Titration of the Iranian White Spot Virus isolate, on Crayfish *Astacus leptodactylus* and *Penaeus semisulcatus*

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Abstract

White Spot Virus (WSV) is currently the most serious viral pathogen of shrimp worldwide; it causes mortality up to 100% within 7-10 days in commercial shrimp farms. Infected Indian white shrimp *Fenneropenaeus indicus* samples were collected from Guatr shrimp site in Sistan and Baluchestan province in south of Iran and WSV infection was confirmed by Nested PCR**.** WSV was isolated from infected shrimp samples by centrifugation and filtration and multiplied in crayfish by intramuscular inoculation, the isolated virus was called WSV/IRN/1/2010. In order to determine the dilution resulting in 90-100% mortality in *Penaeus semiculcatus,* diluted virus stock in steps from 10^0 till 10^5 times in sterile PBS was injected intramuscularly to 14 shrimps in each group. Also the virus stock was diluted in steps from 1/2 till 1/32 times in sterile PBS and injected intramuscularly in *Astacus leptodactylus* crayfish. Therefore the LD_{50} of live virus stock in *Astacus leptodactylus* and *Penaeus semiculcatus* crayfish were calculated by the Karber method $10^{3.29}$ /ml and $10^{5.35}$ /ml, respectively.

Keywords: White Spot Virus, Titration, Karber Formula, *Astacus leptodactylus*, *Penaeus semisulcatus*

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Introduction

White Spot Virus (WSV) belongs to the *Whispovirus* genus, from the *Nimaviridae* family. It can infect not only shrimp but also other marine and freshwater crustaceans, including crab and crayfish (Namikshi et al., 2004). WSV is currently the most serious viral pathogen of shrimp worldwide, causing mortality up to 100% within 7-10 days in commercial shrimp farms. WSV virions are ovoid- to-bacilliform in shape and have a tail-like appendage at one end. The virions can be found throughout the body of infected animals, infecting most tissues and circulating ubiquitously in the hemolymph. Sequencing of the WSV genome revealed a circular sequence of 292967 base pairs, but there is variation in size in geographic isolates of WSV (Witteveldt et al., 2004). WSV infection is now found in most shrimp farming areas of the shrimp farming industry. Preventative measures to control the disease such as vaccinating against the virus would be highly desirable. It is well known that crustaceans lack a truly adaptive immune response system and appear to rely on a variety of innate immune response systems to rapidly and efficiently recognize and destroy non-self materials (Deachamag et al., 2006). In many Asian shrimp species the acute phase of disease is characterized by the presence of white spots on the inner surface of the exoskeleton (Lo et al. 1996) from which the disease name is derived. Several decapod crustaceans (Chang et al. 1998, Sahul-Hameed et al. 2003) and shrimp species (Wongteerasupaya et al. 1996, Chou et al. 1998, Wang et al. 1999) are susceptible to WSV infection. Several experiments have been carried out with WSV to determine its pathogenicity in crustacean hosts using (1) intramuscular (i.m.) inoculation (Jiravanichpaisal et al. 2001), (2) the *per os* route by feeding WSV-infected tissues to experimental animals (Rajendran et al. 1999; Wang et al. 1999) and (3) immersion (Chou et al. 1998, Rajan et al. 2000). A standardized inoculation procedure requires 2 major components: (1) the use of animals with low genetic variability and high susceptibility to the virus (2) a WSV stock with a known titer of infection. Such a standardized procedure is essential (1) to compare the susceptibility of different host species, (2) to determine the virulence of different WSV strains, and (3) to test the efficacy of strategies aimed to control the disease. To date, no shrimp cell cultures are available for *in vitro* titration of WSV; therefore, *in vivo* titration is the only alternative (Escobedo-Bonilla et al., 2005).

The aims of the present study were to determine the lethal dose 50% endpoint (LD50/ml) of an Iranian isolate of WSV (WSV/IRN/1/2010) in two host species (*Astacus leptodactylus* and *Penaeus semisulcatus)* by i.m. inoculation and to establish the relationship between WSV infection and mortality for the two host species.

Materials and methods

Sampling

The infected Indian white shrimp *Fenneropenaeus indicus* samples were collected from Guatr shrimp site in Sistan and Baluchestan province in south of Iran. The sampling was done from two shrimp

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farms and about 50 samples were collected from each farm randomly and moved to the aquaculture laboratory in Karaj.

