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Asian Journal of Animal and Veterinary Advances

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Asian Journal of Animal and Veterinary Advances 4 (6): 297-305, 2009
ISSN 1683-9919

Gross Sign, Histopathology and Polymerase Chain Reaction Observations of White Spot Syndrome Virus in Shrimp Specific Pathogen Free *Litopeneaus vannamei* in Iran

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Abstract: The importation of *Litopenaeus vannamei* to Iran from Hawaii was initiated when Iranian shrimp culture was first affected by WSSV in 2004. The main reason for the importation of *L. vannamei* to Iran was the disease susceptibility and mass mortality of the indigenous species (*P. indicus*) when faced with the first outbreak of WSSV. During the two years of study, it was found out that culturists in Iran preferred cultured *L. vannamei* than the local species (*P. indicus*). In 2008, mass mortality occurred in farmed *L. vannamei* in Khuzestan Province South of Iran. Two hundred shrimps with white spot on the carapace and body were collected and preserved in Davidson fixative for histopathology. A part of samples collected were also preserved in 95% ethyl alcohol for Polymerase Chain Reaction (PCR) technique. Two pair primers from VP24 WSSV genome was identified and used for PCR while identified one pair primer for 18SrRNA gene of shrimp was used as house keeping gene in PCR reaction in both positive and negative PCR reaction. Grossly, the samples showed white spot in the cuticle and body surface and red color on the appendages. Histopathologically, all tissue except hepatopancreas showed the intranuclear Cowdry type-A inclusion bodies. PCR studies using designated primer revealed a band of 414 bp from WSSV and 809 bp of shrimp DNA fragments in positive samples. The negative samples showed just 809 bp. This is the first report of White Spot Syndrome Virus (WSSV) in farmed *L. vannamei* in Iran.

Key words: *Litopenaeus vannamei*, gross sign, histopathological changes, PCR, WSSV, Iran

INTRODUCTION

To date, over 20 viruses have been reported from penaeid shrimp in the world. Viruses that seem to cause little or no disease and seem iunocuous in some shrimp species may cause catastrophic disease in others (Lightner and Redman, 1998). With respect to *L. vannamei* three viruses, WSSV, Tauar Syndrome Virus (TSV) and Infection Hypodermal and Hematopoietic Necrosis Virus (IHHNV) have been detected and they have become a major limiting factor for development of cultured shrimp industry (Flegel, 2006; Brock and Main, 1994; Lightner, 1996).

The WSSV is an enveloped, double stranded DNA virus, ovoid to bacilliform in shape with a tail like extension at one end (Van Hulten *et al*., 2001; Yang *et al*., 2001). The virus is the only member of the family *Nimaviridae*, genus *Whispovirus* (Mayo, 2002). The WSSV is pathogenic to at least 78 species, maiuly to decapods crustaceans including marine and freshwater shrimp, crab, crayfish and

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lobsters (Lightner, 1996; Flegel, 2006). The first outbreak due to WSSV was reported in shrimp farms in Taiwan in 1992 (Chou *et al*., 1995) followed by other shrimp farming countries of South East Asia, Middle East, North, Central and South America (Lightner, 1996; Rosenberry, 2002; Rodríguez *et al*., 2003; Flegel, 2006).

The route of WSSV entry and spreading mechanism among the tissues has recently been shown by Escobedo-Bonilla *et al*. (2007). Gills and cuticular epithelium of foregut in *L. vannamei* are portals of entry after oral inoculation of WSSV. WSSV infected shrimp display clinical signs such as anorexia, lethargia, swollen branchiostegites due to fluid accumulation, white spots in the cuticle, separated loose cuticle from underlying epidermis, yellowish-white and enlarged hepatopancreas, hemolymph which fails to coagulate and reddish discoloration of the moribund shrimp (Lightner, 1996; Sahul Hameed *et al*., 1998; Wang *et al*., 1999; Flegel, 2006). Clinical signs do not allow a diagnosis of WSS (Flegel, 2006) because anorexia is observed in uninfected shrimp before and after molting (Jory *et al*., 2001), white spots in the carapace can also be caused by bacterial infection (Wang *et al*., 2000) and other clinical signs are unspecific and common to other diseases. In laboratory challenge tests, WSSV as sole pathogen may cause disease and mortality in SPF *L. vannamei* and other shrimp and crayfish species. In case of natural infection, several biotic and abiotic factors may influence the course of a WSS outbreak. Co-infections of different viruses including Hepatopancreatic Parvolike Virus (HPV), *Penaeus monodon* Baculovirus (MBV) and IHHNV together with WSSV have been reported (Manivannan *et al*., 2002; Flegel *et al*., 2004; Umesha *et al*., 2006). The aim of this study was to detect WSSV in shrimp SPF *L. vannamei* cultured in Iran.

