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The Status of white spot syndrome virus (WSSV) in Islamic Republic of Iran

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Abstract

White spot syndrome virus (WSSV), the causative virus of disease, is found in most shrimp farming areas in Iran, and it cause large economic losses to the shrimp farming industry. Shrimp aquaculture is an important industry in Iran and plays an important role in aquaculture production. The shrimp production in 2012 was more than 12000 metric tonnes and it is estimated it will reach 20000 tonnes in 2013.White spot syndrome virus is highly virulent in shrimp farms and can spread quickly and - cause up to 100% mortality within 3-7 days. The virus is a very large, enveloped, double stranded DNA (ds DNA) and assigned by ICTV to a new genus *Whispovirus* and belong to *Nimaviridae* family. In Iran WSSV first appeared in Khuzestan Province in the south of Iran and later on it appeared in other provinces such as Bushehr, and Sistan and Baluchestan. The aim of this review is to give current information of WSSV in Iran, host ranges, carriers, biology, clinical signs, histopathology, PCR, with emphasis on the effects of WSSV in shrimp aquaculture.

Keyword: WSSV, Shrimp, Disease, Aquaculture, Iran.

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Introduction

Iran, with an area of about 1.62 million km^2 and a population of about 75 million, is located in arid and semiarid region of West Asia. Iran's aquatic resources include 2,870 km of coastline along the Persian Gulf and Oman Sea (1880 km), Caspian Sea (990 km), (Fig. 1). There are also large expansions of flat, barren and saline lands stretching along southern coasts of Iran, which are unsuitable for agricultural purposes. About 5,000 ha of such coastal land were developed for shrimp culture. The total area allocated for shrimp culture is about 200,000 ha (Fig. 1) (Tokhmafshan, 2001). Farmed shrimp production in Iran grew slowly from 1991 until now and a number of large farms started to come into production. Initial trials were carried out with FAO/UNDP assistance in the years 1991-92, producing post-larvae of local species such as *Penaeus semisulcatus*, *P. merguiensis* and *Metapenaeus affinis* and reviewing favorable areas for shrimp culture. The production in 1993 was 16.5 tonnes. When the postlarvae of *P. monodon* were imported from Malaysia the production increased steadily until 2012 and reached 12000 tonnes (Fig. 2). Iran has ambitious plans for expansion of shrimp culture industry from 10,000 tonnes in 2002 to 100,000 tonnes by 2020 (Salehi, 2010; Afsharnasab, 2012).

Figure 1: Map of Iran with area for shrimp culture in the coastal area of the Persian Gulf and Sea of Oman.

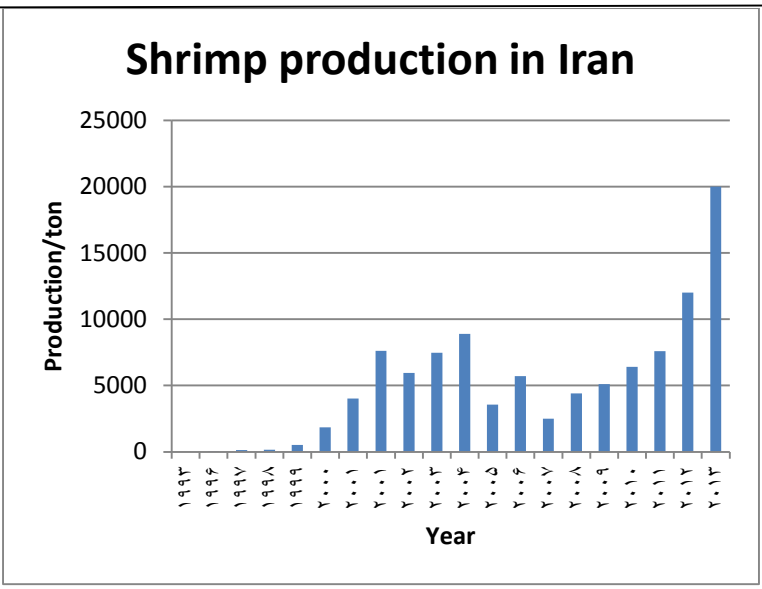


Figure 2: Shrimp production In I. R. Iran.

The expansion of culture of penaeid shrimps in Iran has been accompanied by recognition of penaeid diseases that are of viral etiology. Viral diseases are very important because some of them are accompanied by serious crop and economic losses. White spot syndrome virus is an important invasive pathogen for shrimp (Afsharnasab *et al.*, 2009a; Kakoolaki *et al.*, 2010; Lightner, 2011).White spot syndrome virus was reported first from shrimp farms in Iran in 2001 in Khuzestan Province, followed by other shrimp farming area in Bushehr Province in 2003 and 2005, Sistan and Baluchestan, 2005, 2007, 2008 and 2011 in south of Iran (Afsharnasab *et al.*, 2007b; Afsharnasab *et al.*, 2009a; Kakoolaki *et al.*, 2010; Kakoolaki *et al.*, 2011b). The WSSV can be prevented and its impacts can be mitigated through implementing scientific health management strategies, and application of principles of biosecurity at the pond, farm, national and regional levels. Knowledge about nature of the pathogen, its carriers and routes of entry are very important (Lightner, 1996; Lightner, 2004; Afsharnasab, 2007b; Afsharnasab *et al.*, 2007b;).

Biology of Agent

The causative agent of white spot disease is white spot syndrome virus (WSSV) which is an envelope, double stranded DNA virus. The virion were elliptical to short rods with trilamilar envelop that measured as 248±87×107±11nm and nucleocapsids were 162±15×59±17nm. It seems that the virus at the end have a tail and it is very important for shrimp reproduction (Fig. 3), (Afsharnasab *et al.*, 2005, OIE, 2006). According to the International Committee on Taxonomy of Viruses (ICTV) and Atlas of invertebrate virus, the morphology of virus is similar to that of SEMBV of WSSD group serotype (Flegel, 2006; Afsharnasab *et al.*, 2009a; Lightner, 2011). In the 8th report of the ICTV in 2004, WSSV is assigned as the only member of the genus *Whispovirus* within the *Nimaviridae* family

(OIE, 2006). More than 50 structural and non structural proteins are found in WSSV that most of them are located in the envelope and a few are located in the capsule. Proteins located in the envelop are: VP12, VP19, VP22, VP24, VP28, VP31, VP36B, VP38A, VP39, VP41, VP41A, VP41B, VP51B, VP52A, VP52B, VP53, VP53A, VP68, VP110, VP124, VP150, VP187, VP281, VP292, and VP466 (Van Hulten *et al.*, 2000; Van Hulten *et al.*, 2001a; Van Hulten *et al.*, 2001b; Huang *et al.*, 2002a; Huang *et al.*, 2002b; Van Hulten *et al.*, 2002; Zhang *et al.*, 2002; Tsai *et al.*, 2004; Zhu *et al.*, 2005; Tsai *et al.*, 2006; Xie and Yang, 2006; Xie *et al.*, 2006; Zhu *et al.*, 2006), in the nucleocapsid are VP15, VP35, VP51C, VP60B, VP388, and VP664 (Van Hulten *et al.*, 2002; Tsai *et al.*, 2004; Witteveldt *et al.*, 2005; Tsai *et al.*, 2006), and in the capsule are consisted of VP36A, VP39A, and VP95 (Tsai *et al.*, 2006). This envelope protein is suggested to bind to the shrimp cells, playing a crucial role in the viral penetration (Tsai *et al.*, 2006).

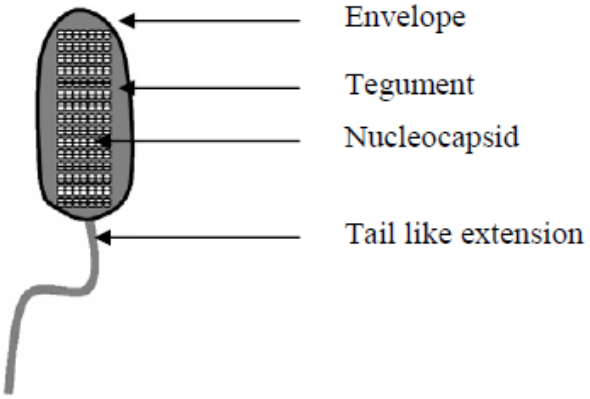


Figure 3: Simplified model of WSSV, Courtesy of (Meezanur Rahman, 2007).