The WSV infection was confirmed in all shrimp samples by Nested PCR (IQ 2000 kit). Briefly, 200 mg of shrimp tissue was mixed by 500 µl Lysis Buffer and homogenized exactly. The prepared sample was incubated at 95^{O}c for 10 minutes and centrifuged at 12000 g for 10 minutes. 200 ul of the upper clear solution was mixed with 400 ul 95% ethanol, centrifuged at 12000 g for 5 minutes, dried the pellet and dissolved by ddH_2O. First PCR reaction reagent mixture was included, 7.5 ul first PCR PreMix and 0.5 ul IQzyme DNA Polymerase (2U/ul). Nested PCR reaction reagent mixture was included, 14 ul Nested PCR PreMix and 1 ul IQzyme DNA Polymerase (2U/ul). For each reaction at least one positive standard and one negative control (ddH2O or Yeast tRNA) was need. Eight ul of first PCR reaction reagent mixture and 2 ul of the extracted sample or standard DNA were added into each reaction mixture and the first PCR reaction took place. After the first PCR was completed, 15 ul of nested PCR reaction reagent mixture was added to each tube, then nested PCR reaction took place. After nested reaction was completed, 5ul of 6X loading dye was added to each tube, mixed well and used in electrophoresis (WSSV instruction manual, 2010).

Isolation of WSV and virus multiplication in crayfish

The infected shrimp samples were homogenized by TN buffer (Tris-HCl 20 mM, NaCl 400 mM, pH 7.4) at ratio 1/5, then centrifuged 1700 g, 10 min at 4 ºC, the supernatant was isolated and filtered by 0.45 µm filter. The filtered supernatants of infected shrimp were used for injection to the crayfish. The *Astacus leptodactylus* crayfish were prepared from Orumyieh in the north western Iran and moved to the aquaculture laboratory in Karaj. Some of the crayfish were examined for WSV by Nested PCR (IQ 2000 kit) randomly. The filtered supernatants which contain WSV were injected intramuscularly to the third and fourth abdomen segments of crayfish by 26-G needles. After 3, 5 and 10 days the haemolymph was withdrawn with anti coagulation solution (20.8 g glucose, 8 g citrate sodium, 3.36 g EDTA, 22 g NaCl per one liter Distilled water). The WSV infection was confirmed in the haemolymph samples by Nested PCR (IQ 2000 kit). The WSV virus stock was produced in the *Astacus leptodactylus* crayfish by intramuscular injection. The infected crayfish haemolymph samples were centrifuged 4000 g, 10 min at 4 ºC, and the supernatant of haemolymph was used as the WSV stock for titration (Huahua et al., 2006).

In vivo virus titration in Penaeus semiculcatus

In order to determine the dilution resulting in 90-100% mortality in the green tiger prawn, *Penaeus semiculcatus*, an *in vivo* virus titration was performed using animals approximately weighing 1 gram. The WSV stock was diluted in steps from 10^0 till 10^5 times in sterile PBS and for each dilution 10 µl was injected intramuscularly into 14 shrimps. The shrimps which were injected with sterile PBS, served as negative control for the

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infection. All shrimps serving as negative control survived, whereas mortality due to WSV infection occurred in all groups with a virus dilution during one week. All the dead shrimps were examined for WSV by Nested PCR (Witteveldt et al., 2004).

In vivo virus titration in Astacus leptodactylus crayfish

In order to determine the dilution resulting in 90-100% mortality in the crayfish, *Astacus leptodactylus*, an *in vivo* virus titration was performed using animals approximately weighing 20 grams. The WSV stock was diluted in steps from 1/2 till 1/32 times in sterile PBS and for each dilution 300 µl was injected intramuscularly into five crayfish. The crayfish injected with sterile PBS, served as negative control for the infection. All crayfish serving as negative control survived, whereas mortality due to virus infection occurred in the groups with a virus dilution during one month. All the dead crayfish were examined for WSV by Nested PCR (IQ 2000 kit).

Karber method

After a virus was propagated in either cell culture or in a suitable animal, the infectivity titer of the virus material was obtained by a 50% endpoint. This was determined in vivo by inoculating increasing dilutions of the virus material to a susceptible host animal or cell culture. Based on mortality seen in different dilutions, the infectivity titer was the reciprocal of the highest dilution showing a 50% mortality in the inoculated animals, expressed as LD_{50} per ml and calculated by using either Karber formula or Reed-Muench (Ravi et al. 2010).

Karber formula is the simple equation; Log LD_{50} = X – D (Sp – 0.5). At first, virus dilution proportion (p) of infected animals was calculated, using a positive fraction in each dilution. In the Kaber formula X is the last dilution index for which all n shrimps are infected (p=1). D is the log of the dilution factor (log 10 = 1). Sp is the summation of p between the last dilution for which all n shrimps are infected (p=1) and the first dilution for which all n shrimps are unaffected (p=0) (Karber, 2002).

