Red-pink colony-producing *Pseudomonas* sp. is the causative agent of mass mortality in larvae and post-larvae of *Litopenaeus vannamei* raised in hatcheries in south Iran

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Abstract

This study aimed to identify the causative of the mass mortality observed in zoeal to post-larval shrimp raised in hatcheries in south Iran. For three consecutive months, samples of nauplii and zoea of *Litopenaeus vannamei* were collected from an affected hatchery located in the province of Bushehr. Upon culture on marine agar, bacterial colonies that produced white, orange, yellow and red pigments were identified. In the hatcheries in which mass mortality was observed, the water columns of the affected tanks exhibited a red-pink color. Therefore, the bacteria that produced red pigment were selected for further phenotypic characterization using polymerase chain reaction (PCR) and virulence bioassays. Our results indicate that this bacterium belonged the genus *Pseudomonas* and that it was identical to *P. mesophilica* and *P. anguilliseptica*. PCR analysis of this bacterium revealed the production of a 150-bp band that was consistent with the *Pseudomonas* genus. To determine the pathogenicity of the isolated bacteria in nauplii and post-larvae of *L. vannamei*, we performed bioassay experiments by bath immersion at 27-28°C. Our results showed that culture of nauplii and post-larvae of *L. vannamei* with the bacteria at a concentration of 1.5-2.0 × 10^5 CFU/mL in marine broth resulted in a 100% mortality rate 24-48 h post-challenge. In contrast, there was no mortality in the nauplii and post-larvae that were cultured in the absence of bacteria. Upon pathological examination, we found that the color of the larvae was abnormal and pink, with acute necrosis of the entire body 48 h post-challenge.

Introduction

Bacterial disease is one of the major causes of mortality in shrimp larviculture worldwide (Lightner, 1983; Daniels, 1993). Since the beginning of shrimp larviculture in 1995 on the coast of the Persian Gulf in southern Iran, more than 30% of larval and post-larval shrimp (mainly at zoeal stages 1-3) eliminations have been attributed to the development of a red-pink color at the bottom of the rearing tanks. In particular, the province of Bushehr has been the most affected. This phenomenon accounts for up to 95% of shrimp mortality. This condition, referred to as "the red spot syndrome," was identified by shrimp farmers several years ago. However, no etiological agent has been found and no treatment has been recommended to eradicate this problem. Some shrimp farmers and aquatic veterinarians who work in the industry suggest that this problem arises from fungal and bacterial infections, while others have suggested that it is related to the quality of the water. In an attempt to ameliorate this problem, some shrimp farmers have used long-term treatment of the rearing tanks with antifungal drugs, such as Treflan, without significant success. Previous reports of the successful treatment of bacterial infections with antibiotics led to the notion that the red-pink color observed at the bottom of the rearing tanks was due to the growth and proliferation of bacteria, which may affect the growth and development of the shrimp at different stages. There are several reports that demonstrate that bacterial infection in larval shrimp leads to high mortality; however, none of these studies report red-pink colony-producing bacteria as the causative agent. For example, Hameed (1993) reported that *Vibrio* sp. are the dominant bacteria that infect shrimp eggs, larvae and post-larvae followed by *Pseudomonas*, *Alcaligenes, Aeromonas* and filamentous bacteria, such as *Flavobacteria, Leucothrix, Thiotrix, Flexibacter* and *Cytophaga* (Sunaryanto and Mariam, 1986; Hameed,

1993; Lightner, 1983 and Mouriño *et al*., 2008). Although some of these bacteria, such as *Pseudomonas*, produce water soluble and insoluble pigments (Holt *et al*., 1994; Buller, 2004), there are no reports to demonstrate that these mass mortality of shrimp during larviculture.

The objective of this study was to identify the causative agent of mass mortality in zoeal to post-larval shrimp raised in rearing tanks of hatcheries in south Iran. We describe the clinical status of the affected rearing tanks and the isolation, biophysical characterization and pathogenicity of *Pseudomonas* sp., which we identified as the bacterial agent responsible for the mass mortality of shrimp.