Distribution, History, Host and carrier

Since 1992, white spot syndrome disease which was reported from China has overshadowed all other viral diseases. The white spot disease (WSD) is also reported from all countries in Asia and America. White spot disease caused by WSSV emerged in east Asia in 1992–1993 and it was quickly dispersed with infected seeds and broodstock across 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, 2011). In Japan white spot syndrome virus was first reported from farmed *M. japonicus* in 1993 (Inouye *et al.*, 1994, Nakano *et al.*, 1994) and the causative agent was named penaeid rod-shaped DNA virus (PRDV) or rod-shaped nuclear virus of *M. japonicus* (RV-PJ). Later, outbreaks of viral disease with similar gross signs caused by similar rod-shaped viruses were reported from elsewhere in Asia and other names were applied: hypodermal and hematopoietic necrosis baculovirus (HHNBV) in the People's Republic of China (Huang *et al.*, 1995), white spot baculovirus (WSBV) and PmNOBIII in Taipei China (Chou *et al.*, 1995), and systemic ectodermal and

mesodermal baculovirus (SEMBV) or PmNOBII in Thailand (Wongteerasupaya *et al.*, 1995). First in July 2001, high mortality occurred in cultured *P. indicus* shrimps in Khuzestan Province, south of Iran by WSD (Tokhmafshan, 2004; Afsharnasab *et al.*, 2009a). High mortality was reported from all farms in Bushehr Province as the main area of shrimp culture in Iran during 2003, 2004 and 2008. In late 2007, 2009 and 2011, Guader area in Sistan and Baluchestan faced WSD and high mortality occurred in shrimp farms during these years (Afsharnasab *et al.*, 2008, Afsharnasab *et al.*, 2009a, Salehi, 2010, Afsharnasab, 2012). White spot syndrome virus can be detected by PCR methods in carrier and reservoir. As described by researchers (Chou *et al.*, 1998, Lightner, 2011, Flegel, 2006) many crabs, lobsters, arthropods, shrimps and insects are carriers of viruses. Many of them as well as the shrimps such as *P. monodon, P. semisulcatus, P. merguiensis, Metapenaeus ensis, P. indicus, Macrobrachium rosenbergii, P. chinensis, P. penicillatus, M. japonicus, Litopenaeus vannamei, L. stylirostris, P. Aztecus, P. californiensis, L. setiferus, P. duorarum, Trachypenaeus curvirostris, Exopalaemon orientalis, Orconectes punctimanus, Procambarus clarkii , Charybdis feriatus, Portunus pelagicus, Portunus sanguinolentus, Scylla serrata, L. occidentalis, Thalamita sp., Panulirus versicolor, Panulirus penicillatus, Cherax quadricarinatus* are very susceptible to virus. In Iran most penaeid shrimps such as *P. Indicus, P. semisulcatus, P. merguensis* are very susceptible to virus (Mohan *et al.*, 1997; Vaseeharan *et al.*, 2003; Gholamhoseini *et al.*, 2013b). The infection can be transmitted vertically transvarian and horizontally by consumption of infected tissue (e.g. cannibalism, predation, etc.), and by water born methods. Birds such as gulls may mechanically transmit infection between ponds by releasing captured, moribund or dead shrimp. Also the role of farm equipments and people should not be neglected in mechanical transmission of virus particles in heavy presence of virus in the environment (Khatibitabar, 2010).

Techniques for WSSV detection

As mentioned by researchers (Lightner, 1996; Flegel, 2006; OIE, 2006; Afsharnasab, 2007a; Afsharnasab, 2007b) different methods such as microscopic observation under light, dark field, phase contrast microscope, bioassay, molecular methods (PCR, Nested PCR, RT-PCR), transmission electron microscopy, immunological and histopathological methods are developed to detect WSSV infection. The selection of a method is dependent on the purpose. For instance, for screening brood stock and nauplii in hatcheries, different detection methods are used on WSSV pathogenesis. The frequently used clinical sign, histopathological, immunological and molecular methods are described below.

Wet mount microscopy

The wet mount methods where a fresh smear of target tissue, organ or feces is prepared or stained using 0.05% malachite green for checking the virus (Lightner, 1996; Flegel, 2006; Afsharnasab, 2007a ,).

The impression smear method is used to compare diagnostic sensitivity to standard histopathological methods applied to the diagnosis of WSSV infection in postlarvae and Juvenile shrimp (Afsharnasab, 2007a; Afsharnasab, 2007b). Staining of feces and HP smear with 0.05% aqueous malachite green or rapid Huang's staining show the inclusion bodies under light microscopy for WSD (Fig. 4) (Huang and Yu, 1995).

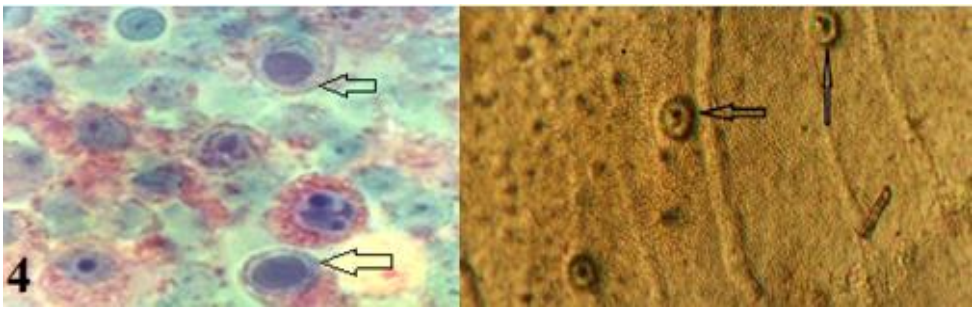


Figure4: Wet mount smear (Right) and T-E Huangs (Left) courtesy from Huang and Yu (1995) staining for rapid WSSV inclusion bodies detection (arrow), Mag:1000X.

Clinical sign of WSD

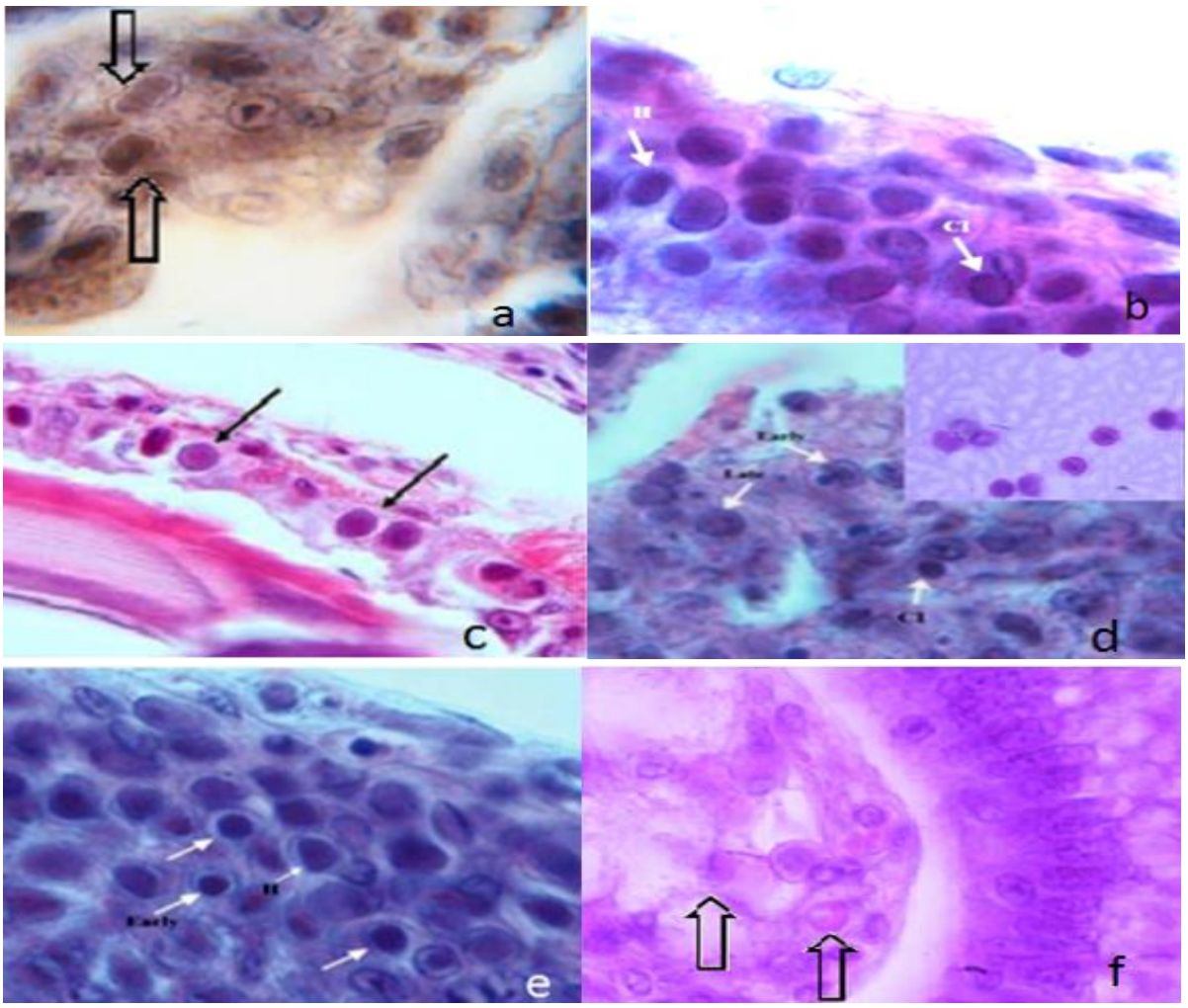
White spot syndrome virus infection in penaeid shrimps typically cause lethargic behavior in affected animals, cessation of feeding followed within a few days by the appearance of moribund shrimp swimming near the surface at the edge of ponds (Fig. 5a) (Lightner, 1985; Jiravanichpaisal *et al.*, 2001; Afsharnasab and Akbari, 2005; Flegel, 2006; Afsharnasab, 2007b; Afsharnasab *et al.*, 2007b, , Kakoolaki *et al.*, 2011a,). Pink to reddish-brown discoloration of the body and white spots of about 0.5-2mm on the cuticle (Fig. 5b), especially on the inner surface of the exoskeleton of cephalothorax and abdomen, are the predominant gross lesions observed and spots under light microscopy in fresh squash show melanin rings (Fig. 5c) (Flegel *et al.*, 2004; Flegel, 2006; Afsharnasab, 2007b; Khatibitabar, 2010; Gholamhoseini *et al.*, 2013a, ,). The cuticulin is easily separated from underlying epidermis, and hepatopancreas become yellowish-white with a swollen and fragile texture (Figs. 5d, 5e). Cuticular deformities such as broken or withered antennae and damaged rostrum, opaque abdominal musculature and melanised gills are consistently observed (Fig. 5f). Gills are infected with epicommensal and fouling organisms, especially with *Zoothamnium* sp. Thereare 70-100% mortality in white spot affected farms within 3-7 days after the onset of the clinical signs (Fig. 5f) (Lightner, 1996; Chou *et al.*, 1998; Jiravanichpaisal *et al.*, 2006; Afsharnasab *et al.*, 2009a; Lightner *et al.*, 2012).

