Rate (ROI) and severity (SOI) of infection of white spot disease in cultured and captured Penaeid shrimps in the Persian Gulf using histopathology and polymerase chain reaction

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Abstract

White Spot Disease (WSD) is well known as a widespread viral disease in shrimps from 1992. Many studies focused on morphological, histopathological and epidemiological characteristic and pathogenecity of the disease but less on the determination of the severity of WSD using the histopathological features in target tissues. A generalized scheme for assigning a numerical qualitative value to severity grade of infection considering to histopathology and counting the inclusion bodies in different level of infection and different microscopic fielding immersion lens was accomplished before. This study was conducted in order to estimate the rate of infection (ROI) and the severity of infection (SOI) of WSD in native shrimps in the Persian Gulf. About 90 live specimens of affected cultured *Penaeus indicus* were collected from Abadan region, south Iran and 150 specimens of native shrimps were captured from the Persian Gulf. Histopathological changes were observed by light microscope in target organs such as: gills, cuticular epidermis, heart, hemolymph, fore stomach and hepatopancreas. ROI and SOI were estimated respectively by standard formulas and grading between 0-4 based on the percentage of white spot syndrome virus WSSV positive cells in selected fields of microscope. The results were confirmed by conducting nested PCR method. The SOI of *Parapenaeopsis stylifera* was estimated in grade four and its ROI was about 85% as the most susceptible species. Histopathologic infection of *Metapenaeus affinis* shrimp by WSD was observed also during this study.

Keywords: White spot disease, Native shrimps, Histopathology, ROI, SOI, Persian Gulf

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Introduction

WSD is one of the most important shrimp diseases. It occurs worldwide and it affects most of the commercially cultured shrimp species (Inouye et al., 1994; Spann and Lester, 1996; Tokhmafshan et al., 2004). The causative agent of WSD is an envelope, non-occluded, rod shaped DNA virus 240-380 nm x 70-159 nm in size (Kimura et al., 1995; Lo et al., 1997; Afsharnasab and Akbari, 2004). It is covered by a double-layered lipid membrane. Analysis of a genomic segment of WSV (white spot virus) suggested that this virus is a member of a Nimaviridae family in a new genus called *Whispovirus* (van Hulten et al., 2001). The disease is characterized by distinct white spots in the cuticle, lethargy, sudden decrease of feed intake, aggregations around the pond with cumulative mortalities often reaching 100% within 2-7 days post-infection (Chou et al., 1995; Wang et al., 1995; Tokhmafshan et al., 2004). The target organs are usually the gills, pleopods, hemolymph, stomach, genital organs, heart, and less commonly the lymphoid organs, the cuticular epithelium, the mid gut and the nerves but there is no evidence of hepatopancreatic cells infection (Chang et al., 1996; Lightner, 1996; Lo et al., 1997). Eosinophilic to basophilic intranuclear inclusion bodies in the hypertrophied nuclei of infected cells are the typical histopathological diagnostic feature of WSSV. The nuclear hypertrophy and the inclusion bodies are due to the development and accumulation of developing virions within the nucleus. In the initial stages of infection, WSSV inclusion is eosinophilic in nature, which becomes basophilic as the infection advances (Lightner, 1996; Vijayan et al., 2003)

WSD has been naturally reported in cultured species like *P. monodon, Litopenaeus vannamei, Fenneropenaeus indicus, Metapenaeus sansis,* while an experimental form of WSD was observed in *L. stylirostris, Fenneropenaeus durarome , L. setiferus,* and *P. aztecus* and following diagnosis by PCR in *P. merguiensis* and *P. orientalis.* There was no report of infection in *M. affinis.* Some families of crabs, lobsters, and crayfish are susceptible to this disease also (Chang et al., 1998; Wang et al., 1998; Vaseeharan et al., 2003; Afsharnasab et al., 2009). It was reported for the first time as epidemic viral disease from Japan, in 1992-1993 (Lightner, 2004; Kakoolaki et al., 2011). In the next 4-6 years it was reported from many other locations worldwide (Lightner et al., 1992; Huang et al., 1995; Subasinghe et al., 2001). The occurrence of a highly contagious disease with rapid mortality like WSD in farmed shrimps was defined during June and July 2002 resulted in an investigation on the presence of virus among native shrimps in Iran (Tokhmafshan et al., 2004). This study was conducted in order to investigate the presence of WSV in native shrimps of Iran*; F.indicus, P. semisulcatus, Parapenaeopsis stylifera,* and *M.affinis* and subsequently to determine ROI and grading SOI of WSD in them by means of PCR and histopathologic methods. Such an approach could be useful to find the

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most resistant native species of a region for rearing as it can lead to reduced losses during disease outbreaks of WSD.

