Multiple infections in shrimp *Litopenaeus vannamei* broodstock in commercial hatcheries in Khouzestan Province

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Abstract

The present study was carried out to investigate the status of health and disease, and their impact on shrimp broodstock (*Litopenaeus vannamei*) in Khouzestan Province (Choeibde Area) in the south of Iran. From March 2012 until April 2013, about 140 broodstock and 5000 postlarvae (PLs) were collected from hatcheries and grow out farms. Clinical signs of samples were documented in take history forms and then the samples were transported to Iranian South Aquaculture Institute (Ahvaz). Bacterial and fungal studies were carried out on hemolymph, hepatopancreas and gill tissue and then the shrimps were preserved in Davidson Fixative for histopathology. A part of uropods was also preserved in ethyl alcohol for PCR study and detecting three viruses, WSSV, TSV and IMNV. A part of PLs was also preserved in ethyl alcohol for PCR and the remaining was preserved in Davidson Fixative for histopathology. The results showed that 5 bacteria consisting of *Vibrio alginolyticus, V. proteolyticus, V. mimicus, A. hydrophila* and *Plesiomonas shigelloides* and one fungi *Aspergillus fumigatus* were identified. The results of PCR exhibited that the broodstock was free of the three viruses and the PL was infected with WSSV. In histopathology some tissues showed the effects of *Vibrio* infection in different organs such as gill and midgut, and the infection of PL tissues showed the Cowdry type A inclusion bodies in WSSV. Two specific signs of abnormality were also exhibited in histopathology that we call them Pseudo inclusion and Reolike particles and we need to conduct a new study to clarify detailed information about these finding. This finding can be used for assessing the health of shrimp culture and prevention of disease in broodstock in Iran.

Keywords: Shrimp broodstock, Health, Disease, Histopathology, PCR

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Introduction

World shrimp productions in the world is growing steadily and continued supply of good quality brooder is one prerequisite for successful operation of commercial hatcheries. As mentioned by FAO (2003) process of broodstock is divided into two broad categories: pre-spawning process and post-spawning process. As recently as a decade ago, much of the world's production of farmed shrimp was directly or indirectly dependent on brood stock shrimp for the "seed" stock used to populate its farms (Lightner, 2011a). The pre-spawning process includes procedures for broodstock selection, screening for disease, maintenance, maturation, acclimatization, spawning, and hatching. As these procedures require different facilities, the facility maintenance guidelines are described under different specific facilities used in the hatchery production process. The main challenge for shrimp broodstock in the future is disease and the culturists annual loss of 22% of shrimp production in the world (Valderrama and Anderson, 2011). With respect to disease agents, 60% of losses were attributed to viruses and about 20% to bacteria. Thus, the majority of our effort on disease control (80%) should clearly be focused on viral and bacterial pathogens (Flegel *et al*., 2008a). The main viral diseases in shrimp brooder are White spot syndrome virus (WSSV), Tura syndrome virus (TSV), Infection myonecrosis virus (IMNV) that have induced huge infections in the stocks. Among bacterial agents, that cause serious loss in shrimp culture as well as in brooders, the best known is *Vibrio* spp., because of the devastating economic effects they have on affected farms and broodstock (Lightner, 2005). White spot disease caused by WSSV has emerged in east Asia in 1992–1993 and it was quickly dispersed with infected seed and broodstock across the Asian continent to SE Asia and India, where it caused a major pandemic and continues to cause significant losses in some regions (Flegel, 2006; Lightner, 2011b).White spot syndrome virus is highly virulent in shrimp farms and can spread quickly and cause up to 100% mortality within 3-7 days (Flegel, 2006; Lightner, 2011b). The virus is very large, enveloped, double stranded DNA (dsDNA) and assigned by ICTV to a new genus *Whispovirus* and is belonged to Nimaviridae family (Walker and Mohan, 2009; OIE, 2010; Lightner, 2011b). White spot syndrome virus infections in penaeid shrimps typically cause lethargic behavior in affected animals, cessation of feeding, followed by the appearance of moribund shrimp swimming near the surface at the edge of pond within a few days (Wang *et al*., 2002; Afsharnasab, 2007; Walker and Mohan, 2009; Afsharnasab, 2012). The histopathology of WSSD in shrimp is dominated by presence of large conspicuous intranuclear eosinophilic cowdry type-A inclusion bodies in the tissue. The tissue section of affected shrimp stained with H&E/Phloxine showed the intranuclear eosinophilic Cowdry type-A inclusion bodies in the gill, midgut, cuticular epidermis, lymphoid organ, hematopoietic tissue, cecum, heart and