Figure 5: Moribund shrimp near the edge of farm and shrimp moribund exhibit the red appearance (Fig. 5a). The comparing infected cuticle with white spot (arrow) and non infected cuticle of shrimp (Fig. 5b). The white spot under light microscopy with melanin area (arrow) and circular rings (Fig. 5c), Mag: 1000X. The comparing infected shrimp with opaque muscles and none infected with transparency muscles (Fig. 5d). The hepatopancreas of infected shrimp with yellow color and fragile texture (arrow) (Fig. 5e). High mortality of shrimp infected with WSSV (Fig. 5f).

Histopathology of WSSV

Histopathological technique by routine paraffin section stained with H and E is adapted in diagnostic procedure (Lightner, 1996; Afsharnasab, 2007a). This technique is normally quite labor intensive, due to detection of the inclusion bodies, which may be masked by extraneous material and difficult to distinguish. However, this technique can serve as a criterion to confirm WSSV. The histopathology of WSSD in shrimp is dominated by presence of large conspicuous intranuclear eosinophilic Cowdry type -A inclusion bodies in the tissues. The tissue section of affected shrimp stained with H and E/Phloxine show intranuclear eosinophilic Cowdry type-A inclusion bodies in gills, midgut, cuticular epidermis, lymphoid organ, hematopoietic tissue, cecum, hearth and connective tissues (Lightner, 1996; Kou *et al.*, 1997; Lo *et al.*, 1997; Afsharnasab *et al.*, 2007a; Lavilla-Pitogo *et al.*, 2007; Meng *et al.*, 2009; Lightner, 2011,) Figs 6a-h). However, the virus does not infect hepatopancreatic epithelial cells (HEC), even in moribund

specimens (Fig. 6i). Although HEC have no viral inclusion, but the hemocytes are highly infected, which are infiltrated with hemolymph fluids in interstitial space (Fig. 6j). The hepatopancreas show cellular vacuolation resulting in diminution of the tubule lumen (Fig. 6i). These might be the reason why hepatopancreas become swollen and fragile (Afsharnasab *et al.*, 2007b; Afsharnasab *et al.*, 2009a). Early stage of infection is generalized epithelial hyperplasia and the hyperplasia cells have nuclear hypertrophy with chromatin margination containing eosinophilic to basophilic intranuclear inclusion body. With the progress of infection, the inclusion body is separated by a halo from marginal chromatin (Fig. 6k). At advance stage of infection the nuclei of cell is disintegrated and the halo space is seen in cells. In moribund shrimps, there are also focal to multifocal necrosis, evidenced by nuclear hypertrophy and vacuolar space around the nucleus containing the inclusion (Chou *et al.*, 1998; Wang *et al.*, 1999; Chakraborty *et al.*, 2002; Wang *et al.*, 2002; Balasubramanian *et al.*, 2006; Afsharnasab *et al.*, 2007b).