Materials and Methods

Sample collection

During three consecutive months, 118 samples of nauplii and 56 shrimp at zoeal stage 1 were collected from an affected hatchery located in the province of Bushehr. The larvae were transferred to sterile screw-capped bottles containing sterile seawater and transported to a diagnostic laboratory. The samples were aseptically homogenized, and 5 µL of each sample was inoculated onto marine agar and incubated at 28°C for 48 h. Samples of zoea and nauplii were quantified according to Ossiander and Wedemeyer at a 2% prevalence rate (OIE, 2003).

Phenotypic and molecular characterization

During the three month collection period and upon bacteriological analysis, four different bacterial colonies of different colors (white, orange, yellow and red) were identified on marine agar (Fig. 1a). Because the rearing tanks with the affected larvae typically develop a red-pink pigment on the tank floor, we isolated and subcultured the red-pink colonies on marine agar in order to obtain a pure culture (Fig. 1b). The bacteria from the pure culture were Gram stained and used for further phenotypic and molecular characterization as described below.

Phenotypic characterization

Phenotypic characterization of the isolated red-pink pigment-producing bacteria was performed according to the criteria described by Balebona et al. (1998; Table 1). In order to obtain a more accurate identification of the isolated bacteria at the species level, an extended phenotypic characterization was undertaken (Table 2).

Molecular assays

To confirm that the isolated bacteria belonged to the genus *Pseudomonas*, we performed PCR assays as described by Purohit, Raje and Kapley (2003). Briefly, primers (forward 5'-CTACGGGAGGCAGCAGTGG-3'; reverse 5' TCGGTAACGTCAAAACAGCAAAGT-3') were used at a concentration of 25 pM in a 25 µL reaction volume (2.5 µL 10X PCR buffer, 1 µL $MgCl_2$ (50 mM), 0.75 µL dNTP (10 mM), 1 µL of each primer, 0.3 µL Taq polymerase (10 Iu), 16.45 µL distilled water, 2 µL DNA template; boiling method). The PCR conditions were as follows: 95°C for 5 min, followed by 35 cycles of 94°C for 30 s, 62°C for 15 s and 72°C for 30 s.

Table 1: Some biochemical characteristics of red-pink colony bacteria isolated from *L. vannamei* hatchery *comparing with other genera of Vibrio, Aeromonas, Photobacterium, Cytophaga and Pseudomonas* (Balebona *et al., 1998*).

Feature	A	B	C	D	E	F
Bipolar staining	–	–	+	–	–	–
Motility in liquid	+	+	–	–	+	+
Gliding motility	–	–	–	+	–	–
Polar flagellum	+	–	–	–	+	+
NaCl required for growth	+	–	+	V	V	–
O/ F test	F	F	F	F	O	–
Gelatinase	V	+	–	V	V	–
Growth on TCBS agar	+	–	–	–	–	–

A= Vibrio, B= Aeromonas, C= Photobacterium, D= Cytophaga, E= Pseudomonas, F=Test isolate, V=Variable, F=Fermentative, O=Oxidative.

Table 2: Phenotypic features of red-pink colony producing bacterium isolated from *L.vannamei* larvae compared with *P. anguilliseptica* and *P. mesophilica* (Michel *et al*., 1992; Berthe *et al*., 1995; Buller, 2004).