connective tissue (Afsharnasab, 2007; Lavilla-Pitogo *et al*., 2007; Meng *et al*., 2009). Taura syndrome is caused by the TSV, a single-stranded RNA virus in the family Picornaviridae. Taura syndrome generally occurs over the course of a single molt in juvenile shrimp, and may have a sudden onset within 5-20 days or a more chronic course of several months. Signs of infection include weakness, a soft shell, an empty digestive tract, and diffused expansion of red chromatophores in the appendages. Mortality can vary from 5-95 percent (Lightner, 2004; Afsharnasab *et al*., 2011; Aranguren *et al*., 2013). In histopathology TSV infections revealed that the epithelium in all tissues such as cuticular epidermis and stomach are separate from cuticular epidermis, and there is a free space between cuticular epidermis and epithelium (Lightner *et al*., 1997; Flegel *et al*., 2004 Afsharnasab, 2007). The cells of epithelium are swollen and hypertrophied and show many spherical inclusion bodies ranging from 2 to 15 µm diameters that color in H&E staining from eosinophilic to basophilic. The infected cell in the lesion showpeppered or buckshot appearance. Within the lesions multiple basophilic spheres that represent intracytoplasmic inclusion bodies, pyknotic and karyorrhectic nuclei also are seen (Lightner, 1996; Flegel, 2006; Lightner, 2011b). The IMNV particles are icosahedral in shape and 40 nm in diameter, with the genome consisting of a single, double-stranded RNA (dsRNA) molecule. Based on these characteristics, IMNV is most similar to members of the Totiviridae (Flegel, 2006; OIE, 2006). In the gross sign large numbers of sick animals and significant mortalities in juveniles and subadults are reported, but the disease progresses to a chronic phase with persistent low-level mortalities (Poulos and Lightner, 2006; Afsharnasab, 2007). With histopathologic techniques the infected shrimps show~~ed~~ characteristics of coagulative necrosis of striated (skeletal) muscle fibers, often with marked edema among affected muscle fibers (Poulos and Lightner, 2006; Lightner, 2011b; Lightner *et al*., 2012). Vibriosis is one of the most prevalent shrimp diseases caused by bacteria belonging to the genus *Vibrio* as well as in hatcheries (Hosseini *et al*., 2004; Jayasree *et al*., 2006). The main *Vibrio* spp. reported from hatcheries and shrimp farms consists of *V. harveyi, V. splendidus, V. parahaemolyticus, V. alginolyticus, V. anguillarum, V. vulnificus, V. campbellii, V. fischeri, V. damsela, V. pelagicus, V. orientalis, V. ordalii, V. mediterranei, V. logei*e (Lightner, 1996; Soltani *et al*., 1998, Jayasree *et al*., 2006). Serious epizootics in penaeid shrimps in juvenile and adult stages are reported (Lightner and Redman, 1998). Diseased post larval shrimp display cloudiness of hepatopancreas while juveniles with infection display cloudiness of muscle in the sixth abdominal segment and broken spot in gill and lymphoid organ (Vaseeharan and Ramasamy, 2003; Jayasree *et al*., 2006). Histopathological study of affected shrimps by *Vibrio* showed extensive necrosis and bacterial invasion of the lymphoid organ, heart, gill, hepatopancreas, antennal gland, cuticular

epidermis and subcutis, and other connective tissues with multiple haemocytic nodules (Lightner, 1996; Hosseini *et al*., 2004). The aims of this study were to assess the viral agents, WSSV, TSV and IMNV and bacteria such as *Vibrio* and fungal agents in shrimp broodstock in Khuzestan Province, Iran.

Material and Methods

One hundred and forty shrimp broodstock of *L. vannamei* and 5000 PLs were collected from hatcheries in Khouzestan Province (Choibde area) during March 2012 until April 2013. The gross sign of samples were taken in farm history record and then the samples were transferred to South Aquaculture Research Institute (Ahvaz). After acclimation of the shrimp, haemolymph was obtained from ventral part of the haemocoel of the second abdominal segment, using a 25 gauge needle and an 1 ml syringe for bacterial and fungal studies as mentioned by Lightner (1996) and Soltani *et al*. (1998). The gill tissue and hepatopancreas were also examined for bacterial and fungal studies. A small part of samples (in the case of PLs, whole animals) were preserved in ethyl alcohol for PCR assay and detecting WSSV, TSV and IMNV according to ~~a~~ the guideline of IQ2000 kite (Afsharnasab *et al*., 2007). The samples also were fixed in Davidson's fixative for 48 hours, and transferred to 70% ethanol. After processing and hydration of the tissues, wax impregnation was done. The paraffin wax embedded samples were sectioned, mounted on slides, stained with Meyer-bennet haematoxylin and phloxine/eosin and viewed under the Nikon microscope according to Bell and Lightner (1998). Estimation of bacterial prevalence was calculate by the formula mentioned by de la Peña *et al*. (2008).