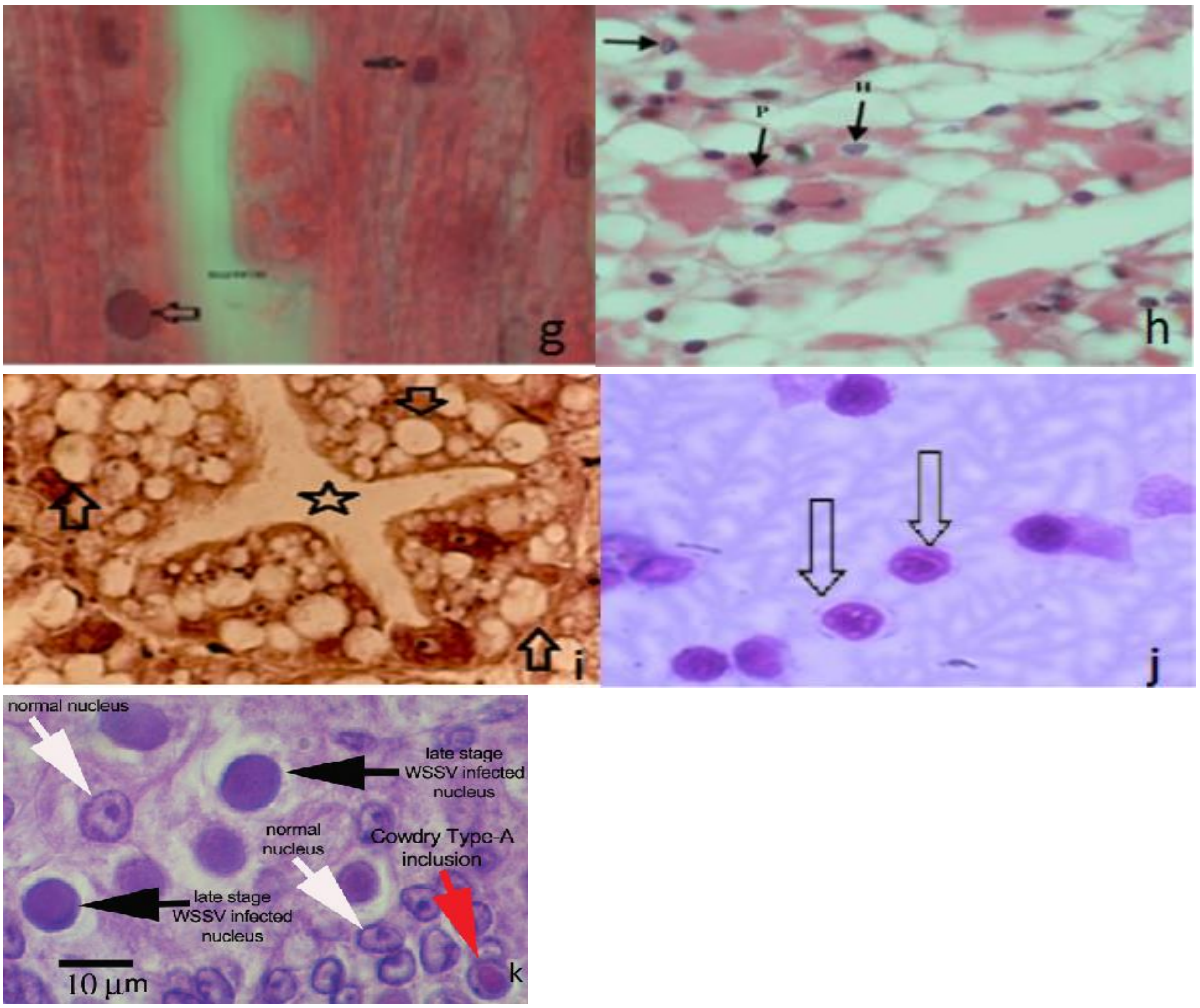


Figure 6: The gill of *P. indicus* infected with WSSV and Cowdry type A in the cells (arrows) (Fig. 6a) HandE/Pheloxin, Mag: 1000X. Midgut of shrimp *L. vannamei* infected with WSSV and abundant Cowdery type A in the cell (arrows) (Fig. 6b) HandE/Pheloxin, Mag: 1000X. The cuticular epithelium of *L. vannamei* infected with WSSV and advances Cowdery type A (arrows) (Fig. 6c), HandE/Pheloxin, Mag: 1000X. Lymphoid organ of shrimp *L. vannamei* infected with WSSV and the tissue and hemocyte (Right angle) showed intranuclear Cowdery type A, (Fig. 6d) HandE/Pheloxin, Mag: 1000X. Hematopoeitic tissue of *L. vannamei* with heavily infected with WSSV and the cells showed intranuclear Cpwdery type A, (Fig. 6e) HandE/Pheloxin, Mag: 1000X. The longitudinal section of cecum with cells infected of WSSV (arrows), (Fig. 6f) HandE/Pheloxin, Mag: 1000X. Section of heart muscles and connective tissue with infected cells (arrows) (Fig. 6gandh),HandE/Pheloxin, Mag: 1000X. Hepatopancratic epithelial cell (HEC) with cellular vacuolization (arrows) and lumen (star) without any infection, (Fig. 6i) HandE/Pheloxin, Mag: 1000X. Hemocyte infected with WSSV in shrimp. *L.vannamei* SPF, (Fig. 6j) HandE/Pheloxin, Mag: 1000X. Different stage of WSSV in shrimp infected with Cowdry type A inclusion body Courtesy by Flegel (2006) (Fig. 6k), Bar:10µm.

Transmission electron microscopy

Transmission electron microscopy (TEM) is a powerful tool in the diagnosis of WSSV. Ultra thin section of gill, exoskeleton, heart and hepatopancreas from shrimp suspected for white spot disease are processed for TEM studies. Under TEM the nuclei of infected cells show slight hypertrophy, chromatin is marginated along the nuclear membrane. The nucleolus and chromatin are fused, causing the central area of nucleus to become thin and hemogenous (Fig. 7)(Wang *et al.*, 1995; Wang *et al.*, 1999;

Afsharnasab and Akbari, 2005; Flegel, 2006; Lightner *et al.*, 2012; Tang *et al.*, 2013,). The nuclei are rounded due to gross hypertrophy and very electron-dense marginated chromatin is embedded in a less electron-dense zone of granular material that formed a continuous dark layer (ring-zone or RZ) adjacent to the nuclear membrane (Fig. 8). More electron-lucent central virogenic stroma (VS) contain distinct viral envelope material in membranous or vesicular form. In some cells, the central virogenic stroma is denser, with the appearance of numerous viral particles. The progressive infection cells are revealed ovoid, elongated or elliptical virions, typically non-occluded baculo-like forms within markedly hypertrophy nuclei (Fig. 9). The virions, which are both longitudinally and crossly cut, are measured about 248 ± 87 nm×162 ±15 nm and the nucleocapsids are about 162 ± 15 × 59±27 nm, with a well development trilaminar envelope. In some hypertrophied nuclei, the immature virus with empty capsid, nucleocapsid and circular envelope along with fully mature virions are detected in the nucleoplasm (Fig. 10) (Wang *et al.*, 2002; Afsharnasab and Akbari, 2005; Flegel, 2006; Lightner *et al.*, 2012).

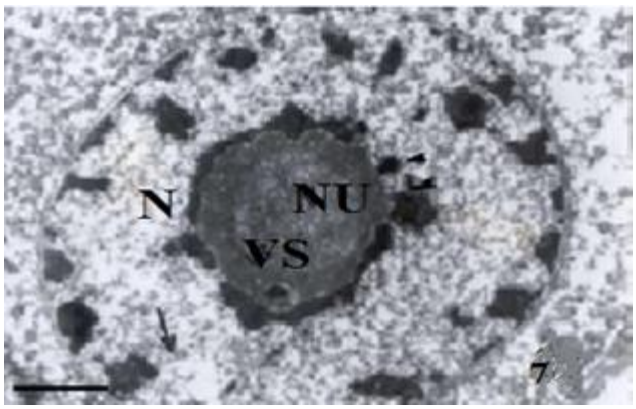


Figure 7: The cuticlar epithelium cell of shrimp P. indicus infected with WSSV and the chromatine marginated(arrow) and nucles (N) and nuclelus (NU) hyoertrophy, the cytoplasm compressed and has a narrow shape. Lead citrate and uranyl acetate.Scale bar:1.5µm.

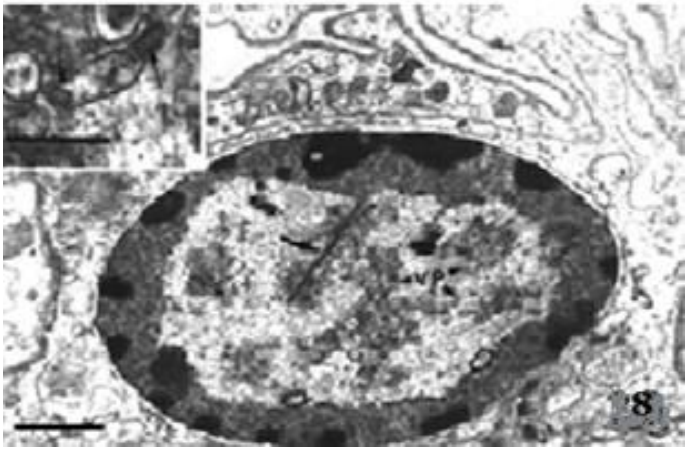


Figure 8: WSSV-infected cuticular epidermal cellscourtesy from Wang etal, (1999). The marginated chromatln is transformed into a dense ring-zone (blank arrows). The central virogenic stroma is less dense, vesicular in form, and shows many viral particles (VP) undergoing assembly. Note a viral nucleosome (arrow) in the nucleus. Scale bar = 1.4 pm. Inset: Higher magnificatonof the viraal particle (*) indicating 2 portions of a capsid separated at the ends (arrows).Lead citrate and uranyl acetate. Scale bar = 340 nm.

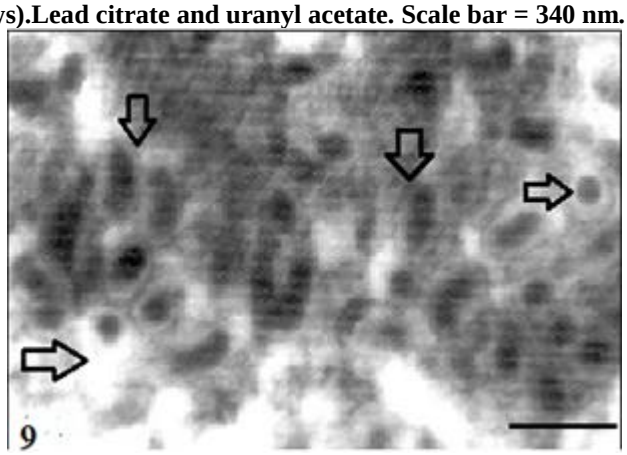


Figure 9:Ultrathin section of WSSV showed longitudinal and horizontal view of virus (arrow).Lead citrate and uranyl acetate. Scale bar: 200 nm.

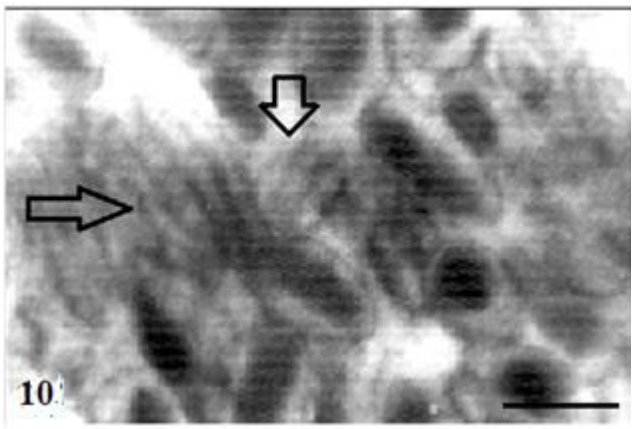


Figure10: High magnification of WSSV virion with showed trilamiar envelop and empty capside (arrow). Lead citrate and uranyl acetate. Scale bar: 230 nm.