Results

The WSV infection was confirmed by Nested PCR (IQ 2000 kit) in the infected Indian white shrimp *Fenneropenaeus indicus* samples which were collected from Guatr shrimp site in Sistan and Baluchestan province (Fig. 1). According to Fig. 1 and guidance of IQ 2000 Nested PCR kit, the positive control in Lane 1 showed 20 copies of WSV DNA templates per reaction. Also lanes 2, 3, 4, 6, 7 and 8 were the light positive shrimp samples, and lane 5 was Molecular weight marker of IQ 2000 kit; 848 bp, 630 bp, 333 bp. Some of the crayfish tissues were examined for WSV by Nested PCR (IQ 2000 kit) randomly (Fig. 2). In Fig. 2, sever and light positive control showed 2020 copies of WSV DNA template per reaction, respectively. Also the uninfected crayfish tissues didn't show any DNA bands like negative control.

All the haemolymph samples of infected crayfish were examined by Nested PCR (IQ 2000 kit) (Fig. 3). According to Fig. 3, the haemolyph and tissue samples of infected crayfish in the third and fifth days

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post injection of WSV stock were negative just like negative control, but the haemolyph and tissue samples of infected crayfish in ten days post injection were positive. Also mortality was started on the 25_{th} day post infection in crayfish, and proceeded till the 30_{th} day. Therefore WSV propagation in *Astacus leptodactylus* crayfish need ten days, at least, and the infected crayfish haemolymph must be collected between 10 and 25 days post infection as virus stock. This WSV virus stock which was isolated from Iran is called WSV/IRN/1/2010.

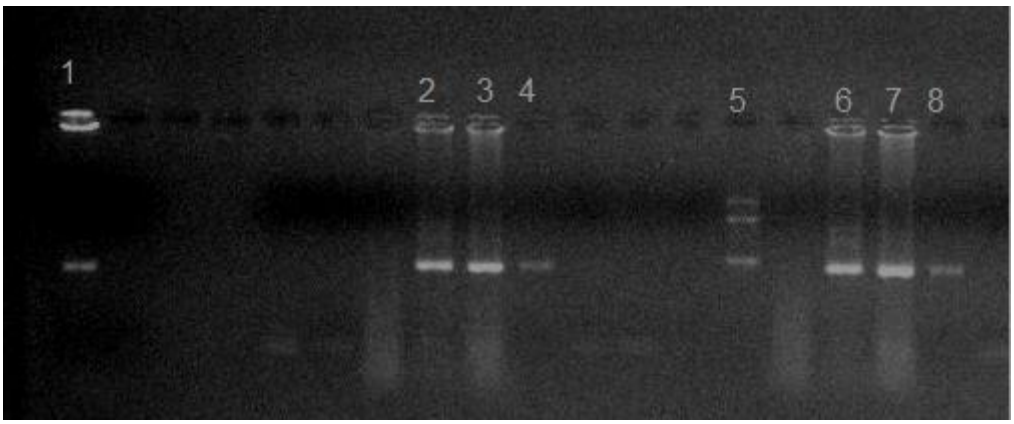

Figure 1: The infected Indian white shrimp, *Fenneropenaeus indicus* samples were collected from Guatr shrimp site in Sistan and Baluchestan province, lane 1: positive control, lanes 2, 3, 4, 6, 7 and 8 are the shrimp samples lane 5: Molecular weight marker of IQ 2000 kit; 848 bp, 630 bp, 333 bp.

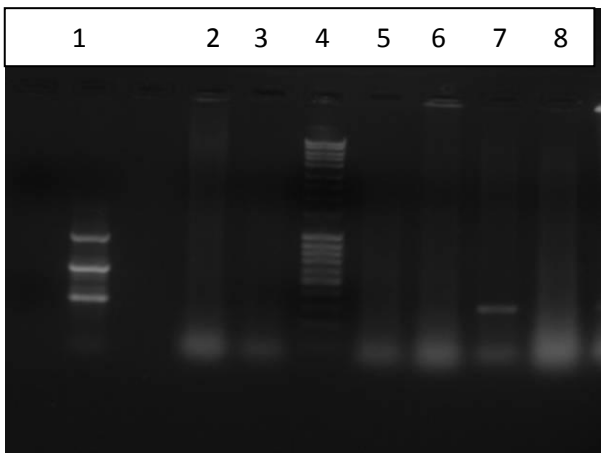

Figure 2: Uninfected crayfish, lane 1: severe positive controls; lanes 2, 3, 5 and 6 are the uninfected crayfish tissues; lane 4: DNA ladder (10000 – 80 bp, 1 kb Fermentase DNA Ladder # 0403); lane 7 is light positive control and lane 8 is negative control.