Results

Bacteriological studies

According to the diagnostic scheme for *Vibrio* species, and biochemical and physiological characteristics, which are presented in table 1, five *Vibrio* spp. were isolated from haemolymph, gill and hepatopancreas. The *Vibrio* spp. consisted of *V. alginolyticus, V. proteolyticus, Plesiomonas shigelloides, V. mimicus* and *A. hydrophila* isolated from gill and in the hepatopancrease only *V. mimicus and Plesiomonas shigelloides* were identified. Hemolymph did not show any bacterial agents (Table 2). As mentioned in Table 2 the prevalence of *V. mimicus* was 57% in hepatopancreas and 28.5% in gill. The prevalence of *Plesiomonas shigelloides* was seen to be 14.2% in gill and hepatopancreas, *V. proteolyticus, V. alginolyticus* and *A. hydrophila* showed a 14.2% prevalence respectively. In this study only one fungi (*Asp. fumigatus)* with 28.5% prevalence was identified.

Table 1: Phenotypic characteristics of the *Vibrio* strains evaluated in this study.

Test	Species				
	V. alginolyticus	***V. mimicus***	***V. proteolyticus***	***A. hydrophila***	***P. shigelloides***
Gram stain	G-	G-	G-	G-	G-
TCBS colony stain	Y	G	G	Y	G
Salt tolerance 0%	+	+	+	+	+
Salt tolerance 3%	+	+	+	+	+
Salt tolerance 6%	-	-	+	+	+
Salt tolerance 8%	-	-	+	+	+
Salt tolerance 10%	-	-	+	+	+
Arginine dihydrolase	-	-	-	+	-
Lysine decarboxylase	+	-	+	+	-
Ornithine decarboxylase	+	+	+	+	-
Oxidase	+	+	+	+	+
Lactase	-	+	-	-	-
Sacarose	+	+	-	-	-
Cellobiose	-	+	-	+	-
Inositol	+	-	+	+	-
Mannitol	-	-	-	-	-
Simon citrate	+	+	+	-	-
Nitrate	+	+	+	+	+
Gelatinase	+	+	V	-	+
MR-VP	+	-	V	+	_
O/129	+	-	-	-	_
Luminoscence	+	-	-	-	-

Table 2: Bacterial prevalence in different tissues.

***Bacterial* spp.**	**Tissues**		
	Gill	**Hepatopancreas**	**Hemolymph**
V. mimicus	40 (28.5%)	80 (57%)	-
P. shigelloides	20 (14.2%)	20 (14.2%)	-
V. alginolyticus	20 (14.2%)	-	-
V. proteolyticus	20 (14.2%)	-	-
A.hydrophila	20 (14.2%)	-	-

PCR studies

For detecting and prevention of WSSV, TSV and IMNV viruses we used IQ 2000 kit TM. The results showed, that the broodstock were negative for the three viruses (Figs. 1, 3 and 4). The PCR for WSSV just showed a 848 bp band and its housekeeping gene from shrimp, and showed control negative in the kit (Fig. 1). It means that 11 samples from the broodstock were negative for WSSV, but the PLs in the case of WSSV were positive and the samples exhibited 296 bp and 550

bp bands that means the PL samples were positive (Fig. 2). The results of PCR for detecting presence of TSV and IMNV are shown in Figs. 3 and 4, and a single band of 680 bp was observed indicating that the samples for TSV and IMNV were also negative.

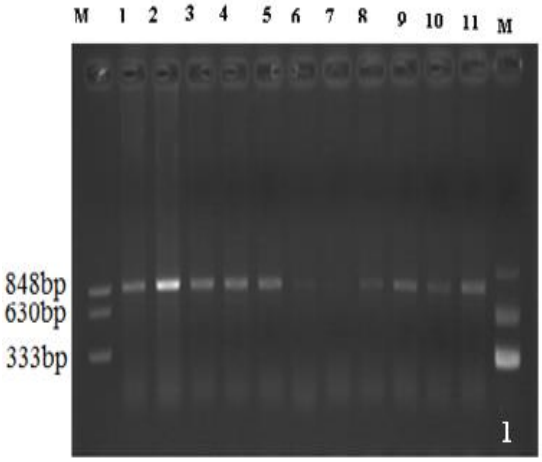

Figure 1: The results from IQ2000 TM for Detection and Prevention of WSSV kit in 11 samples of the broodstock from Khouzestan Province. The results showed that just 848 bp was amplified, and the 296 bp and 550 bp were not amplified that implicate that the samples were negative for WSSV.

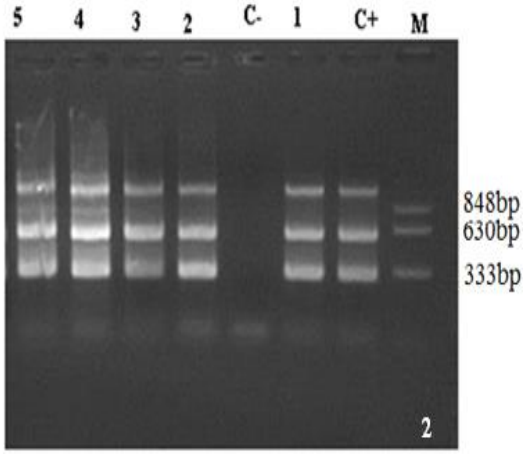

Figure 2: The result from IQ2000 TM for Detection and Prevention of WSSV kit in 5 samples of PLs were suspected to WSSV from Khouzestan Province. The results showed that the samples amplified 848 bp, 296 bp and 550 bp, it means that the PL samples were positive for WSSV.