Polymerase chain reaction

This method can be a highly sensitive means for detection of WSSV in both the shrimp tissues and the culture environment. Molecular methods also are used to demonstrate that the organism is definitely a particular species or strain. In Iran as mentioned by researchers (Afsharnasab *et al.*, 2005; Saberi *et al.*, 2008) two pairs of primers are designed (CLC Bio software) using viral VP24 gen for nested PCR. Also a pair of primers is designed for host of 18SrRNA as a PCR control on both positive and negative samples. Primers: Shri F 5´-GTA GGT ACG CCT ACA ATG G-3´, and Shri R 5´CCG GAA CTC AAA GAC TTT GGT T-3´ are used as PCR control for sampling of 809 nucleotides from shrimp 18SrRNA GEN. Primers WSS F1 5´-CAC CTG GGT TTG ACT ACA ATA-3´, and WSS R1 5´-TCT GTT TTT TTC TCT CAT GAC-3´ are designed to amplify 785 nucleotide (product of PCRI) from viral VP24 gene. Primers: WSS F2 5´- TCC AAA CAC AAG TGT GTT GAT C-3´, WSS R2 5´AAG ACG CCT ACC CTG TTG AAT C-3´ amplify 414 nucleotides (products of PCRII) of the first PCR product (Saberi *et al.*, 2008). DNA extraction is done as described by (Lightner, 1996) with some brief modifications. 50 mg of mixture of hard and soft tissues of shrimp are poured into a microfuge and 200 µL of lyses buffer containing 20 µg Ml^{-1} proteinase K is added to it and Kept in 37ºC overnight. Then, it is boiled and centrifuged. The supernatant containing DNA is transferred to a new tube and extracted using phenol and chloroform. DNA precipitate is dissolved in 50 µL of deionized water. The thermocycler program and electrophoresis are done as mentioned by Saberi *et al.* (2008). The results of PCR to detect the presence of WSSD genomic DNA is shown in Fig. 11. A single band of 414 bp fragment is observed after 30 cycles of PCR amplifications of viral genomic of WSSV. According to the procedure of the kit a single band of 414 bp of unknown samples is representative of WSSV in the shrimp examined. The appearance of 809 bp shrimp DNA product (false negative control) and 414 bp WSSD DNA product in the positive control confirm the validity of the results (Afsharnasab *et al.*, 2005; Saberi *et al.*, 2008) (Fig. 12).

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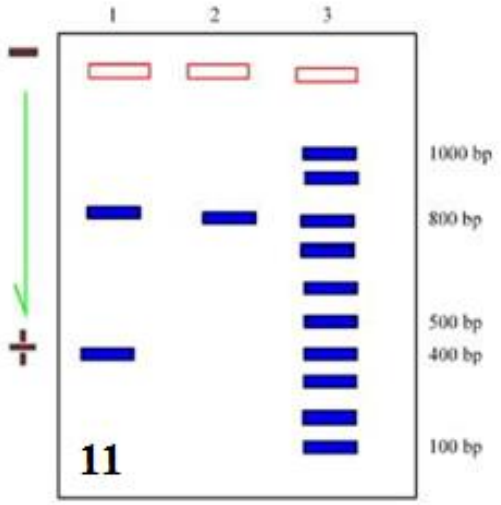

Figure 11: Electrophoresis pattern of PCR product in positive and negative samples in compare with DNA ladder marker. In negative samples only PCR product of shrimp will observe and in positive samples both of PCR products of virus and shrimp will observe. The shrimp PCR product is a control device for PCR system.

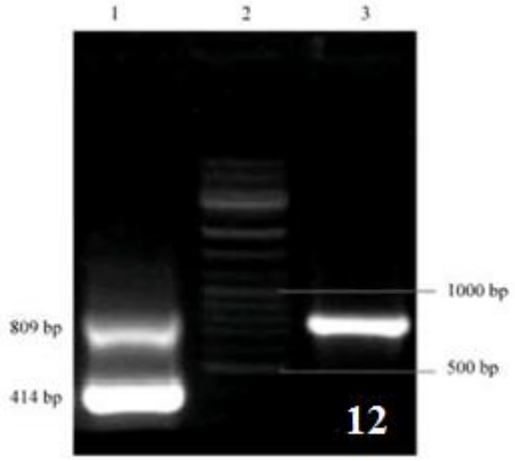

Figure 12: Agarose gel electrophoresis of positive and negative samples. Lane 1 show PCR product of positive sample because PCR product of host and pathogen are seen and lane 3 shows negative sample because only PCR product of host is seen.

Other researchers from different areas also introduced different protocol for detection of WSSV. These methods are based on primers designed against a specific part of the genome sequence of WSSV. PCR methods include one step PCR (Lightner, 1996; Lo *et al.*, 1997), semi nested PCR (Kiatpathomchai *et al.*, 2001), two step PCR (Tapay, 1999; Hossain *et al.*, 2004), quantitative competitive PCR (Tang, 2000) and real time PCR (Durand and Lightner, 2002). One step PCR detects WSSV in shrimps containing a substantial concentration of viral DNA which is usually the case in animals displaying gross signs of disease (Otta, 1999; Jian, 2005). Two step PCR can detect light infections in brood stock, nauplii, postlarvae and juveniles (Lo, 1996; Lo, 1997), and quantitative PCR can be used for the quantification of viral load. The disadvantages are misdiagnosis (false positive) (Claydon, 2004; Sritunyalucksana *et al.*, 2006), inability to confirm whether the detected DNA is infectious or not, the fact that sensitivity depends on the primer used (Hossain *et al.*, 2004), lack of localization of the infection in tissues and possible presence of inhibitory factors in some tissues (false negative) (Shekhar, 2006). The protocols of multiplex PCR to detect WSSV and other viruses such as IHHNV (Quéré, 2002), TSV (Tsai, 2002), MBV (Natividad *et al.*, 2006) or IHHNV and TSV simultaneously have also been developed (Xie, 2007). A new protocol called in situ PCR can detect light infection in tissues at early stage of infection (Jian, 2005). Another method named loop mediated isothermal amplification (LAMP) is claimed to have more sensitivity than other PCR protocols. It can detect up to 1 femtogram (fg) of virus (Kono, 2004).

Sampling and sample size for study WSSV

Laboratory procedures should comply with the Manual of Diagnostic Tests for Aquatic Animals (OIE, 2010). The purpose for specimen collection is observing the symptom, study the pathogen, and isolate it or conduct epidemiological survey. There recommended minimum number of specimens to collect for diagnosis is 100 for larval stages of most crustaceans; 50 for post larval stages; and 10 for juveniles and adults, with preference for individuals with signs and/or gross lesions. There are two situations in which WSSV infection requires detection: For confirmation of suspect clinical WSD and in targeted surveillance (screening) to establish the infection status of asymptomatic populations. As mentioned by Lightner (1996) the sample size in statistical analysis should be collected as Table 1.

Table 1: Sample size based on assumed pathogen prevalence in a population

Population Size	Size of Sample Needed at Prevalence						
	2%	5%	10%	20%	30%	40%	50%
50	50	35	20	10	7	5	2
100	75	45	23	11	9	7	6
250	110	50	25	10	9	8	7
500	130	55	26	10	9	8	7
1,000	140	55	27	10	9	9	8
1,500	140	55	27	10	9	9	8
2,000	145	60	27	10	9	9	8
4,000	145	60	27	10	9	9	8
10,000	145	60	27	10	9	9	8
>/=100,000	150	60	30	10	9	9	8

Confirmation of suspect clinical WSD

For confirmation of a suspected outbreak, animals that are representative of those showing clinical and/or gross signs should be sampled. Whole animals, haemolymph, gills, stomach, abdominal muscles and pleopods provide suitable specimens for examination. Although dead animals can sometimes provide useful diagnostic information, they are often unsuitable for examination because of the rapid onset of postmortem changes (Lightner, 1996; Afsharnasab, 2007a). There is a higher probability of detecting the virus in crabs than in shrimp. The best life stages of crustaceans for detection are late PL stages, juveniles and adults. Probability of detection can be increased by exposure to stressful conditions (e.g. eye-stalk ablation, spawning, moulting, changes in salinity, temperature or pH, and during plankton blooms, (Lightner, 1996; OIE, 2010).

Two-Step PCR is the preferred test and follow-up bioassay to confirm the presence of viable virus in PCR-positive samples may be required (Lightner, 2005; Sritunyalucksana *et al.*, 2006). Two-step PCR and sequencing are recommended methods as well for declaring freedom of a country/zone/compartment, only for juveniles and adults and possibly PLs. For such purpose, Two-step PCR negative results are required. Where a two-step PCR positive result cannot be confirmed by sequencing, this counts also as a negative result (OIE, 2010).