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In vivo WSV titration in *Penaeus semiculcatus* and *Astacus leptodactylus* crayfish were calculated by the Karber method and reported $10^{5.35}$ per ml and $10^{3.29}$ per ml, respectively (Karber, 2002).

The virus dilution proportion (p) in the infected *Penaeus semiculcatus* groups is shown in table 1. According to virus dilution proportion (p) in table 1 and the Karber formula, LD_{50} of WSV stock in *Penaeus semiculcatus* was calculated; Log LD_{50} = -2 – 1 (1.92-0.5) =-3.42 $LD_{50} = 10^{3.42}$ / 0.01 ml = $10^{5.42}$ / ml.

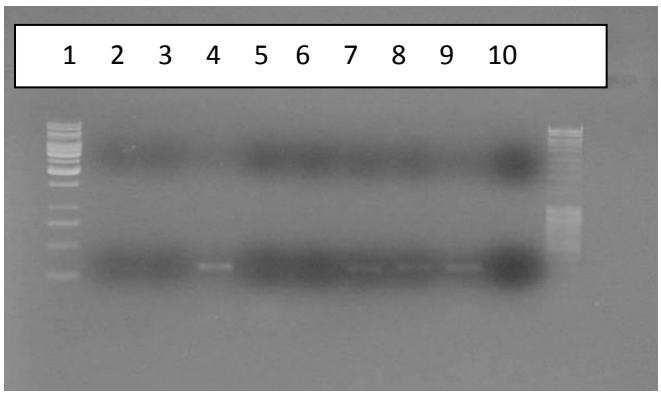

Fig 3: The haemolymph of infected crayfish, lane 1: DNA adder (10000 – 250 bp, 1 kb Fermentase DNA Ladder # 0313), lanes 2, 3 and 4: infected haemolyph in third, fifth and tenth days post injection; lanes 5, 6 and 7: tissue of infected crayfishes in third, fifth and tenth days post injection; Lanes 8 and 9: light positive controls and lane 10: negative control.

Virus dilution proportion (p) in the infected *Astacus leptodactylus* crayfish groups is shown in table 2. LD_{50} of WSV stock in *Astacus leptodactylus* was obtained by virus dilution proportion (p) in table 2 and the Karber formula; Log LD_{50} = - 0.9 – 0.3 (4.8-0.5) =-2.19 $LD_{50} = 10^{2.19}$ / 0.1 ml = $10^{3.19}$ / ml

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Table 1: Virus dilution proportion (p) in the infected *Penaeus semiculcatus*

Dilution	Number in each group (n)	Positive fraction	Virus dilution proportion (p)
1	14	14/14	1
10^{-1}	14	14/14	1
10^{-2}	14	14/14	1
10^{-3}	14	9/14	0.64
10^{-4}	14	3/14	0.21
10^{-5}	14	1/14	0.07

Table 2: Virus dilution proportion (p) in the infected *Astacus leptodactylus* crayfish

Dilution	Number in each group (n)	Positive fraction	Virus dilution proportion (p)
1	5	5/5	1
1/2	5	5/5	1
1/4	5	5/5	1
1/8	5	5/5	1
1/16	5	2/5	0.4
1/32	5	2/5	0.4

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Discussion

One of the most important procedures in virology is measuring the concentration of a virus in a sample, or the virus titer (Flint et al., 2004). The *in vivo* titration of viral stocks using the 50% endpoint dilution assay is commonly used when virus titers cannot be calculated *in vitro* (Flint et al., 2004). In any biological quantitation, the most desirable endpoint is one representing a situation in which half of the inoculated animals or cells show the reaction (death in the case of animals and CPE for cells) and the other half do not. In other words, the endpoint is taken as the highest dilution of the biological material, which produces desired reaction in 50% of the animals or cells. The 50% endpoint can be based on several types of reactions. The most widely used endpoint, based on mortality, is the LD_{50} (50% lethal dose). This terminology can also be applied to other host systems for example, tissue cultures in which the $TCID_{50}$ represents the dose that gives rise to cytopathic effects in 50% of inoculated cultures. When computing, if closely-placed dilutions are used and in each dilution large numbers of animals or cells are used, it may be possible to interpolate a correct 50% end point dilution, but it is neither practical nor economical. Reed and Muench and Karber devised a simple method for estimation of 50% endpoints based on the large total number of animals, which gives the effect of using at the two critical dilutions between which the endpoint lies, larger groups of animals than were actually included in these dilutions (Ravi et al., 2010)

Escobedo-Bonilla et al. determined the virus infection and mortality titers of a WSV stock inoculated by i.m. and oral routes. This was the first study to describe the relationship between routes of exposure (i.m. vs. oral) and virus infectivity of a WSV stock in *Litopenaeus* vannamei. The relationship between the virus infection and mortality titers using the Thai isolate of WSV by the i.m. or oral route was 1:1 only in experiments which were terminated at 120 hpi or later. Thus, every shrimp that became infected with this strain of WSV by either of these routes of inoculation died within 120 hpi (Escobedo-Bonilla et al., 2005).