MATERIALS AND METHODS

During the period of August to December 2008, mass mortality occurred in farmed *L. vannamei* in Khuzestan Province along the coast of Persian Gulf of the I. R. Iran (Fig. 1).

About two hundred moribund specimens of cultured *L. vannamei* were collected randomly from 25% (20 from 80 farms) of all growouts farms according to Lightner (1996).

All the specimens collected were transported in a container with aerators to the South Aquaculture Research Centre (SARC) in Khuzestan Province. Samples for histolopathology had been

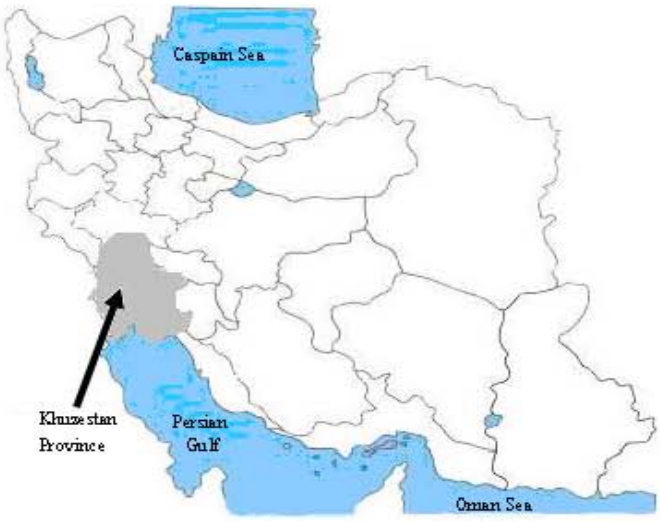

Fig. 1: The map of Iran and area of WSSV outbreak in Khuzestan Province

subcollected randomly and preserved in Davidson's fixation (Humason, 1979). After 24 to 48 h in Davidson's fixative, preserved shrimp were transferred to 50% ethyl alcohol for storage. The hepatopancreas, lymphoid organ, gills and midgut had been prepared for light microscopy using the routine paraffin techniques, sectioned at 5-6 μm thickness and stained with H and E (Bell and Lightner, 1988; Humason, 1979; Luna, 1968).

The used primers, PCR reaction and amplification program were carried out as described by Saberi *et al*. (2008) (No. DQ196431).

RESULTS

In this study based on the gross sign, target-affected organs, locations of the Inclusion Bodies (IBs) as the specification of the known virus and PCR indicate the presence of WSSV in the obtained samples. The gross sign of WSSV in *L. vannamei* observed include lethargic behavior in affected animal, cessation of feeding, followed within a few days by the appearance of moribund shrimp swimming near the surface at the edge of pond. Pink to reddish-brown discoloration of the body and white spot of about 0.5-2 mm on the cuticle (Fig. 2) especially on the inner surface of the exoskeleton of cephalothorax and abdomen. The cuticle easily separate from the underlying epidermis and the hepatopancreas become yellowish-white with a swollen and fragile texture (Fig. 3). Cuticular deformities such as broken or withered antennae and damage rostrum, opaque abdominal musculature and melanised gill were consistently observed. There was 70-100% mortality in white spot disease affected farms within 7-30 days after the onset of the clinical signs.