In non-destructive screening by PCR, it is recommended by OIE aquatic manual

(2010) to submit (a small piece of) gill, (a small aliquot of) haemolymph or (a small piece of) pleopod. There is also some evidence to suggest that an ablated eyestalk would be a good alternative, provided that the compound eye is removed prior to submission since it may contain a PCR inhibitor (Vaseeharan *et al.*, 2003).

Definition of a suspect case of WSD, according to OIE aquatic manual (2010), for juvenile and adult shrimps are gross signs of WSD, which for shrimp at any life stage (larva to adult) is mortality, and for shrimp and crab at any life stage (larva to adult) are hypertrophied nuclei in squash preparations of gill and/or cuticular epithelium; unusual aggregates in hemolymph by dark-field microscopy, inclusion bodies in histological sections in target tissues. Suspect cases should first be checked by PCR. If in a previously WSSV-free country/zone/compartment, PCR results were positive, then, they should be confirmed by sequencing. Histopathology, probes and electron microscopy can also be used to confirm the cases.

Environmental factors and WSSV infection

Infection and disease caused by WSSV in both cultured and wild penaeid shrimps are influenced by environment parameters (Afsharnasab *et al.*, 2006; Annies and Rosamma, 2007; Afsharnasab *et al.*, 2009b). Consequently, manipulation of these parameters can control or eliminate infections, benefiting production of cultured penaeids. Salinity, temperature and pH are important water quality variables, but they also strongly affect other water quality variables (Cheng and Chen, 2000; Chen W, 2012). Shrimp larvae are produced or captured in water with salinity of 28 to 35 ppt., but post larval stages are often stocked in ponds where salinity is much lower. When shrimp postlarvae are stocked into ponds, they should be acclimated gradually to lower salinity to reduce stress and mortality. The acclimation rate should not exceed 1 or 2 ppt change in salinity per hour (Boyd, 1990). Two species of shrimp, *L. vannamei* (90%) and *P. indicus* (10%) are commonly cultured in Iran. Best survival and growth of *L. vannamei* is at salinities above 20 ppt, but *P. indicus* will survive and grow well at higher salinity as mentioned by (Tokhmafshan, 2001; Afsharnasab *et al.*, 2008).

The smallness of the size of the ponds, cause alterations in physico-chemical properties, especially salinity and temperature, which consequently prolong the culture period in shrimp farms resulting in few epizootics of WSD in Iran in the last decade (Soltani *et al.*, 1998; Kakoolaki, 2004; Kakoolaki *et al.*, 2011b). So far, the effective impact of temperature on WSSV outbreak has not been well known. Rahman *et al.* (2007, 2006) showed that higher temperature of water with 33°C could be used to control the mortality of WSSV infected shrimps in the field. Due to the effects of temperature on metabolism, growth, survival rate and immunological criteria, it becomes one of the most important environmental factors in shrimp farms (Wyban *et al.*, 1995). Esparza-Leal *et al.* (2010) showed that if days of shrimp culture extend to autumn, the susceptibility to WSSV among exposed shrimps could

increase. The risk of WSSV outbreak is reduced when water temperature goes up and salinity fluctuation is small (Tendencia *et al.*, 2010). In contrary to the results of Sahoo *et al.* (2005), the association between WSSV outbreak and environmental risk factors such as temperature (Corsin *et al.*, 2002; Peinado-Guevara and López-Meyer, 2006), ammonia (Corsin *et al.*, 2001) and salinity (Kakoolaki *et al.*, 2011a) are evident . Load of the virus is reduced in *L. vannamei* in waters with a temperature of 32ºC (Granja *et al.*, 2006). As Kakoolaki *et al.* (2011a) mentioned minimum and maximum counts of mortality for shrimps exposed to WSSV at salinities of 30, 40 and 50 ppt were 3.5, 8.5; 0.5, 4.5 and 1.5, 7.5, respectively. No mortality was observed in untreated control groups of 30, 40 and 50 ppt during the experiment (Fig. 13). It is concluded that the higher and the lower salinities, lesser or greater than the normal condition, in which the shrimps are exposed to WSV could lead to severe mortality of WSD (Liu *et al.*, 2006; Peinado-Guevara and Lopez-Meyer, 2006). The outbreak of the WSD in Iran in 2008 (Sistan and Baluchestan Province) had occurred within a week after a heavy raining which made a rapid change in salinity (Afsharnasab *et al.*, 2009b). Water temperature has a profound effect on disease expression, with average water temperatures of below ~30°C being conducive to WSD outbreaks. Increasing the density of shrimps per pond also can act as a stressor if the ponds' environmental factors are poorly managed. In most cases of WSD outbreak in Iran, poor management in farms is believed to be the main stressor for occurrence of the disease.

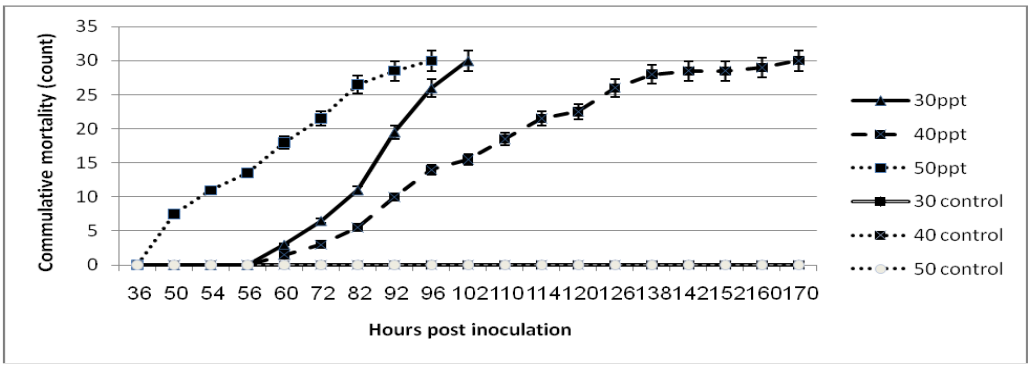

Figure 13: Cumulative mortality of each group after distinct hours post inoculation.

The optimal pH range for most species is between pH 6.5 and 8.5; outside this range direct toxic effects can occur and stress levels are high. High pH and low water temperature might be the reason for WSSV in shrimps and ultimately mass mortality. In Vietnam, although no significant association was detected with salinity, alkalinity and other water quality variables that might have acted as stressors as mentioned by Corsin *et al.*, (2001). They found also that WSD outbreaks were preceded or coincided with higher pH and un-ionized [cb4] ammonia.

Extreme pH conditions of 1.2 and 12.4 reduced the infectivity of Heliothis nuclear polyhedrosis virus (Ignoffo and Garcia, 1966), and heat inactivated cytoplasmic-

polyhedrosis viruses of *Bombyx mori* (Argua *et al.*, 1963) and *Colias eurytheme* (Tanada and Chang, 1968). Gudauskas and Canerday (1968) further demonstrated reduced infectivity of Heliothis NPV and T. ni NPV when exposed to extreme acid or alkaline conditions, UV light, and heat. In contrast, little information is available about the effects of chemical and physical conditions on the infectivity of aquatic shrimp's *Whispovirus*.

Immunostimulants and vaccine

Afsharnasab (2008) explained aquatic crustacean immunity such as shrimp, marine and fresh crab. He mentioned that the immunosystem of crustaceans is consisted of three defense mechanisms: 1) Physical and chemical defense; 2) Cellular defense; and 3) Humeral defense. The physical and chemical defense containing cuticle and skin, and both have to do with secretion, but this defense system is not sufficient to prevent enter of particles to the body, particularly the crustaceans have open circulating system. During ecdysis, crustaceans are easy targets for the attack of organisms, so they need the immunosystem to fend. Because crustaceans have open circulating system, the blood or lymph coagulation is very important. The coagulation system helps the animal to control the missing lymph and prevents diseases (Mohajeri *et al.*, 2011). In crustaceans blood is called haemolymph and blood cells are called hemocyte. The hemocyte is comprised of hyaline, granular and semigranular cells. Each of them has a main role in disease prevention (Kakoolaki *et al.*, 2010{van de Braak, 2002 #572). In addition crustaceans have a humeral defense mechanism that is based on different enzyme production and has a main role to defend the animal against microorganism (Balasubramanian *et al.*, 2008). The crustacean's innate immune system recognizes molecular patterns shared by large groups of pathogens, such as beta-glucans from fungi and lipopolysaccharides and peptidoglycans from bacteria. Also reports have shown that beta-glucan, Vitamin C, seaweed extracts (fucoidan) and other immunostimulants may improve resistance to WSD (Afsharnasab *et al.*, 2010). Several studies have shown that resistance of prawns to WSSV can be enhanced by exposure to these compounds. As their efficacy and methods of administration become better defined, immunostimulants may be used to improve the resistance of farmed crustaceans to WSSV and other pathogens in an attempt to reduce the risk of disease outbreaks. However, any benefit they may confer is likely to be minimal in adverse environments or in the absence of appropriate disease prevention strategies. Although recent studies of crustacean immunology suggest some capacity for acquired immunity, but currently no consistently effective vaccination methods have been developed. Ghaednia *et al.* (2012) reported the potency of dietary β 1,3 and 1,6 glucan (BG), derived from *Saccharomyces cerevisiae*, in stimulating the non-specific immunity of white Indian shrimp, *P. indicus* and improving its resistance to white spot syndrome disease. They also reported significant increase of parameters such as total hemocyte count