In vivo titrations are important to evaluate differences in susceptibility between life stages within a host species or between related species (Plumb and Zilberg 1999). In shrimp, there are indications that susceptibility to WSV may differ between life stages (Pramod-Kiran et al., 2002, Yoganandhan et al., 2003), shrimp species (Lightner et al., 1998, Wang et al., 1999) and different decapods (Wang et al., 1998, Sahul-Hameed et al., 2003). Wang et al. (1999) indicated wild crabs such as *Calappa lophos*, *Portunus sanguinolentus*, *Charybdis granulate* and *C. feriata* were infected by the *White spot baculovirus* (WSBV) experimentally. The wild crabs were fed by infected *Penaeus monodon* and the WSBV infection was detected by PCR 20 days post infection (Wang et al., 1998). Wang et al. reported which WSSV was specifically detected by PCR in *Penaeus merguiensis* hemocytes, hemolymph and plasma. This suggested a

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close association between the shrimp hemolymph and the virus (Wang et al., 2002).

The quantity of virus in a specified suspension volume that will kill 50% of a number of infected animals is termed the LD_{50}. The two best-known methods of estimating the LD_{50} in quantal response data are those of Karber and Reed and Muench (Thompson, 1947; Reed and Muench, 1938). The Reed-Muench and Karber methods unfortunately lead to a bias in the estimation of the LD_{50} if the logarithms of the doses are not spaced symmetrically about the true log LD_{50}, a situation which is at times unavoidable. Reed and Muench suggested a modification by which this bias could be effectively removed, and a similar modification is available in Karber's method (Armitage and Allen, 1950).

According to the results of this study the LD_{50} in WSV- infected *Penaeus semiculcatus* was about two logarithmic cycles more than *Astacus leptodactylus* crayfish. Therefore production of progeny virions in the WSV- infected *Penaeus semiculcatus* was faster and more efficient than WSV- infected *Astacus leptodactylus* crayfish. Also WSV infection was latent in *Astacus leptodactylus* crayfish till the 25_{th} day post infection and mortality preceded 100% till the 30_{th} day post infection**.**

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تعیین تیتر ویروس لکه سفید جداسازی شده از ایران در خرچنگ دراز (*Astacus leptodactylus*) و میگو ببری سبز (*Penaeus semisulcatus*)

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چکیده

ویروس لکه سفید اخیراً یکی از مهمترین عوامل بیماریزای میگو در دنیا شناخته شده است که باعث ۱۰۰٪ مرگ و میر در طی ۷-۱۰ روز در مزارع میگو می گردد. نمونه های میگو سفید هندی (*Fenneropenaeus indicus*) عفونی شده از منطقه گواتر در استان سیستان و بلوچستان در جنوب ایران جمع آوری شده و عفونت ویروس لکه سفید آنها به روش Nested PCR تایید شد. با استفاده از روش های سانتریفوژ و فیلتراسیون ویروس لکه سفید از نمونه های میگو عفونی شده جداسازی گردیده و به صورت درون عضلانی به خرچنگ دراز تزریق شد، این ویروس جداسازی شده به نام WSV/IRN/1/2010 نامیده شد. جهت تعیین رقتی از ویروس که باعث ۹۰-۱۰۰٪ مرگ و میر در میگو ببری سبز شود، رقتهای ویروسی 10^{0} تا 10^{5} در بافر فسفات استریل تهیه و هر رقت به یک گروه ۱۴ تائی میگو به صورت درون عضلانی تزریق شد. همچنین رقتهای ویروسی ۱/۲ تا ۱/۳۲ در بافر فسفات استریل تهیه و به صورت درون عضلانی به خرچنگ دراز (*Astacus leptodactylus*) تزریق شدند. بنابراین دز کشنده ۵۰٪ (LD_{50}) استوک ویروسی زنده در خرچنگ دراز و میگوی ببری سبز به روش کربر محاسبه شده و به ترتیب $10^{3,29}$ و $10^{5,35}$ در هر میلی لیتر تعیین گردیدند.

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