Materials and methods

Sampling

About 90 live specimens of affected cultured *P.indicus* according to their clinical signs and behavioral changes were collected from 10 different contaminated ponds in Abadan region, south Iran, with supposing the 30% of WSD prevalence in ponds with 95% level of confidence. 150 samples were required to give a 95% confidence of detecting an infection at 2% prevalence in the population (Ossiander and Wedermeyer, 1973). Specimens of native shrimps including: 75 *P. semisulcatus,*40 *Parapenaeopsis stylifera*, and 35 *M. affinis* were captured from the Persian Gulf. Samples after recording their color, size, number of rostrum spines, the region and the date of capturing , were fixed by injection of Davidson's fixative , in both sides of carapace and ventral segments then kept in the same fixative for 24-72 hours, they were transferred to 50% ethyl alcohol until required for routine histological process (Bell and Lightner,1988).

Histopathology

Each cephalothorax divided into two parts from dorsal to ventral, then routine procedures of preparing, sectioning and staining were followed; sections of paraffin embedded tissues were cut with rotary microtome at 5 µ. Sections were mounted on slides and kept in 40 °C incubator for one hour then stained with modified Mayer's hematoxylin and eosine pheloxine staining (Bell and Lightner, 1988). Histopathological changes were observed by light microscope in tissues by routine diagnostic protocol of Lightner (1996). Nuclear inclusion bodies from small eosinophilic to large basophilic ones were observed in cells of all experimented tissues; gill, cuticular epidermis, heart, hemolymph, and forestomach except hepatopancreas. Each slides were studied and severity of infection were graded as G1(<10%), G2(30-40%), G3(40-50%), G4(>80%) based on percentage of WSSV positive cells according to level of their inclusion bodies from small central red to large basophilic one (level1,2,3) in a selected field of 100 emersion lens in gill tissues (Tables 1 and 2).

PCR

Gills of samples were also collected and preserved in 70% ethanol for PCR studies. Gills only should be separated without adherence of the other tissues. IQ2000TM WSV commercial kit was used for detection of WSSV according to the manufacture's protocol. Briefly, 2ml of prepared homogenous gills solution put in 75°C for 5 minutes, 0.7 ml chloroform was added, after doing vortex; it was centrifuged 12000 rpm for 5 minutes. PCR products were analyzed by electrophoresis on 2% agarose gel containing 1 mg of ethidium bromide solution and observed under UV by UVidoc, UVitec in waves of 365 and 312 nm. WSSV DNA fragments in 269 and 550 bp were expected to amplify as positive results (Afsharnasab et al., 2009; Kakoolaki et al., 2011).

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Rate of infection (ROI) and severity of infection (SOI) estimation

ROI were estimated by using the formula described by Natividad and Lightner (1992) below:

ROI= 100*(Number of infected samples/total samples)

SOI were graded by using a generalized scheme for assigning a numerical qualitative value to severity grade of infection which was provided by Lightner (1996), in consideration to histopathology and counting the inclusion bodies in different level of infection and different microscopic fielding immersion lens. At first, three levels of WSV nuclear inclusion bodies were identified. The next step was identifying grades of WSV infection (G0-4) to show the severity of infection as it has been shown in Tables 1, 2 and Figures 1 to 5. The result was confirmed by conducting nested PCR method (Table 3).

Table 1: Identifications of different levels of white spot disease(WSV) infection

Microscopic finding	level of WSV inclusion bodies
Injured cell nucleus small red central inclusion, has hypertrophy, marginated chromatin	1
Larger basophilic inclusion in cow dry type A with vacuolated hepatopancreatic cytoplasm could be seen	2
Big basophilic inclusion covered all the nucleus With disappeared cell wall and necrosis appear	3

Table 2: Identifications of different grades of WSV infection

How to estimate	Severity grades of WSV infection
All tissues are normal without any sign	G0
Level of 1,2 could be seen in less than 10% of nucleus of each microscopic view(100 immersion lens)	G1
Level of 1,2 could be counted in 30-40%	G2
Level of 1,2,3 could be counted in 40-50%	G3
Level of 2,3 could be counted in 80%	G4

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Results

The histological examination revealed intra nuclear eosinophilic cowardry type-A inclusion bodies in the gills, midgut, cuticular epidermis, lymphoid organ, heart and connective tissue in all species examined (Figs.7, 8, 9, 10).