Feature	Test isolate	*P. anguilliseptica*	*P. mesophilica*
Gram reaction	–	–	–
Cell shape	coccobacill	rod	rod
Polar flagella	one	one	
Oxidase	+	+	+
Catalase	+	+	+
Motility	+	+	+
Pigment produced	red-pink		coral-pink
Lysine decarboxylase	–	–	
Nitrate reduction	–	–	variable
Indole		–	–
Citrate	–		–
Methyl red	–		
Voges-proskauer	–		
Gelatinase	–	variable	–
Oxidative /fermentative	-	inert reaction	oxidative
Hydrogen sulphide	–	–	–
Triple sugar iron agar	alkaline/alkaline		
Glucose	–	–	weak
Arabinose	–	–	
Inositole		–	
Lactose	–	–	–
Maltose	–	–	–
Manose	–	–	–
Salicin	–	–	
Sorbitol	–	–	
Growth in TCBS agar	–	variable	
Growth at 0- 40°C	+	5-30	25-35
Growth in NaCl (0-10%)	–	0-3	–

Confirmation of red-pink pigment production by bacteria in the water column and at the bottom of the tanks

Two red colonies of the isolated bacteria were inoculated into 200 mL sterile plastic vessels containing 80 mg of sterile nutrient broth dissolved in 100 mL of

sterile seawater, with a salinity of 30 g/L, and incubated at 28°C for 24 h. Three replicates of this culture were used for each trial. The production of pigment by the bacteria was analyzed at 12 and 24 h post-inoculation by gross observation of a red-pink color in the water column or at the bottom of the containers.

Pathogenicity assays

To determine the virulence level of the isolated red-pink pigment-producing bacteria, we performed bioassay experiments using nauplii and post-larvae of *L. vannamei.*

Healthy stage 4 nauplii (n=150) were aliquoted in triplicate into 3-L sterile vessels containing either diluted sterile marine broth or sterile seawater at 28°C. The nauplii were then challenged with a fresh culture of bacteria (grown for 24 h) at a final concentration of 1.5×10^5 CFU/mL. Control groups of nauplii were also cultured under the same conditions but without bacteria. Mortality of the nauplii was recorded daily, and the production of red-pink pigment was monitored.

Healthy stage 2 post-larvae (n=70) were aliquoted in triplicate into 3-L sterile vessels at 28°C. The post-larvae were then challenged with a fresh culture of bacteria (grown for 24 h) at final concentration of 2×10^5 CFU/mL. Control groups of post-larvae were also cultured under the same conditions but without bacteria. Clinical signs and mortality of the post-larvae were monitored and recorded daily. The cause of mortality was confirmed by Gram staining of bacterial samples and by confirming the appearance of red-pink pigment-producing bacteria upon culture of the dead post-larvae on marine agar.

Statistical analysis

All of the data were statistically analyzed using the Student's t-test or the one-way ANOVA followed by a Tukey test. P-values of <0.05 were considered to be statistically significant.

Results

Phenotypic features of isolated bacteria

The phenotypic characteristics of the isolated red-pink pigment-producing bacteria are shown in Tables 1 and 2. According to Balebona *et al*. (1998), these bacteria are Gram-negative, motile coccobacilli with a single polar flagellum and do not have the ability to grow on thiosulphate citrate bile salts sucrose (TCBS.) The bacteria that we isolated produced red-pink colonies on marine and nutrient agar (Fig. 1a and 1b), did not perform oxidation or fermentation, lacked the enzyme gelatinase and were unable to grow in media containing less than 5% seawater. In addition, the bacteria did not grow in media containing only NaCl. Furthermore, we found that the isolated bacteria shared many characteristics with members of the genus *Pseudomonas*, particularly with *P. anguilliseptica* and *P. mesophilica* (Michel *et al*., 1992; Berthe *et al*., 1995; Buller, 2004; Tables 1 and 2)

Molecular characteristics

PCR analysis of the bacteria revealed the production of a 150-bp product, consistent with the genus *Pseudomonas* (Fig. 2). The expected 150-bp product obtained from the *Pseudomonas* DNA control confirmed the specificity of the primers.

Confirmation of red-pink pigment production by bacteria in the water column and at the bottom of the tanks

After culture in sterile seawater, the isolated bacteria produced a red-pink pigment 16 h post-inoculation. In addition, the bacteria grew to a density of 2.1×10^5 CFU/mL of seawater without any contamination (Fig. 3). Moreover, the red-pink pigment produced by the bacteria settled at the bottom of vessels (Fig. 3).