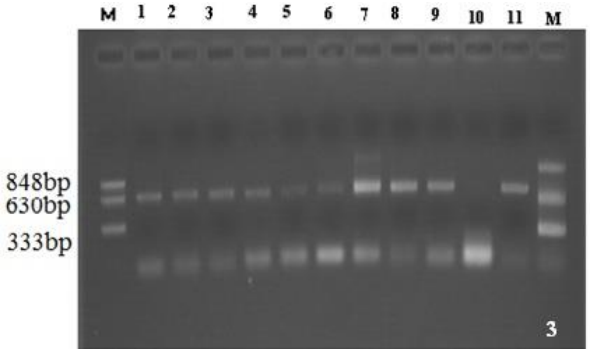

Figure 3: The results from IQ2000 TM for Detection and Prevention of IMNV kit in 11 samples of the broodstock from Khouzestan Province. The results showed that just 680 bp was amplified, and 255 bp and 510 bp were not amplified that implicate that the samples were negative for IMNV.

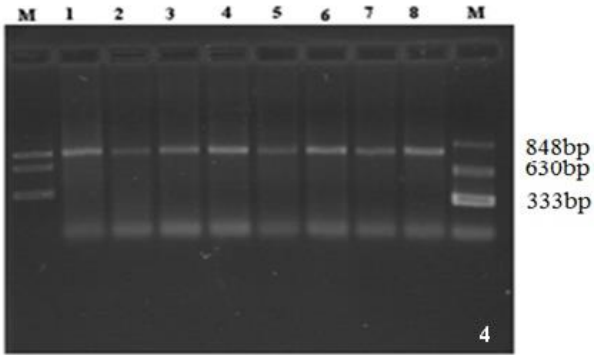

Figure 4: The results from IQ2000 TM for Detection and Prevention of TSV kit in 11 samples of the broodstock from Khouzestan Province. The results showed that just 680 bp was amplified, and 284 bp and 470 bp were not amplified that implicate that the samples were negative for TSV.

Histopathology results In histopathology the bacterial infection from *Vibrio* spp. was obvious. The gill samples of the broodstock showed melanization as well as in the secondary lamella (Figs. 5 a and b). This finding indicated that the shrimps are under stress and when stress is increased, the symptoms of disease would appear. The bacterial infection in mussels (arrow) (Fig. 5c) and midgut (arrow) were seen and the epithelium is separated from the midgut wall (white arrow) (Fig. 5d). Granulomatosis (black arrow) is also seen in some tissue (Fig. 5e), and with high magnification the bacillus form bacteria (arrow) with flagellum tail (white arrow) appeared clearly in the tissue (Fig. 5f).