Such inclusion bodies were not found in the hepatopancreatic epithelial cells of the same samples (Fig.6), but the infiltrated hemocytes within interstitial spaces were highly infected. The hepatopancreas exhibited cellular vacuolation. The early stage of infection was generalized by hypertrophic nuclei with marginated chromatins and eosinophilic inclusion which turns to basophilic color in late stage of infection. By the progress of infection the inclusion bodies were separated by a crescent space from the marginal chromatin. There was also focal to multifocal necrosis together with nuclear hypertrophy and vacuolar space around the nucleus containing the inclusion.

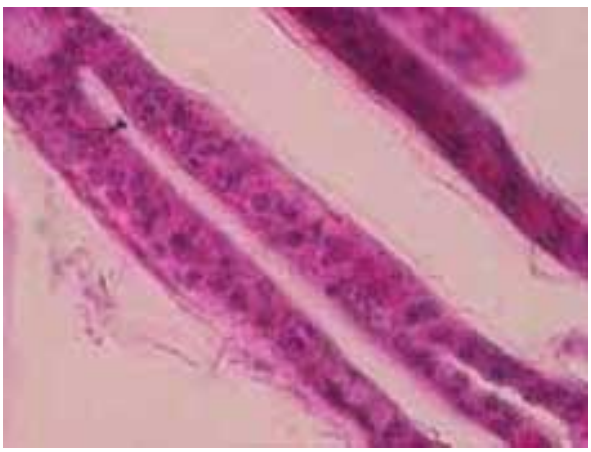


Figure1: Gill of the shrimp, normal tissue, showing Go of SOI it means there is no sign of WSD, H&E, X10

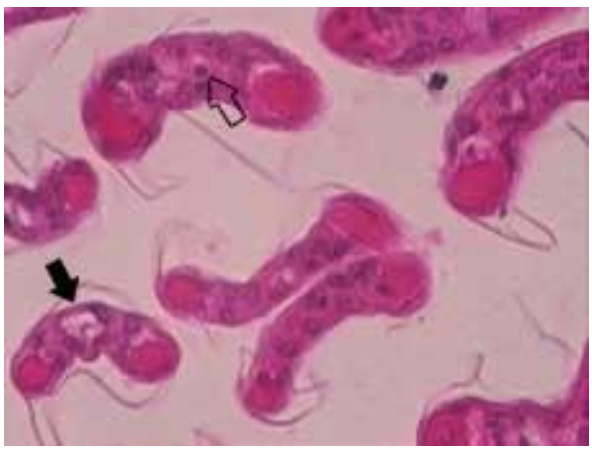


Figure2:Gill of the shrimp, increasing vacuolation space (dark arrow) and a basophilic inclusion body (hallow arrow), showing G1, H&E, X10

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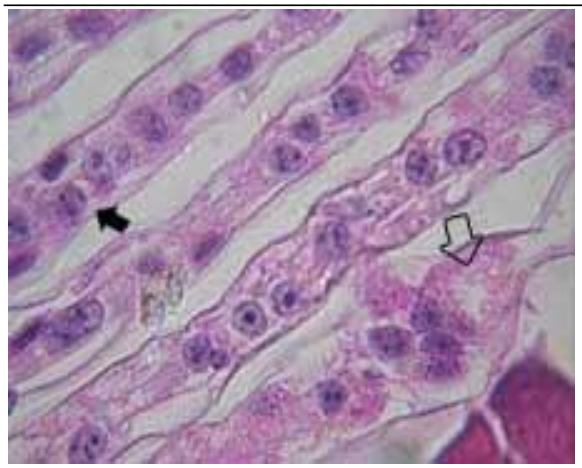