Bacterial pathogenicity in nauplii

The level of mortality in larvae challenged with bacteria in marine broth reached 100% within 24 h post-challenge. In contrast, the control group cultured without bacteria exhibited only 19% mortality after nine days of culture ($p < 0.05$; Fig. 4). The affected larvae exhibited acute necrosis of the entire body, as observed by lysis of all tissue cells 24 h post-challenge (Fig. 5). The level of mortality in larvae challenged with bacteria in the absence of marine broth reached 81.1% within 48 h post-challenge, while the control group cultured in sterile seawater without bacteria exhibited only 5.5% mortality after nine days of culture ($p < 0.05$; Fig. 4). Acute necrosis and lysis of all tissue cells was also seen in the larvae exposed to bacteria in the absence of marine broth (Fig. 6). In addition, both the dead nauplii and the tank water exhibited a red-pink color 24 h after challenge. In contrast, no color change was observed in the control group.

Bacterial pathogenicity in post-larvae

The level of mortality in post-larvae challenged with bacteria in the absence of marine broth reached 100% 24 h post-challenge, while no mortality occurred in the control group ($p < 0.05$). In addition, the affected post-larvae exhibited acute necrosis and lysis of tissue cells 24 h post-challenge (Fig. 6). As observed in nauplii, the dead post-larvae and the water in the holding tanks exhibited a red-pink color (Fig. 7).

Discussion

Some bacterial genera, including Vibrio (e.g., *V. harveyi*, *V. parahaemolyticus*, *V. alginolyticus*, *V. vulnificus*, *V. fluvialis and V. anguillarum*), Aeromonas,

Figure 1: (a) Production of red-pink pigment on marine agar by the Pseudomonas sp. isolated from affected rearing tanks; (b) purified red-pink pigment-producing bacteria after subculturing on marine agar for 24 h at 28°C.

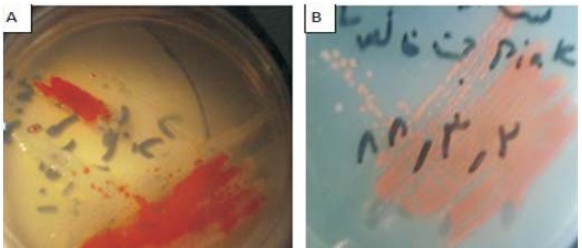


Figure 2: Amplification of the PCR products from the DNA template of Pseudomonas sp. isolated from shrimp hatcheries. Lanes 1-2: positive control (*Pseudomonas* sp.); lanes 3-4: test bacteria; and lane 5: 100-bp ladder.

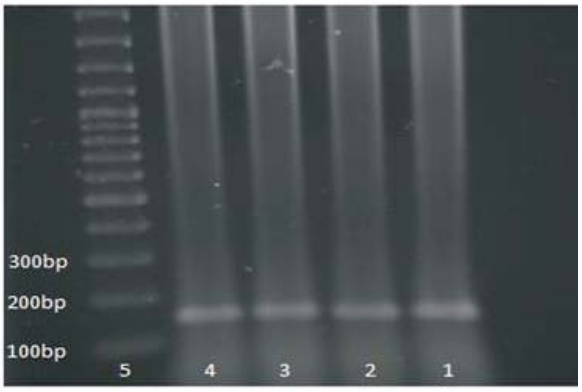
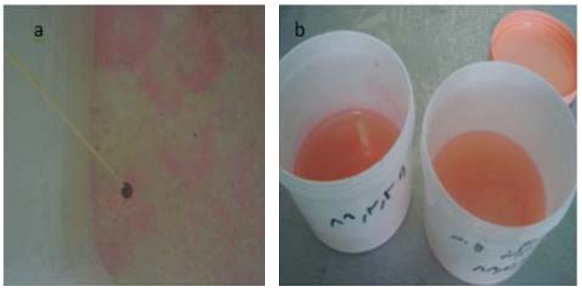


Figure 3: (a) Sedimentation of red-pink pigment at the bottom of the tanks from affected hatcheries; (b) Production of red-pink pigment by isolated Pseudomonas sp. inoculated into seawater at 28°C.