The histopathology of WSSV in the *L. vannamei* was dominated by the presence of large conspicuous intranuclear eosinophilc Cowdry A-type inclusion bodies in the tissue. The tissue section of cuticular epithelium of shrimp stained with H and E showed the intranuclear eosinophilic Cowdry A-type inclusion bodies. The cuticular epithelium was separated from the connective tissue and inclusion bodies are centronuclear and segregated from the membrane. With the progress of infection the inclusion bodies were separated by a halo from the marginal chromatin (Fig. 4). The section of the gill from *L. vannamei* infected with WSSV revealed hypertrophoied cells and the presence of intranuclear Cowdry A type also was present. The presence of the Cowdry A-type and many basophilic nuclei in this section is typical of early stage of WSSV infection (Fig. 5). The high magnification of tissue section from heart displays many free inclusion bodies in tissue. In the late stage of WSSV infection the infected cell ruptured and inclusion bodies release from cell (Fig. 6). The lymphoid organ of shrimp is a target tissue for WSSV and the section of this tissue revealed many

Fig. 2: White spot on the cuticle of infected shrimp (arrows)

Fig. 3: Comparing the infected shrimp *L. vannamei* with normal shrimp. The infected shrimp showed opaque mussels and the carapace separate easily from cuticle

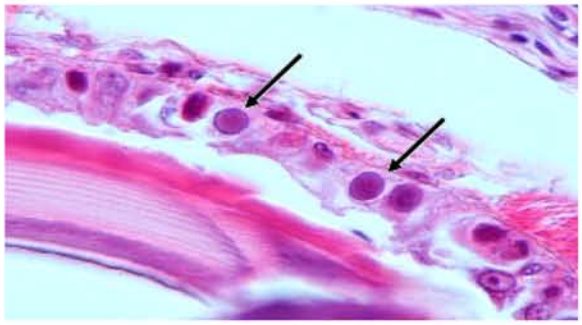

Fig. 4: Cuticular epithelium with large basophilc intranuclear inclusion bodies (arrows) characteristic of white spot syndrome virus (WSSV) in *L. vannamei* in Iran (H and E.100X)

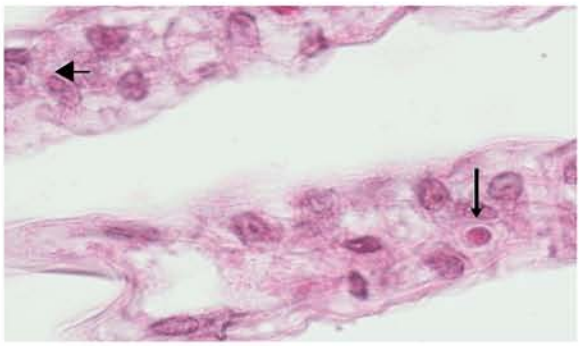

Fig. 5: Intranuclear inclusion bodes characteristic of WSSV infection (arrow) in the gill tissue cells of *L. vannamei* showing signs of WSSV (H and E.100X)

larger, more fully developed, without halo inclusion bodies and contains a single inclusion body (Fig. 7). However, the virus did not infect the Hepatopancreatic Epithelial Cell (HEC), even in moribund specimen.

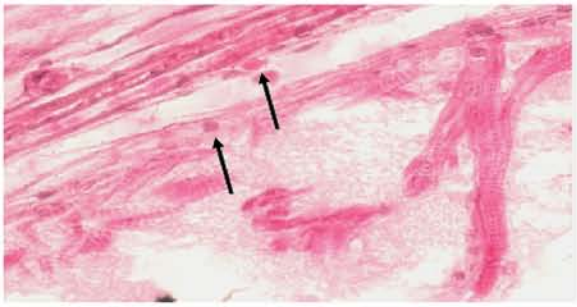

Fig. 6: Intranuclear inclusion bodes characteristic of WSSV infection (arrow) in the heart tissue cells of *L. vannamei* showing signs of WSSV (H and E.100X)