(THC), differential hemocyte count (DHC), total plasma protein (TPP), Phagocytic activity (PA), bacterial clearance efficiency (BCE) and bactericidal activity (BE) when *P. indicus* shrimps (11.32 ±1.20 g) were immersed in seawater (39 ppt and 25 ±1○C) containing hot-water extracts of the brown alga, *Sargassum glaucescens*, at 100, 300 and 500 mg/l, and *Laminaria digitata* (p<0.05, Ghaednia *et al.*, 2011). Dashtyannasab *et al.* (2009) studied complementary feedstuff extracts from *Ascophyllum nodosum* containing 1% alginic acid as shrimps stimulating immune system for the control of WSSV. The *L. vannamei* shrimps in larval stages (Z1-PL1), post larval stages (PL1-PL10) and juvenile (from 30th day to 40th day) were also fed by complimentary feedstuff as control group. Their results showed that the survival rate of exposed groups were significantly higher than that of the control group (p<0/05). Their results also showed that mortality in the exposed groups was observed 48 hours later than the control group. Many articles focused on vaccine use for controlling WSSV in shrimp. DNA vaccination, recombinant and oral vaccine, and gene therapy are some of the methods for WSSV vaccine use in shrimp. Many structural viral envelop proteins from WSSV genome are used as vaccine for protection of shrimp against the virus. The main vaccines used as mentioned in the literature consist of VP28 (Van Hulten *et al.*, 2001a; Witteveldt *et al.*, 2004a; Witteveldt *et al.*, 2004b),VP19 (Witteveldt *et al.*, 2004b), VP15 (Van Hulten *et al.*, 2001a), DNA vaccines (Rout *et al.*, 2007; Li *et al.*, 2010), or with dsRNA vaccines (Robalino *et al.*, 2006; Kim *et al.*, 2007). In Iran, Afsharnasab *et al.* (2010) explained nuclear and non nuclear methods for production of vaccine against WSSV. He used three methods, Gama radiation, electron beam and formalin with 50% protection dose for controlling WSSV in *P. indicus* shrimps. Their results showed that Gama radiation has better protection against WSSV comparing with other methods.

Past and Future of WSSV in Iran

According to FAO and GOAL 2001 (Valderrama and Anderson, 2011), world production of cultivated shrimp has increased steadily since early 1990's and reached 4.2 million tonnes in 2013 (Fig. 14). It is also known that the world shrimp fishery is not growing, while the demand for shrimp is increasing steadily. Only aquaculture can meet this increasing demand.

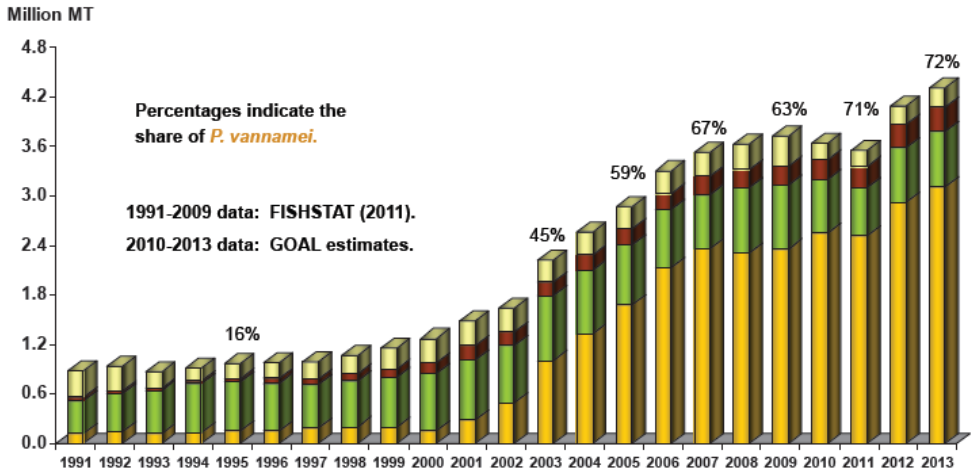

Figure 14: GOAL 2011 survey world shrimp aquaculture (Including *M. rosenbergii*) by specious. Source: FAO, 2011; GOAL, 2011.

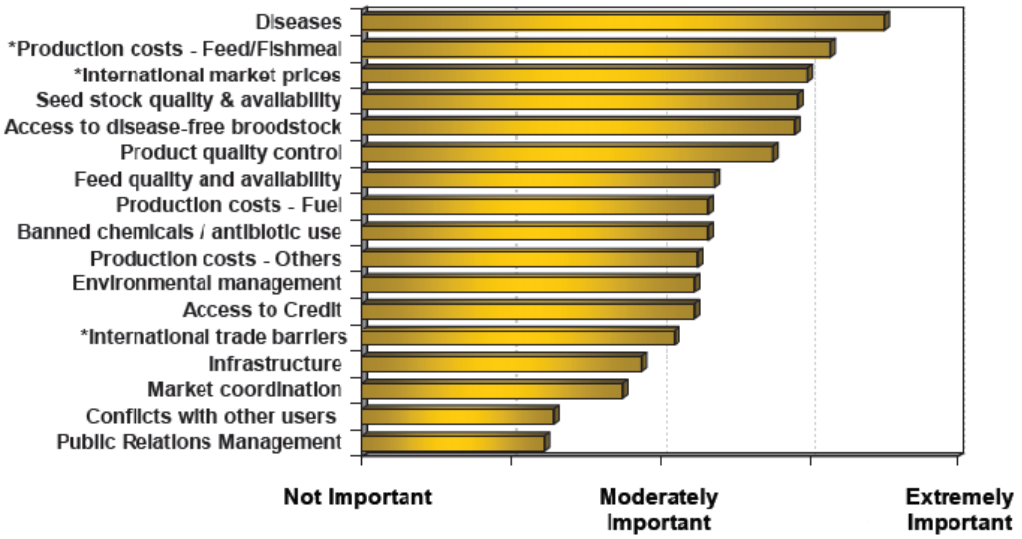

Figure 15: Issue and challenge in shrimp aquaculture in 2011 in all countries. Source: FAO, 2011;GOAL, 2011.

According FAO and GOAL (Valderrama and Anderson, 2011) shrimp production in the world in the future will face many issues and challenges (Fig. 15) that the main and extremely important issue of them is disease. Despite the explosive growth in world production of cultivated shrimp, there have also been staggering, periodic losses due to disease. A global shrimp survey by the Global Aquaculture Alliance (GAA) in 2011 revealed a rough overall loss of approximately 22% in a single year due to diseases. Given a total production of 4.2 million tonnes in 2013 with a value of roughly $ 7 per kg, this is translated into an estimate of about US$ 6 billion loss in a year. This is probably a conservative estimate, since farms with very bad results may not have responded to the survey. Thus, a conservative estimate for the total loss to disease over the past 15 years may be in the order of $ 50 billion. This

illustrates the importance of disease control to the industry. With respect to disease agents, the GAA survey revealed that 60% of losses were attributed to viruses and the rest from bacteria, fungi and protozoa. In Iran the shrimp production and impact of WSSV in this industry is divided into three phases (Fig. 16). At the first phase, from 1992 until 2001, shrimp cultivation systems were semi extensive and stocking densities were low, disease problems were relatively few and production were relatively low. In these years, there were few disease specialists available to help shrimp farmers, and diagnostic capabilities in most regions were limited. This was a vulnerable situation as the industry was growing exponentially with trends towards increasing farm densities in suitable farming areas and increasing rearing (stocking) intensity in individual ponds. In these years shrimp production increased steadily and from 16 tonnes in 1991 the total production reached 7600 tonnes in 2001. The second phase started from 2001 (Fig. 16), explosive and large-scale shrimp production of shrimp was made possible by development of eyestalk ablation technique to stimulate maturation of captured female broodstock and stocking density gradually increased, especially in Khuzestan Province. As mentioned by Afsharnasab *et al.*, (2005) and (2012) and Salehi (2010) an outbreak of WSD of cultured Indian white shrimp occurred in 2001 in semi-intensive farms in Abadan (Khuzestan Province), southwestern Iran, where it caused losses of almost 100 percent of the production. Later WSSV disease occurred in all shrimp sites in Bushehr Province, where it severely affected small-scale farms practicing high stocking density, resulting in great economic loss. In 2007, an outbreak of disease of cultured Indian white shrimp occurred in semi-intensive farms in shrimp sites of Goader (Sistan and Baluchestan Province), southeastern Iran. During these years Iran Fisheries Research Organization (IFRO) imported Specific Pathogen Free *L. vannamei* from Hawaii and used this species instead of *P. indicus* (Afsharnasab *et al.*, 2008). Many research projects have been done on this species and the results showed that this is a good shrimp species for culture in Iran's conditions (Afsharnasab *et al.*, 2007c; Afsharnasab *et al.*, 2008). Serious WSD disease outbreaks revealed during 2002 - 2008 that the shrimp industry was forced to be better prepared with more knowledge about shrimps and their pathogens so that disease prevention methods could be improved. This need shifted attention to biosecurity, that is, possible methods of cultivating shrimp in restricted systems designed to prevent entry of potential pathogens. The industry also realized that there is a good number of disease outbreaks originated from careless transboundary movement of contaminated but grossly normal aquaculture stocks. More than any other problem, the WSSV pandemic served as a "wake up" call that shocked the industry into concerted actions. The catastrophic losses had serious impacts on whole national economy as well.