Pseudomonas and filamentous bacteria, such as Flexibacter, Cytophaga and Flexitrix, have been shown to cause mortality of shrimp raised in hatcheries (Sunaryanto and Mariam 1986; Baticados *et al*., 1990; Lavilla-Pitogo *et al*., 1990; Karunasagar *et al*., 1994, Lightner, 1983, Mournio *et al*., 2008). In terms of *Pseudomonas,* however, little is known regarding its ability to cause such mortality. There is also no such data available for the pathogenic red-pink pigment-producing Pseudomonas found in shrimp hatcheries. Here we show that the phenotypic and molecular

Figure 4: Pathogenicity of Pseudomonas sp. in *L. vannamei* nauplii. G1: nauplii inoculated with bacteria prepared in marine broth; G2: nauplii prepared in sterile marine broth (control group); G3: nauplii inoculated with bacteria prepared without marine broth; and G4: nauplii prepared in sterile seawater (control group). Mean ± SE, n=150, three replicates.

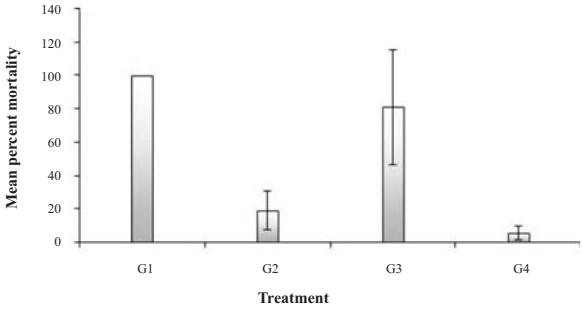
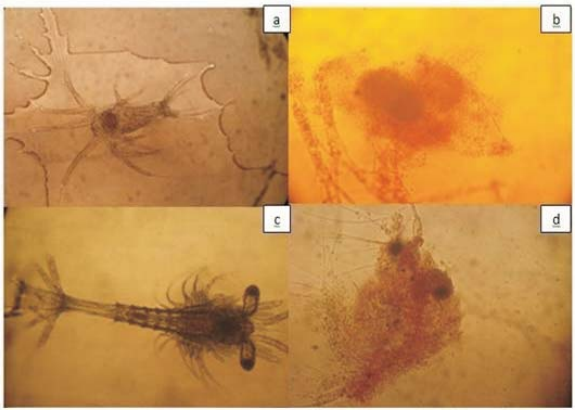


Figure 5: *L. vannamei* nauplii challenged with *Pseudomonas* sp. (a) Nauplii prepared in marine broth without *Pseudomonas* sp. (control); (b) nauplii challenged with Pseudomonas sp. in sterile seawater and marine broth exhibit general acute necrosis and a red-pink color throughout the body; (c) larva of *L. vannamei* nine days post-culture (control group); (d) *L. vannamei* challenged with *Pseudomonas* sp. for 48 h in seawater without marine broth.



features, pathogenicity and production of red-pink pigment are consistent with *Pseudomonas* sp. infection and confirm that this organism is the main cause of mortality of *L. vannamei* raised in hatcheries in south Iran. We showed that *Pseudomonas* sp. produced a red-pink pigment in the water column in the presence or absence of larval or post-larval shrimp, which indicates that the water column contains the necessary, albeit unknown, elements required for its growth and reproduction. Nauplii, zoea, mysis and post-larvae exposed to the bacteria failed to survive, whereas control organisms that were not exposed to the bacteria did not exhibit any mortality.