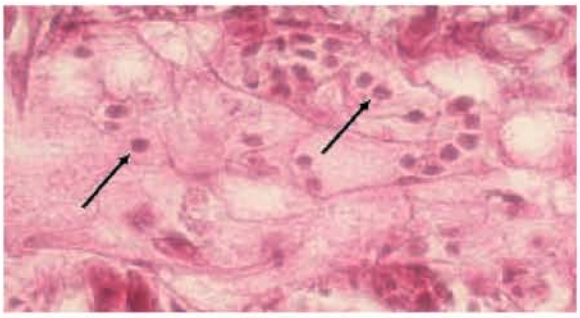

Fig. 7: Intranuclear inclusion bodes characteristic of WSSV infection (arrow) in the haematopoeitic tissue cells of *L. vannamei* showing signs of WSSV (H and E.100X)

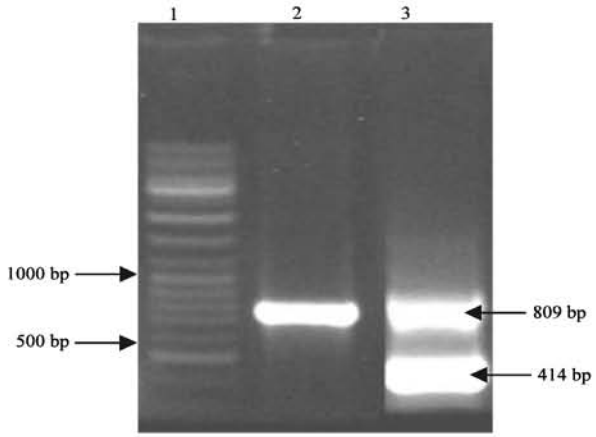

Fig. 8: Photograph of agarose gel electrophoresis of PCR product of the gill and hepatopancreas samples obtained from affected *L. vannamei*, Lane 1: 100 bp DNA ladder, Lane 2: Negative sample, Lane 3: Positive sample

The result from PCR showed two bands of 414 and 809 bp fragments after 30 cycles of amplification of the viral genomics (Fig. 8). Positive samples infected with WSSV show amplification

of a 414 bp viral DNA band for infected samples, while the 809 bp shrimp DNA product (false negative control) in the positive samples confirms the validity of the obtained result.

DISCUSSION

Iran has ambitious plans for expansion for the shrimp culture industry from 10,000 MT in the year 2002 to 100,000 MT by 2020. The rapid expansion of culture of the penaeid shrimp in Iran has been accompanied by the recognition of penaeid disease that are of viral etiology. In 2004 and 2006, Iranian shrimp industry was challenged by White Spot Syndrome Virus (WSSV) in shrimp *P. indicus* and mass mortality occurred during these years (Afsharnasab *et al*., 2006). Comparing the mortality rate and virulence of WSSV in *P. indicus* as occurred in 2004 and 2006 with *L. vannamei* in 2008, the time to reach peak mortality of 100% in *L. vanamei* was longer (7-30 days) than the *P. indicus* (3-7 days). As mentioned by Sudha *et al*. (1998) natural outbreaks of WSSV are categorized into peracute, acute to subacute and chronic forms, where mortality occurs within 2-3 days, 7-10 days and 15-28 days, respectively. In this regard the outbreak of WSSV in *P. indicus* as occurred in 2004 and 2006 was the acute form and outbreak of WSSV in 2008 in *L vannamei* was the chronic form. Earlier study showed 70-100% mortality in white spot disease affected farms within 3 days after onset of the clinical signs in *P. monodon* and *P. japonicus* (Momoyama *et al*., 1994; Takahashi *et al*., 1994; Wang *et al*., 1995). Different in virulent between WSSV in *L. vannamei* and *P. indicus* in Iran may be due to the susceptibility of different species, the defense mechanism in these species and environmental factors in Iranian condition. The studied by Granja *et al*. (2003) showed number of apoptotic cells in shrimp *L. vannamei*, reduce viral replication, allowing the shrimp to control the disease and survive. Briggs *et al*. (2004) also reported *L. vannamei* is generally considered to be more resistance than other shrimp to WSSV. The present findings agree with previous works where mortality time was longer in WSSV-infected *L. vannamei* than other species (Vidal *et al*., 2001; Granja *et al*., 2003, 2006).