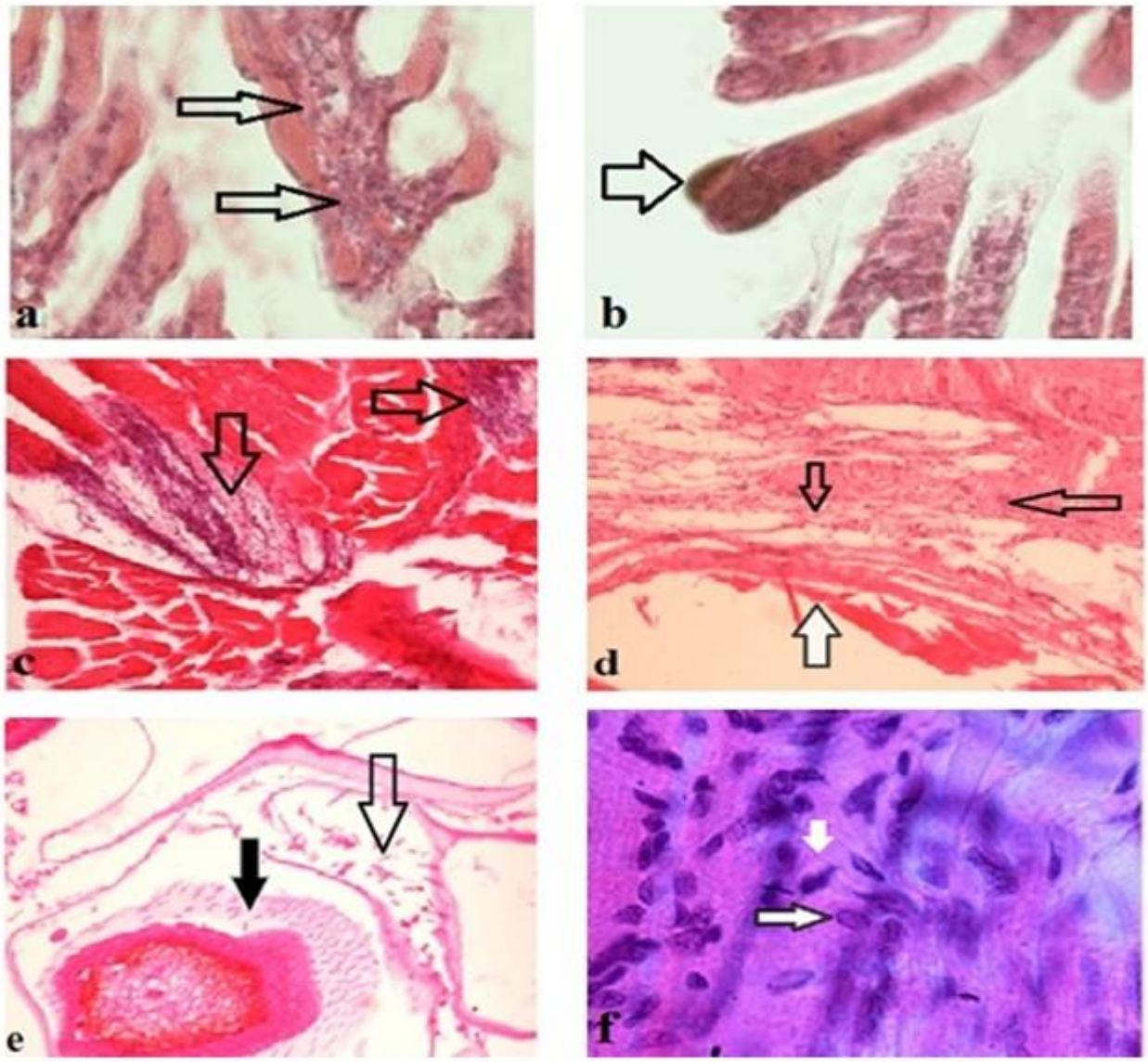

Figure 5: Bacterial infection in shrimp broodstock with *Vibrio* spp. and the secondary lamella exhibited melanization and appeared black (arrow) (Figes 5-a, 5-b) H&E/Phloxine: X400. Aggregation of bacteria was seen in muscle (Fig. 5 c) (arrow) and midgut wall (Fig. 5d) (arrow), and in midgut the epithelium was separate from the wall (white arrow) (Fig. 5d) H&E/ Phloxine: X400. With high magnification granulomatosis is seen in the tissue (arrow) (Fig. 5e), and the bacillus form bacteria (arrow) with large tail are seen in the tissue (Fig. 5f) H&E/ Phloxine: X1000.

Samples of PL showed histopathological evidence of WSSV. Cowdry type A inclusion bodies was seen in hypoderm and interstitial hepatopancreas cell (Figs. 6a and b). The infected cells also showed different stages of infection, hepatopancrease was swollen and cells showed vacuoles in different sizes (Figs. 6b and c). Abdominal muscle also showed multiple inclusion bodies and the cells were destructed (Fig. 6d), this is the main reason that shrimp died during this study in Khouzestan Province.