The third phase of shrimp culture in Iran started from 2008 until now (Fig. 16), during which the production increased rapidly and reached to an estimated 20000 tonnes of *L. vannamei* in 2013 (Afsharnasab, 2012). We believe that the Iranian shrimp industry will be dominated

by cultivation of domesticated lines of shrimp that are free of most, if not all, of the significant shrimp diseases. Most of the stocks used will also be improved by genetic selections for growth rate and other desirable traits like disease tolerance. We already know from experience with *L. vannamei* that such stocks are highly successful when reared with good biosecurity and management of feed and pond environment. The latter can be achieved by following Good Aquaculture Practices recommended by the Global Aquaculture Alliance. Iran is also going to produce SPF *L. vannamei* shrimp and the combination of using SPF stocks and proper management would greatly reduce the risk of disease outbreaks and essentially eliminate the need for chemotherapy in the future.

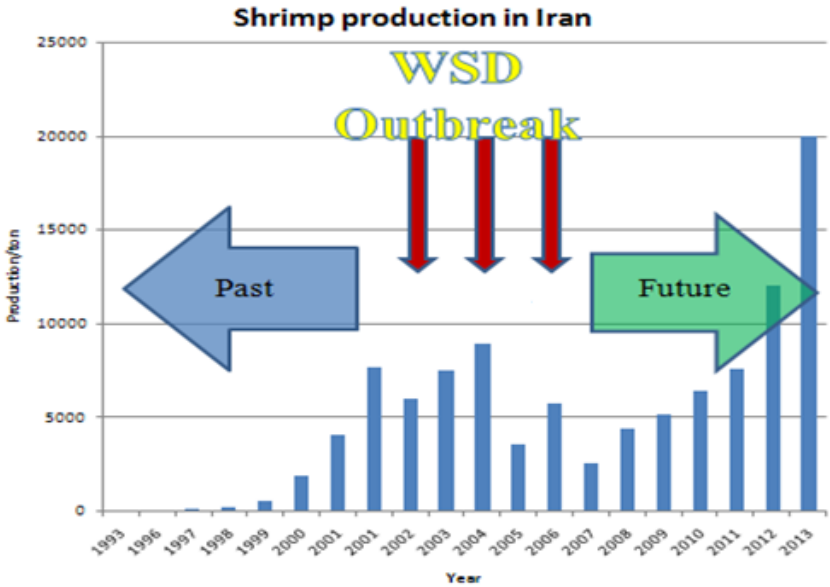

Figure 16: Past and future impact of WSSV in shrimp industry of Iran.

Discussion

The clinical signs in white spot disease affected shrimps in Iran, closely match published descriptions of white spot disease such as white spots on the carapace and appendages, and signs of lethargic and yellowish discoloration of hepatopancreas with 70-100% mortality (Chou *et al.*, 1995; Wang *et al.*, 1995; Wongteerasupaya *et al.*, 1995; Wang *et al.*, 1999). Additionally, cuticular deformities such as broken or withered antennae and damage rostrum, opaque abdominal musculature and melanised gill were observed in these shrimps. There was 70-100% mortality in white spot disease affected farms within 3-7 days after onset of the clinical signs, which was considered a characteristic of WSD (Wang *et al.*, 1995; Wongteerasupaya *et al.*, 1995; Wang *et al.*, 1999).

The diagnosis of WSSV by gross sign is difficult, because some diseases show the same symptoms. The IHHNV is a viral shrimp disease that show white spots in the body but the white spots in IHHNV first appear on the body and then on the carapace (Afsharnasab, 2007b). High pH and vibriosis also show white spots on the cuticle of shrimp (Afsharnasab, 2007b).

Diseased shrimp show widespread focal to diffused cellular degenerations and unclear hypertrophy in most tissues of ectodermal and mesodermal origin. Additionally necrosis is observed in the hepatopancreas. Necrosis, hemocytic infiltration and hypertrophied nuclei eosinophilic to basophilic inclusion bodies within the hypertrophied nuclei of affected cells, are considered as characteristics of white spot disease (Chou *et al.*, 1995; Wang *et al.*, 1999; Pazir *et al.*, 2011).

There is no treatment for viral diseases, and it's latent in healthy shrimp. The disease agents are virus that are latent and are also found in the adult stages. If the shrimp is unhealthy or in a stressed condition, caused by overcrowding, diseases may occur (Afsharnasab *et al.*, 2009a). As vaccines are not available for shrimp viruses and use of immunostimulants is far fetched in practical scale at the moment in shrimp ponds (Afsharnasab *et al.*, 2010), thus disinfectants are useful to eliminate viruses in culture systems. Based on the present study, proper management of pond water and infected shrimps are recommended as follows. (1) Drainage system with long drainage canal, and sedimentation and treatment ponds should be established as a part of farm construction. During normal culture period, all discharged water must pass through drainage system because numerous zooplankton including small wild shrimp and copepods often coexist with cultured shrimp in the ponds, and they were also found to be carriers of viruses. (2) Shrimps confirmed with viruses such as MBV and WSSV infection should be removed using a seine net. The pond water must be disinfected, and then passed to the drainage system for storage. (3) If there is no drainage system, affected shrimps and the pond water must be disinfected at once, and kept in the pond for a few days (5-10 days) before discarding. An important point: do not directly release diseased shrimp and pond water into public environment as it plays an important role in spread of the disease.

Because treatment of viral diseases in grow out ponds is expensive, best is to prevent the occurrence of these diseases. Preventing diseases are much more economic then providing expensive treatments following a disease outbreak. However, there is not a single, ideal and universal preventive program that can be applied in every procedure. With respect to prevention we recommended:

1. Development of a rapid, simple and accurate method for detection of virus infection such as PCR diagnostic kit. At present, MBV and WSSV infections may be detected for their characteristic inclusions in either wet mounts or histological preparations of hepatopancreas or midgut contents.
2. Establish virus-free broodstocks (SPF) in order to hatch free postlarvae for shrimp production farms.
3. Low stocking density, intensive culture systems encourage development and transmission of many diseases including viruses.
4. Proper feeding management, feeds must be controlled to a minimum. feed residues which increase organic wastes which adversely affect the water quality and the production
5. Use of disinfectants or chemicals to

prevent or treat bacterial sepsis or protozoal epicommensals if necessary.

6. Educate shrimp culturists and farm technicians on disease epizootiology and preventive measures.

We strongly believe that many of the disease problems encountered in aquaculture can be avoided with good management practices. In Iran the two major causes of problems for shrimp farmers are due to poor management practices and environmental stresses. Management problems are usually solved through assistance provided by government extension workers or from technical sales staff of various feed companies. Environmental problems are more difficult to solve especially when they result from natural phenomena such as red tide. When environmental problems are caused by poor farming practices, then it would be necessary to gain co-operation among all farmers in the area if disaster is to be avoided. And in some cases, government legislation may be necessary to limit practices that may threaten sustainability of the industry. Regarding sustainable shrimp aquaculture in Iran, regulations are required to control stocking density, use of chemicals and drugs in aquaculture, pond design, control water inlets and outlets, and shrimp feeding regimes. Currently Iranian Fisheries Organization (Shilat) with assistance of the government is drafting legislations for the aquaculture industry. In the larger interest of developing of a sustainable shrimp culture industry in Iran, the following measures are recommended: i) establish diagnostic laboratories, ii) provide training for the farmers, iii) establish practical guidelines and regulations for farmers and iv) conduct regular monitoring on the use of chemicals.

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