In histopathological finding of WSSV outbreak of *L. vannamei*, Cowdry type A inclusions are present, which are characterized by marginated chromatin separated from nucleoplasm. As mentioned by Flegel (2006) histological signs of WSSV infection include enlarged nuclei in tissues of ectodermal and mesodermal origin. The most convenient tissue for diagnosis is the subcuticular epithelinm. In the current work, the subcuticular epithelium of stomach provides excellent view of inclusion bodies (Fig. 4). These finding have been described as some microscopic characteristics of white spot syndrome disease by Nash and Akarajamon (1995), Chou *et al*. (1995) and Wang *et al*. (1999). In histopathology WSSV differs from the other penaeid viruses as well IHHNV that showed the Cowdry type A inclusion body and white spot in the cuticle. In histopathology of IHHNV with H and E staining, the Cowdry type-A inclusion occurred in eularged nuclei as eosinophilic often haloed inclusion surrounded by marginated chromatin in tissue of ectodermal and mesodermal origin (Alday de Graindorge and Flegel, 1999; Lightner, 1996), while the Cowdry type-A inclusion body in WSSV is basophilic (Flegel, 2006; Lightner, 1996). The white spot in WSSV is found on the carapace and cuticle of body surface, while in the IHHNV the white spot is on the 3 to 6 segment of the shrimp's body (Lightner, 1996).

The identification of WSSV by PCR has been developed through a nnmber of researchers, from different countries such as Taiwan, Thailand, Japan and India by designing the different primers. Wongteerasupaya *et al*. (1995) reported an average size of about 168 kbp for SEMBV genomic DNA fragment in the agarose gel. Similar result was obtained by Wang *et al*. (1995), who estimated the genomic DNA above 150 kbp for the viral agent associated with WSSV in *P. monodon*. In Thailand the Thai National Centre for Genetic Engineering and Biotechnology, Bangkok design a primer with sequence of 232 bp WSSV fragment. In the current work we identified the WSSV in sample with a primer design originally by VP24 with 414 bp and the result from PCR support the histopathology and gross sign of WSSV in shrimps in Iran.

Beside the advantages of *L. vannamei* for Iranian shrimp culture, many risk factors and threats must be considered during introduction. As mentioned by Lightner *et al*. (1989), most of the shrimp viruses have been extensively distributed geographically due to the frequent transfer of shrimp from place to place for aquaculture purposes. It is widely believed that viruses such as Taura Syndrome Virus (TSV) and Infectious Myonecrosis Virus (IMNV) have been introduced to Asian countries through the uncontrolled introduction of *L. vannamei* (Briggs *et al*., 2004). Iran imported the SPF *L. vannamei* for culture study, but there is significant confusion in Asian country regarding the exact meaning of SPF. As mention by Lotz (1997) SPF refers ouly to the present pathogen status for specific pathogen and not to pathogen resistance or future pathogen status.

As mentioned by Briggs *et al*. (2004), there is no mortality evidence from WSSV in SPF *L. vannamei* cultured in Asian countries such as Thailand, but in Iran the mortality in shrimp farm SPF *L. vannamei* might be due to climatic condition or the evolution of new strain of WSSV and this calls for further studies.

The careless importation or poor management practices in the culture industry could induced stress in shrimp population and if the virus is latent in the population or present in the environment, there may be outbreak of the disease. Therefore, the following prevention measure may be embark upon to avoid future disease outbreak and to ensure maximum productivity with its attendant economic gains.

- Bringing of Specific Pathogen Free (SPF) and genetically improved (selective breeding method) brood stock from other countries should be first priority to produce post larvae
- Screening of virus throughout the hatchery cycle from broodstock to post larvae prior to stock in cultured pond by two-step PCR technique
- Maintain the proper quarantine under biosecurity principle during hatchery productions
- Proper pond preparation should be done prior to stock of post larvae

ACKNOWLEDGMENTS

This study was supported by Iranian Fisheries Research Organization. The authors would like to thank Dr. Abbas Ali Motalebi, Head of Iranian Fisheries Research Organization, Dr. Jasem Merameza, Head of South Aquaculture Research Center and Dr. Khosro Aeinjamshied, Head of Shrimp Research Center from Iran, for their support and encouragement.

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