Figure 3: Gill of the shrimp, small eosinophilic inclusion bodies (black arrow), marginated chromatin in the most of the nucleus, and disappearing of the cell membranes (hallow arrow), showing G2 of SOI, H&E, X40

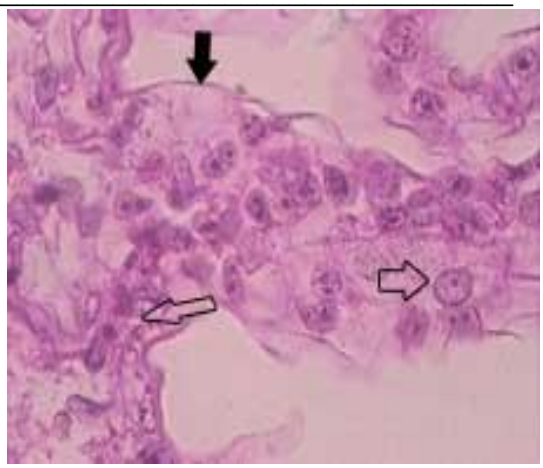
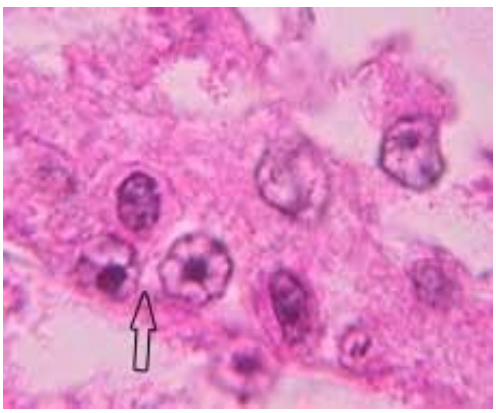


Figure 4: Gill of the shrimp, hypertrophic nuclei are scattered, marginated chromatin (thick hallow arrow), Inclusion bodies (thin hallow arrow), destruction the cell membrane (black arrow) are showing G3, H&E, X40



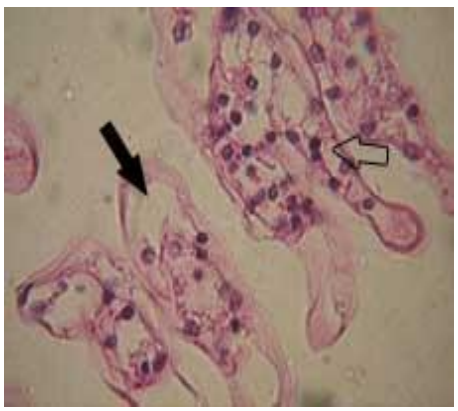


Figure5: Left, marginated chromatin with eosinophilic inclusion body in the cells of the gill of the shrimp (hallow arrow), H&E, X100, Right, Gill of the shrimp, necrosis presents in most of the tissue(black arrow) and basophilic Cowadry type inclusion bodies(hallow arrow) are showing G4 of SOI , H&E, X40.

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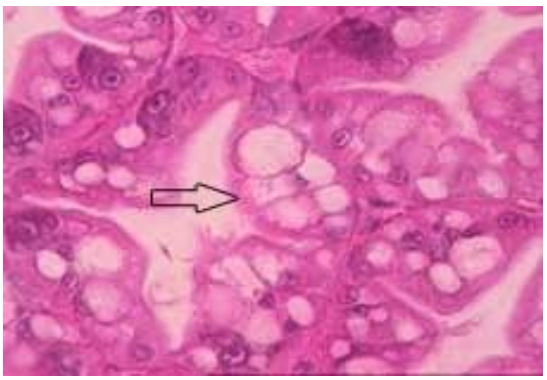


Figure 6: Hepatopancreas tissue of the affected shrimp by WSD and there are many vacuolation forms in it (hallow arrow), H&E, X40

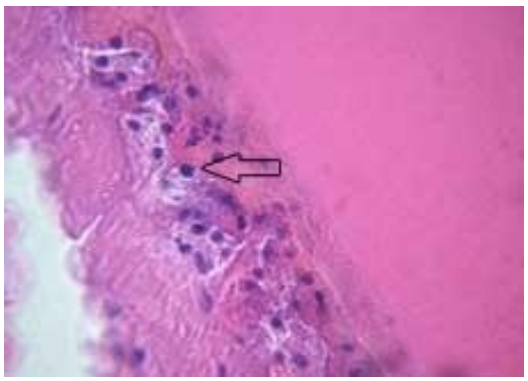


Figure 7: Cuticular epidermis of the infected shrimp, dense and basophilic nuclei(hallow arrow) present WSD infection, H&E, X40

