention and frequent review of these waters for isolation of different pathogenic bacteria from this area must be at the top of environmental microbiological agenda. Although Pourbabaei et al. (2013) has been reported isolation of different genus of vibrio from coastal waters of Bandar abbas (31); for the first time *Photobacterium damselae* subsp. *damselae* has been isolated from *Caranx sexfasciatus* (big-eye trevally) fish which was caught from Persian Gulf, Iran.

The results obtained from this study indicated that totally two *Photobacterium damselae* were isolated from skin and intestine of big-eye trevally fish (*Caranx sexfasciatus*). This is the first time isolation of this bacterium from big-eye trevally fish which is one of indigenous Persian Gulf edible fish. The member of *Photobacterium* has been reported from various types of fish worldwide. In 2006 Labella and his colleagues were reported isolation of *Photobacterium damselae* subsp. *damselae* from redbanded seabream after 2 massive outbreaks (12). Afterward, the organism were isolated from Asian seabass (19) and in recent years, this bacterium isolated from *Scomber australasicus* and *Rachycentron canadum* in 2013 in Taiwan (32). The fish had exophthalmia, hemorrhaging in skin and gills, dark pigments on skin, accumulation of acidic fluid or Mucus in abdominal area, hemorrhagic liver, enlarge spleen and bladder fulfill of bile symptoms (12,19). But in this project the big-eye trevally showed hemorrhaging in eyes, skin and area around

gills and accumulation of blood vessels in abdominal cavity.

In addition, isolation of this bacterium from cultured fish with high economic value and also fish which is recently cultured, make the bacterium as emerging phenomena in aquaculture industry (16, 23, 33, 34). Presence of this bacterium in fish, proves its existence in fish surrounding water and indicate that *Photobacterium damselae* subsp. *damselae* can transfer via water to other fish. Attachment and colonization on fish skin is one of the ways for its pathogenicity. Different reports indicated that this way of spreading (via water) depend on temperature and water salinity and frequently occur in salty water with temperature around 22 to 25°C (20). Therefore, isolation of *Photobacterium damselae* from skin of big-eye trevally (*Caranx sexfasciatus*) in this geographical area confirmed the infection reason of fish in this region.

The results obtained from antibiotic pattern of *Photobacterium damselae* in Egypt in some cases were parallel to our results, but there is two differences regarding erythromycin and cotrimaxazole. Their isolates were resistance to cotrimaxazole and intermediate susceptibility to erythromycin while our results were vice versa (23). It is obvious that this bacterium have  $\beta$ -lactamase enzyme which could be protect it from  $\beta$ -lactam antibiotic and most antibiotics effect on bacterial DNA.

Hemolytic activity is one of virulence factor for *Photobacterium damselae* subsp. *damselae* and the reports showed that most of pathogenic isolates from various fish had  $\beta$  hemolytic activity (19, 35, 36). But both *Photobacterium* isolates from this study showed  $\alpha$  hemolytic on Blood Agar which indicates their pathogenic potential, but  $\alpha$  hemolytic type brings up the hypothesis that may be their degree of pathogenicity is lower than other isolates with  $\beta$  hemolysis. In addition, poly  $\beta$  hydroxybutyrate is another factor that plays role in *Photobacterium damselae* pathogenicity. Researchers who isolated *Photobacterium damselae* from Asian sea bass stated that existence of poly  $\beta$  hydroxybutyrate fatty granules cause enables growth of this species in high water temperature (19). Consequently, after presence of these granules in both isolates from fish it could be concluded that the existence of this granule is one of the reasons for their growth in warm and salty water of Persian Gulf. On the other hand as Dedkova and Blatter explained in 2014 there are correlation between PHB and inorganic polyphosphate in mammalian health and disease

(37). Indeed,  $\beta$  OHB is a metabolic intermediate that constitutes 70% of ketone bodies produced during ketosis, which is generally considered as an unfavorable pathologic state and elevated level of PHB are associated with pathological state (37). Therefore, it could be concluded that there is a relationship between presence of PHB in infected fish and disease in human which needs more experiment in different aspect.

## CONCLUSION

In conclusion, existence of pathogenic species of *Photobacterium* genus in edible Persian Gulf fish is confirmed for the first time by isolation of 2 case of *Photobacterium damsela* subsp. *damsela* from big-eye trevally fish (*Caranx sexfasciatus*). But more research should be done on different fish species to gain full awareness about *Photobacterium* population in Persian Gulf. Since fish is one of important food for humans, more attention and supervision is required from the government to fishing industry in Persian Gulf area, Iran.

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