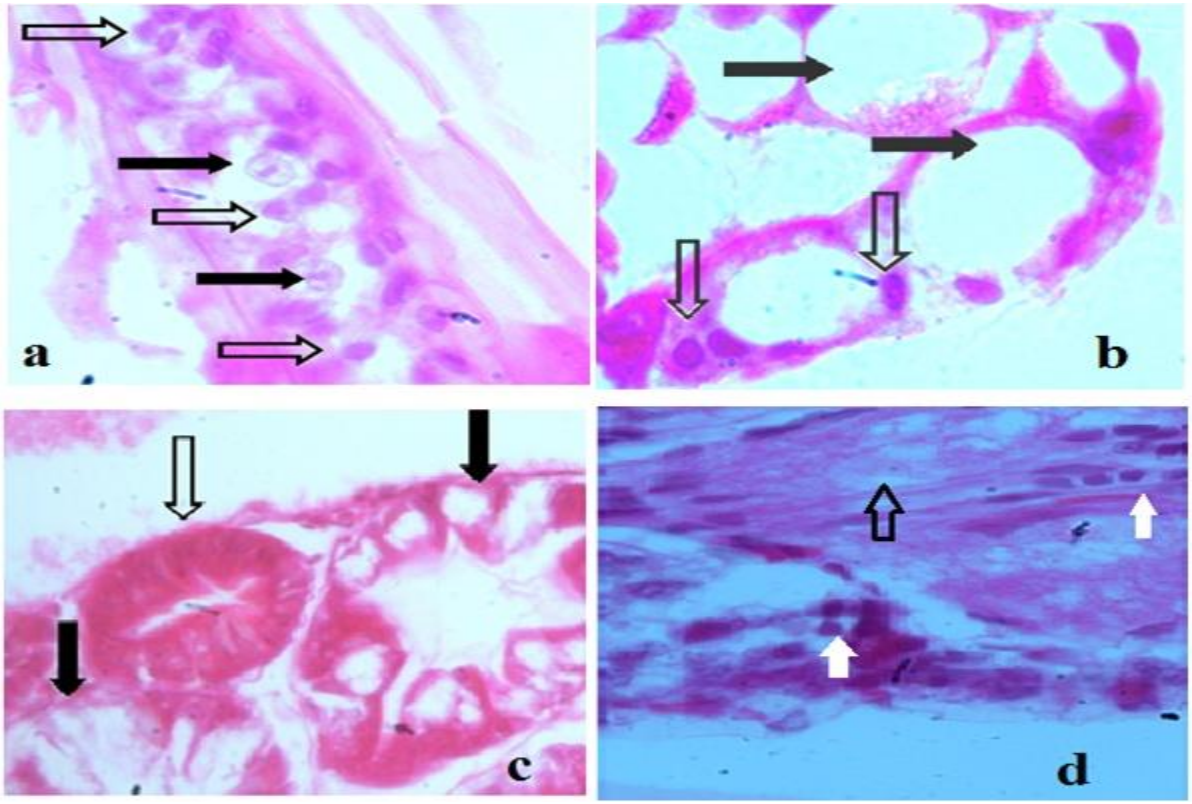

Figure 6: Observation of Cowdry type A inclusion bodies in tissue of PLs of *L. vannamei* from Khozestan Province. Cuticular epithelium (Fig. 6a) showed Cowdry type inclusion bodies in late stage (arrow) and in early stage (black arrow) H&E/Phloxine: X1000. The inter-tubular space of hepatopancreas showed late Cowdry type A inclusion bodies (arrow) in PL samples with basophilic color (Fig. 6b) H&E/Phloxine: X1000. Fig. 6c showed the comparison of the two parts of hepatopancreas, left was not infected (arrow) while right part ~~is~~ was vacuolated and infected (black arrow) H&E/Phloxine: X1000. Abdominal muscle was also infected and exhibited ~~the~~ many Cowdry type A inclusion bodies (arrow) (Fig. 6d) H&E/Phloxine: X1000.

During this study we identified Pseudo Like Inclusion (PLI) in the hepatopancreatic tissue. These samples were negative in PCR for WSSV by IQ 2000 kit and the PLI were just found in the hepatopancreas cells. The figure of PLI is like WSSV inclusion but there were no sign of WSD in the samples (Fig. 7). In part of samples, the cells of hepatopancreas also showed the Reolike particles (RLP). The evidence of this sign (RLP) is the first report from Iranian shrimp aquaculture. In these cells of the

hepatopancreas, large basophilic inclusion bodies were consisting of many basophilic granules and the appearance of RLP was the net shape (Fig. 8).

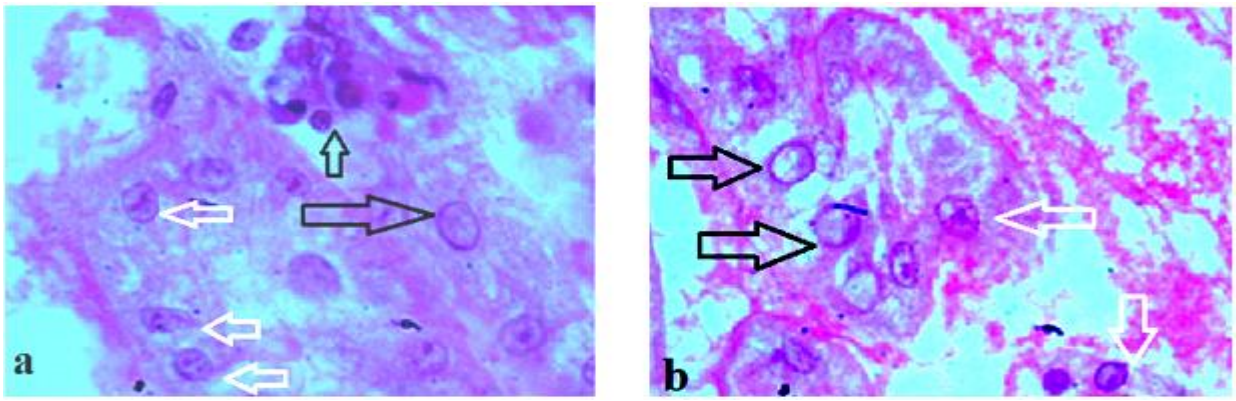

Figure 7: Observation of Pseudo Like Inclusion (PLI) in the hepatopancreas of the shrimp, *L. vannamei*, from Khouzestan Province. Fig. 7 a and b show the cells of hepatopancreas in suspected shrimp (arrows) and the hypertrophy of the infected cells (white arrow) was obvious H&E/Phloxine: X1000.