It is important to note that the source of the bacteria is unknown. It may enter the hatcheries via transportation of brood shrimp within the hatchery, although the farmers usually disinfect the brood

Figure 6: *L. vannamei* post-larvae challenged with *Pseudomonas* sp. at 28°C. (a) Control group; (b, c and d) test groups showing acute necrosis 24 h post-challenge with *Pseudomonas* sp.

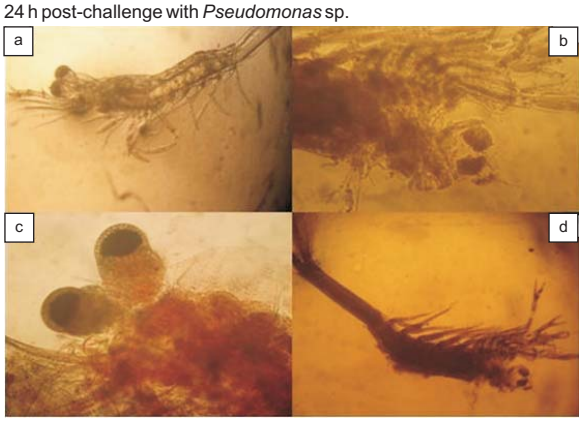
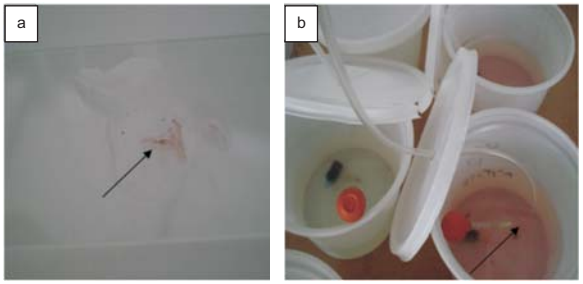


Figure 7: *Pseudomonas* sp. produces a red-pink pigment at the bottom of the shrimp rearing tanks.



shrimp, with formalin for example, prior to moving them within the hatchery. Another possibility is that the food used to nourish the brood shrimp may be contaminated with bacteria, which act as a vector for the transmission of infection. This situation is further aggravated when shrimp hatcheries do not follow the proper health criteria during the spawning and production of shrimp larvae. In addition, shrimp handlers may also be vectors for transmission of infection within the hatcheries. Epidemiological studies performed in south Iran have demonstrated that those hatcheries with adequate sanitary conditions do not see infection or mortality of their shrimp cultures. Therefore, improving the health criteria, particularly with respect to sanitary conditions, can eliminate infections in shrimp hatcheries. In particular, it is important that the inlet water source is properly disinfected.

Pseudomonas is a chitinoclastic microorganism that can invade chitin-based substances including the shrimp cuticle, causing degeneration and hydrolysis (Getchell, 1989; Gooday, 1990; Kobayashi *et al*., 1995; Zhou *et al*., 1999 and Vogan *et al*., 2002). Our experiments show that *Pseudomonas* sp. cause acute necrosis and hydrolysis of the larvae and post-larvae shrimp, which indicates that it is highly pathogenic. In addition, following extensive damage to the cuticle, crustaceans can die as a result of unsuccessful molting (Smolowitz *et al*., 1992). Molting normally occurs every 6-24 h in shrimp larvae, so interference with this process can lead to death (Smolowitz, 1992).

Most *Pseudomonas* sp. can produce extracellular products, such as lipases and proteolytic enzymes, which cause the degradation of connective tissues (Franzetti and Scarpellini, 2007). For instance, injection of *Pseudoalteromonas atlantica* and *P. aeruginosa* lipopolysaccharides into the edible crab *Cancer pagurus* causes 100% and 80% mortality, respectively, 60-90 min postinjection (Costa-Ramos and Rowely, 2004). It is likely that the acute necrosis that we observed in affected larvae and post-larvae shrimp may be mediated, in part, by such extracellular toxins. However, it is not clear whether the red-pink pigment produced by *Pseudomonas* sp. is involved in its pathogenicity and, therefore, further studies are warranted in this field.

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