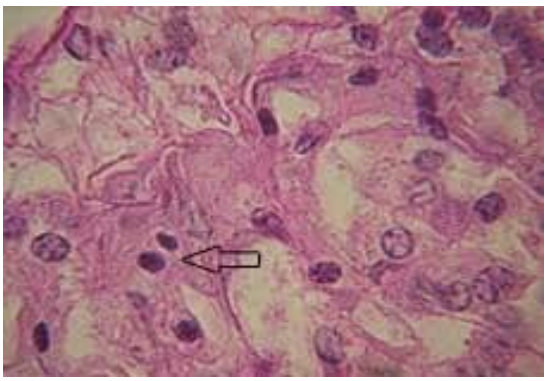


Figure 8: Heart tissue of the shrimp, intranuclearcowadry Type-A inclusion (arrow), H&E, X40

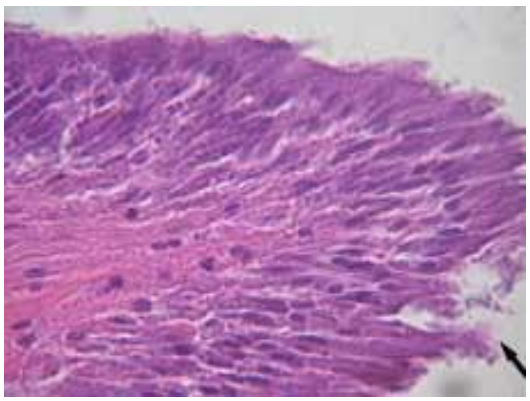
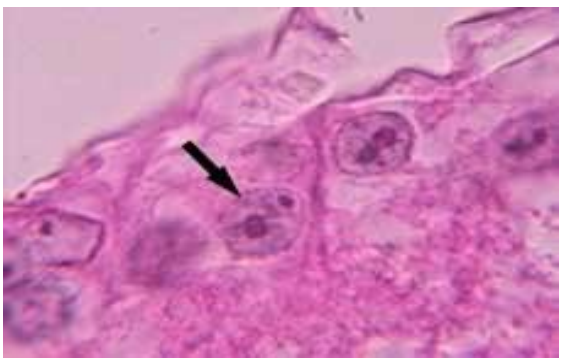


Figure 9:Midgut of the shrimp, destroyed brush border are shown by black arrow, H&E, X40



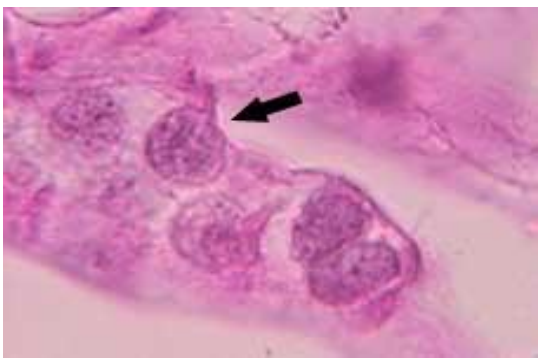


Figure 10: Gill of the shrimp, Left, eosinophilic central intranuclear inclusion body (black arrow), Right, hypertrophic nuclei of gill cells (black arrow), H&E, X100

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PCR results for the WSV infection of the gill samples showed in Figures 11 and 12; when three bands formed in 296, 550 bp and one above the band in 848 bp of the marker, it shows sever WSSV infection of sample, tow bands formed in 296 and 550 bp shows moderate infection and only one band in 296 bp, demonstrates light WSSV infection. Only one band in 848 bp presents negative result (based on the IQ2000, WSV commercial kit). PCR results are shown in Table 3.