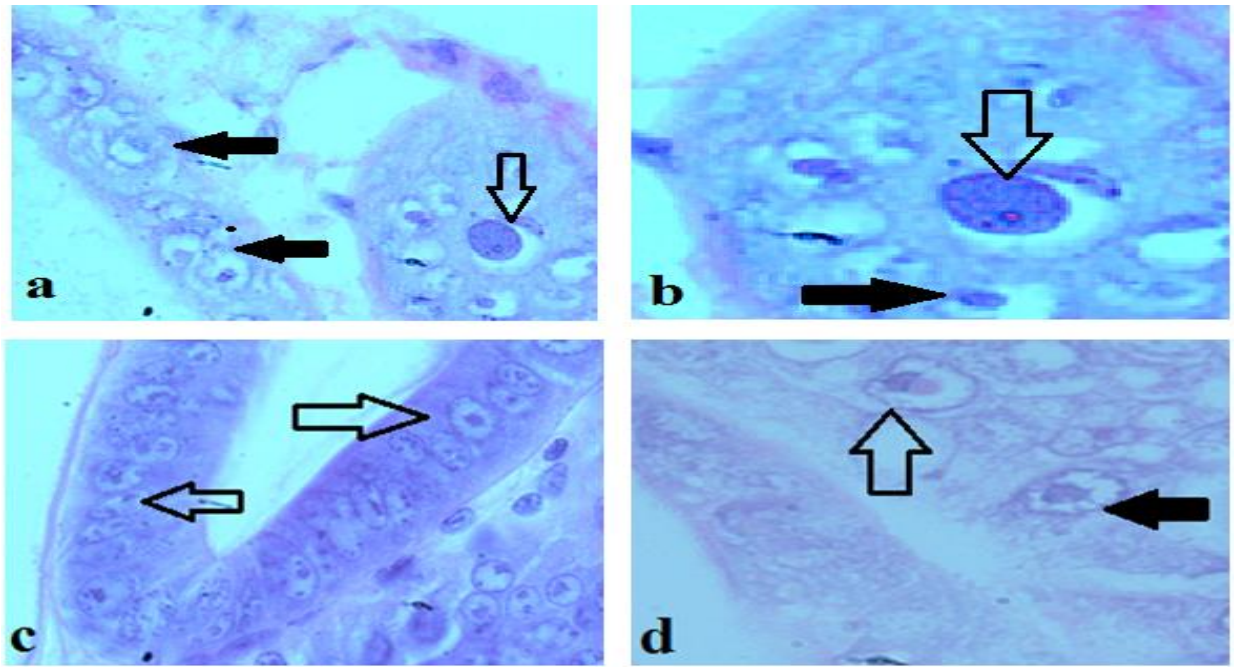

Figure 8: Observation of Reo like Particles (RLP) in hepatopancreas cells. The cells showed hypertrophy (black arrow) and net appearance (arrow) (a and b) H&E/Phloxine: X1000. In Fig. 8c and d abnormality of hepatopancreas cells also indicate the chromatins of cells which also immigrate and bind to RLP (arrow) H&E/Phloxine: X1000.

Discussion

Results obtained in the present study showed that the bacteria isolated in the broodstock of the shrimp, *L. vannamei*, was much lower than that of previous studies have done by researcher in this area. As mentioned by Mortezaei *et al*. (2008) and Afsharnasab *et al*. (2009b) the identified bacteria were 15 spp., while in the present study we found 5 bacteria of them. Among the bacteria isolated in the present study *V. alginolyticus, V. proteolyticus, P. shigelloides, V. mimicus* and *A. hydrophila* were similar with the previous research findings, however the bacteria *V. splendidus, Pasteurella, V. anguillarum, V. vulnificus, V. pelagicus, V. nereis, V. gazogenes, V. campbellii, V. parahaemolyticus, V. flavious* were not found in this study. The fungi reported in this study was *A. fumigatus,* on the contrary of the previous researchers (Mortezaei *et al*., 2008; Afsharnasab *et al.*, 2009b) that found 6 spp. of the following fungi *Aspergillus niger, A. fumigatus, Cladosporium* sp., *Fusarium* sp., *Trichophyton* sp., *Penicillium* sp. in this area. The difference between results of Mortezaei *et al*. (2008) and Afsharnasab *et al*. (2009b) and those observed in our study may be due to biosecurity status in broodstock production and the prevention methods used in this area during 2008 and 2009, and also the time that we examined the shrimp broodstock. Vibriosis has been implicated as the cause of major mortality in shrimp farms (Lightner and Redman, 1998). *Vibrio spp*. are among the normal bacterial flora of both natural and cultured populations of shrimp in the culture environment (Hosseini *et al*., 2004; Jayasree *et al*., 2006), but often act as opportunistic or secondary pathogen that can cause mortality ranging from a few to 100% in under stress affected populations (Lightner and Redman, 1998; Rahimi *et al*., 2010). The present study did not observe any mortality due to vibriosis and as Flegel and Alday-Sanz (1998) mentioned the mainspring use the biosecurity protocol in broodstock farms. In our study histopathologic observation of shrimps infected by *Vibrio* was similar to *Vibrio* reports of Lightner (1996) and Mohajeri *et al*. (2011) in USA and Iran. We found melanization in gill and separated epithelium layer in the midgut, these findings are similarly described by Lightner (1996) and Mohajeri *et al*. (2011).