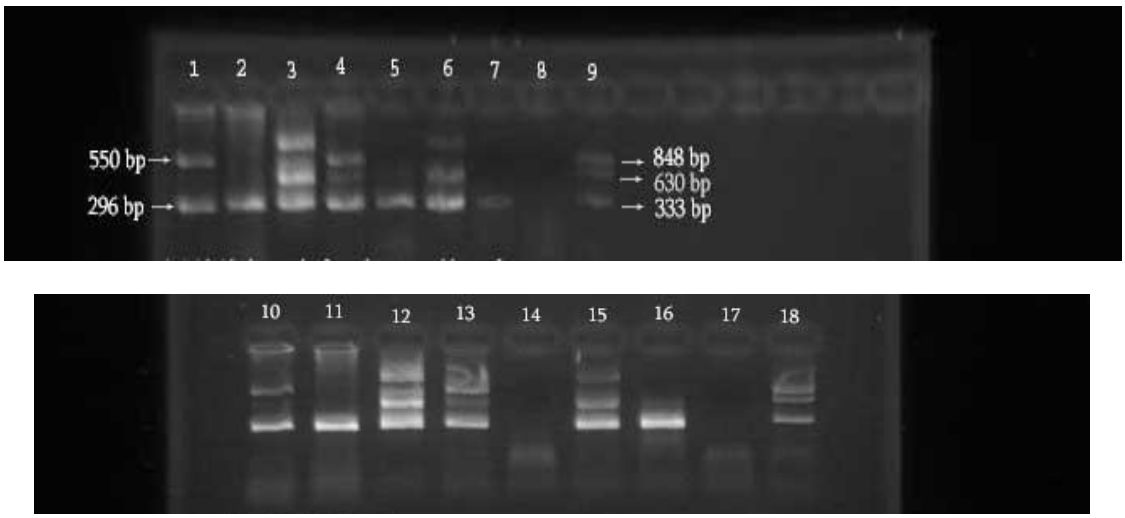


Figure11: The positive result of PCR for the WSV of the gill samples collected from the infected shrimps, 1,4,10,13 samples of moderate WSSV infection, 2,5,7,11,16 samples of light WSSV infection, 3,6,12,15 samples of severe WSSV infection, 8,14,17 double distilled water, 9,18 molecular weight marker in 848,630 and 333bp, diagnostic procedure : bands formed in 296bp and /or 550bp showed positive WSSV infection and bands only in 848 were negative(IQ2000, WSV commercial kit)

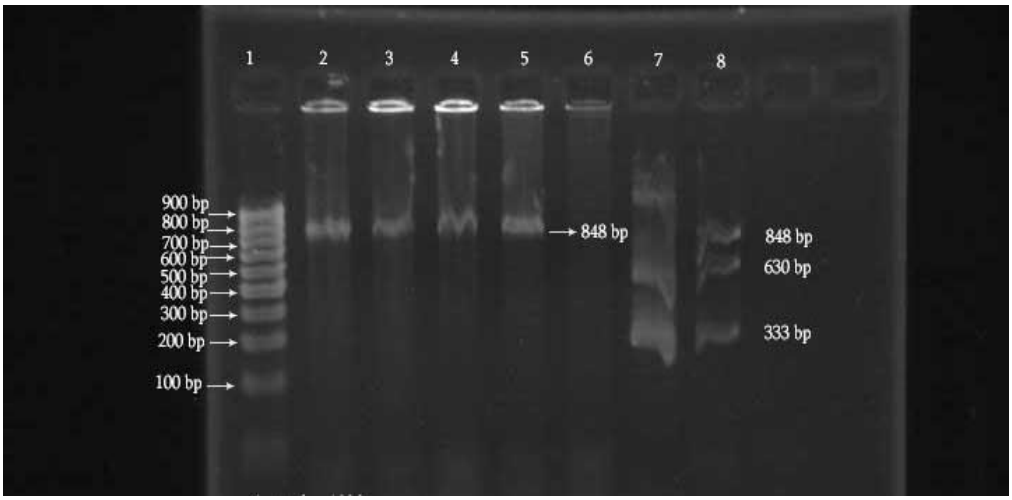


Figure12: The negative results of PCR for the WSV of the samples of the gills, 1 marker100bp, 2,3,4,5 negative sample WSSV, 6 double distilled water, 7 positive control (2000 copies/reaction), 8 marker 848,630, and 333bp (IQ2000, WSV commercial kit).

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Table 3: PCR positive sample number between one + to three +and negative sample number

Shrimp species	negative	+	++	+++
M. affinis	9	17	6	6
P. semisulcatus	12	21	36	36
P. stylifera	6	9	9	9
F. indicus	27	21	21	27

Grades of severity of infection and rate of infection in each species, which was sampled, in each slide were estimated as explained in material and method and the results are shown in table 4 and 5.*P.stylifera* has been shown the highest ROI, was about 85 percent, with high grade of G4 in slides. *Metapenaeus affinis* and *Penaeus indicus* had similar result in their ROI. Grade of SOI in them were more G0.

Table 4: Grades of Severity of WSV Infection between 0-4 among 4 native shrimps

Grades of severity / Species	G0	G1	G2	G3	G4	Total
M. affinis	13	6	3	10	3	35
P. semisulcatus	16	11	6	26	16	75
P. stylifera	6	1	3	7	23	40
F. indicus	28	23	20	14	5	90

Table 5: Rate of WSV Infection(%) in 4 native shrimps

Species	ROI%
M. affinis	68.57
P. semisulcatus	78.66
P. stylifera	85.00
F. indicus	68.88

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Discussion

The purpose of this study was estimating the ROI and SOI of WSD in native shrimps from the Persian Gulf. SOI grading for WSD is designed in this study for the first time from 0 to 4 based on the percentage of infected cells as described in Tables 1 and 2 to find the most resistant and susceptible species of this study with ROI and PCR results.

Parapenaeopsis stylifera has been shown the most susceptible to WSV infection as its ROI was about 85%, with high grade of G4 in slides that confirms this species is the most susceptible among the species were investigated. *Metapenaeus affinis* and *Fenneropenaeus indicus* had similar result in their ROI. Infection of *M. affinis* was observed for the first time in this study.

Clinical signs can lead to rapid diagnosis of WSD but there are three other diseases with white spots on carapace, so detection of specific inclusion bodies are pathogenomonic and histopathology was recognized as one of the accurate diagnosis method (Bell and Lightner, 1988). Widespread focal or diffused cellular degeneration, clear hypertrophy in most tissues of ectodermal and mesodermal origins like gill and heart, necrosis of hepatopancreas, hemocytic infiltration and hypertrophic nuclei containing eosinophilic or basophilic inclusion bodies within the affected cells have been described as some of the main histopathological findings of white spot syndrome disease (Lightner, 1996; Wang et al., 1997; Vijayan et al., 2003). The nuclear hypertrophy and the presence of inclusion bodies are due to the development and accumulation of developing virions within the nucleus. In the initial stages of infection,WSSV inclusion is eosinophilic in nature, which becomes basophilic as the infection get more advances (Lightner, 1996; Vijayan et al., 2003). Histopathological findings in this study clearly were similar with previous findings of Chou et al. (1995); and Inouye et al. (1994) in *Penaeus chinensis, F.indicus, Penaeus merguiensis, and Penaeus monodon*species. There were inclusion bodies in all tissues except hepatopancreas, it may be due to lack of cell receptor for virus. Lo et al. (1997) have no signs of intestinal cell destruction but in this study infection of intestinal epithelial tissue, subepithelial cuticle, foregut and hemolymphatic tissues were observed in wild species of *P.semisulcatus*, *P. stylifera* , and *M. affinis*. These wild species can act as virus carrier and transfer virus to ponds through sea water.

PCR method has been used as an accurate and susceptible method of diagnosis which has been used by Takahashi et al. (1996) for WSV identification. Nested PCR is more accurate than PCR and it decreases false negative results. In this study just 20% of positive results showed clinical signs, it confirms that Nested PCR has accuracy. Vijayan et al. (2003) graded severity of WSV infection as low (< 30%), medium (30–70%) and high (>70%) based on the percentage of WSSV positive cells in a selected field of ×40 by means of Lightner protocol (1996). The causative agents of

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WSD from different locations appeared to be very similar to disease features, histopathology, morphology and PCR. Therefore they have been considered as the same strains of WSV. However, there are the other methods of diagnosis which are quicker and more useful on field, but basic researches in controlling and prevention programs need more confidential and an accurate method to find resistant species for culturing in outbreaks of disease. This study suggested that histopathology with estimating of ROI and SOI can be beneficial in this regard. We recommend further studies using more accurate and modern technology in these four species to investigate the susceptibility of different species by means of ROI and SOI of white spot syndrome virus (WSSV).

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