In other parts of Iran, identification of *Vibrio* spp. is done. Soltani *et al*. (1998) identified *V. parahaemolyticus, V. alginolyticus, V. marinus, V. mimicus, V. vulnificus* and *V. fluvialis* in shrimp farms in Bousher Province, South of Iran. The diseases and health status in Tiab area in shrimp farms of Hormozgan Province were studied (Salehi, 1999). He found and identified *V. anguillarum, V. parahaemolyticus, V. alginolyticus* and *Aeromonas hydrophila* in this area. In comparison of the other's results and ours, the identified variation in *Vibrio* species, could be due to environmental factors, management methods, preventive protocol and feeding habitats.

The PCR results examined by IQ 2000 kit for detection of WSSV, TSV and IMNV

in broodstock of *L. vannamei* collected in Khouzestan Province was negative, while PCR showed that WSSV was detected in the PLs collected in this area. As mentioned by Flegel *et al*. (2008b) shrimps interact with bacterial and fungal pathogens and many new shrimp genes have been discovered, and there is a hope that some of these will lead to new products for disease control. One group may be apoptosis and another group viral accommodation. These factor improved by growing shrimp and this may be the fact that explains why shrimp broodstock were negative in the PCR while PLs were positive. Work on this area of research is just beginning, but it is developing rapidly and many interesting discoveries have been made that may lead to development of new disease control mechanisms.

The PLs of *L. vannamei* which were collected from hatcheries of Khouzestan Provinces showed typical symptoms of white spots on histopathology described by several researchers (Chou *et al*., 1998; Wang *et al*., 1999; Hossain *et al*., 2004), but the shrimps broodstock were eating normally and no death had occurred, which may be caused by acclimation of the host with the virus (Afsharnasab *et al*., 2009a). Post larvae had low mortality due to medium virulence virus but there were no signs of white spots on their carapace. The other signs were eating reduction and emptiness of their intestines, increased lethargy, swimming slowly near the tank surface and reddish body discoloration on the moribund PLs which were similar to the report of Momoyama (2003). However, positive PCR IQ 2000 samples indicated histopathological lesions due to the white spot virus. Results obtained from hatcheries and shrimp farms from Khouzeatan Province were similar to the results of previous studies. Virulence studies showed that broodstock of *L. vannamei* has little resistance to WSD, so when the virus has a high virulence, it could be associated with mass death (100%) (Lightner *et al*., 1997; Wang *et al*., 2002) but in this study were observed no deaths in samples which may be caused by medium virulence. As mentioned by Witteveldt *et al*. (2004) the interfere on factor play an important role in viral defense in shrimp broodstock of *L. vannamei* and this factor slowly increased with shrimp growth, but in the PLs could not find interfere on factor. This finding is significant among broodstock and PLs in WSSV outbreak. Histopathological studies implied the existence of intranuclear inclusion bodies Cowdry type A in cells of PLs which in advanced stages basophilic (H&E\Ph) was observed, similar results were reported by Kou *et al*. (1997); Hossain *et al*. (2004) and Lightner *et al*. (2012).

Two new finding in histopathology also were identified in the present study. Pseudo Like inclusion (PLI) was seen in the hepatopancreas cells of the broodstock, and in these cells chromatin margination and prominent basophilic pseudo inclusion was observed in the basophilic cells. This PLI is similar to inclusion bodies in WSSV (Flegel, 2006; Lightner, 2011a,) and IHHNV (Nunan *et al*., 2001; Garcia-Orozco *et al*., 2012). But shrimps infected by WSSV produced gross sign of white

spot in the carapace and midgut and the inclusion bodies distributed in all tissues except hepatopancreas cells (Wang *et al*., 2002; Pazir *et al*., 2011) and the IHHNV showed white spot in the body surface and inclusion bodies observed in gill tissue (Martorelli *et al*., 2010; Pazir *et al*., 2011; Lightner, 2011b), while the shrimp with PLI did not show any clinical sign of WSSV and IHHNV. The differences between our study and the others may be due to different strains of pathogen or different strains of virus or the resistance of shrimp to pathogens. Another finding in histopathology was Reo-Like Particles (RLP) in shrimps examined in this study. Reo-like virus infection in every reported case, were found in shrimps with mixed infection by other viruses such as MBV (Nash *et al*., 1988) or other serious syndromes (Lightner, 1996). Histological lesion of shrimps infected with reo-like particles were consistent with Nash *et al* (1988) and Lightner (1996) who observed degeneration and necrosis of hepatopancreatic epithelial cells involving the F or R type cells (Lightner, 1996). These observations were similar to our finding for RLP. In this regard it's necessary to check the broodstock for this infection, before being introduced to the hatcheries using modern and accurate techniques such as PCR.

Regarding sustainable aquaculture in Iran, we recommend that regulations are required to control the broodstock, control the water inlet and outlet, establish SPF broodstock in order to hatch disease free PLs, develop rapid diagnostic methods and educate the shrimp culturist.

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