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Protection of *Litopenaeus vannamei* against white spot syndrome virus by electron-irradiated inactivated vaccine and prebiotic immunogen

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ABSTRACT

WSSV[1] is an infectious agent of shrimp and other crustaceans. Neither effective vaccines nor treatments are currently available for this disease. Although the immune system of shrimps is not comparable to that of vertebrates, shrimps can acquire protection against pathogenic challenge by building up immunity. This study for the production of radio vaccines against WSSV used a radiation facility providing 10-Mev electron beams and a 2-mA current. In this study WSSV was isolated from infected shrimp samples and multiplied in *Astacus leptodactylus crayfish* hemolymph and called WSSV/IRN/1/2010. Titration of WSSV was obtained in post-larvae by Karber method as $10^{5.4}$ LD_{50}[2]/mL and the virus was inactivated by the electron beam irradiation, the D_{10} value and optimum dose of electron beam were obtained at 1.85 and 13 kGy, respectively. Electron beam-irradiated WSSV (EBI[3]-WSSV) was used as a radio vaccine to immunize L. *vannamei* by intramuscular injection and bath-immersion routes. Prebiotic Immunogen as a diet additive was used to improve immune responses. PD_{50}[4] was calculated at 5.62 and 6.02 for the shrimp groups vaccinated with EBI–WSSV vaccine and EBI-WSSV vaccine + Prebiotic Immunogen, respectively. Calculated RPS values were 75%, 91% and 25% for the EBI-WSSV vaccine alone, EBI-WSSV + Prebiotic Immunogen and Prebiotic Immunogen alone (PID[5]) groups vaccinated by immersion route, and 73%, 82% and 18.5% by injection route, respectively. Significant difference in cumulative mortalities was observed between both vaccination groups (EBI-WSSV and EBI-WSSV+ immunogen), and the control groups ($P < 0.05$). No significant difference in cumulative mortalities was observed between the two vaccination groups ($P > 0.05$). Therefore, EBI-WSSV vaccine induced immunity responses in shrimp against WSSV infection and Prebiotic Immunogen enhanced this response.

1. Introduction

White spot syndrome virus (WSSV) belongs to *Nimaviridae* family and *Whispovirus* genus; it has a double-stranded DNA genome (Namikoshi et al., 2004; Afsharnasab et al., 2014). The virion is elliptical to short rod with an envelope measuring 248 ± 87×107 ± 11 nm, nucleocapsids measuring 162 ± 15×59 ± 17 nm and with a tail at the end (Afsharnasab et al., 2014; CDC, 2016). The virus is among the greatest threats to the worldwide shrimp aquaculture industry (Edgar et al., 2011). WSSV causes high mortality and large economic losses in cultured shrimp. WSSV was reported first from shrimp farms in 2001 in Khuzestan Province, Iran, followed by other shrimp farming areas in Bushehr Province in 2003 and 2005, and in Sistan and Baluchestan in 2005, 2007, 2008 and 2011 in the south of Iran (Afsharnasab et al., 2007, 2009). Vaccination with immune-stimulation is widely used to prevent diseases in mammals and other vertebrates but it is not suitable for invertebrates such as shrimps, which are thought to possess only innate immunity and do not have the ability to produce antibodies. The plasma of shrimp exposed to inactivated WSSV or its sub-units contains virus neutralizing activity (Venegas et al., 2000) and exhibits reduced mortality upon challenge (Namikoshi et al., 2004; Bright-Singh et al., 2005), suggesting the presence of an

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[1] White Spot Syndrome Virus.
[2] Lethal $Dose_{50}$.
[3] Electron Beam Irradiated.
[4] Protective $Dose_{50}$.
[5] Probiotic Immunogen Diet.

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inducible immunity that can inhibit subsequent infection by the same pathogen. Previous exposure to a recombinant viral envelope protein (rVP28) can also protect shrimp from WSSV (Witteveldt et al., 2004a; Caipang et al., 2008). In this study electron-irradiated inactivated whole viral particles of WSSV and Prebiotic Immunogen as a dietary additive were used to enhance L.*Vannamy* protection against WSSV.

2. Materials and methods

2.1. Virus stock

The infected shrimp samples with signs of WSSV were collected from a farm in Bushehr in the south of Iran and transmitted to the Virology Laboratory in Karaj. The WSSV infection was confirmed by Nested PCR (IQ 2000 Kit) (OIE, 2006; Motamedi-Sedeh et al., 2012). The infected gill, stomach, muscle and hepato-pancreas tissues of shrimp samples were homogenized by TN buffer (Tris-HCl 20 mM, NaCl 400 mM, pH=7.4) at a ratio of 1:5, centrifuged at ×1700*g* for 10 min at 4 °C; the supernatant was filtered with 0.45-μm filter. The filtered supernatant was used for injection into the crayfish. The *Astacus leptodactylus* crayfish was procured from Orumyieh in the northwest of Iran and transmitted to the aquaculture laboratory in Karaj. The filtered supernatant containing WSSV was injected intramuscularly into the third and fourth abdomen segments of crayfish using a 26-G needle. After 4–5 days, the hemolymph was withdrawn with an anti-coagulation solution (20.8*g* of glucose, 8*g* of citrate sodium, 3.36*g* of EDTA and 22*g* of NaCl per one liter of distilled water). The homogenized suspension of infected crayfish hemolymph was confirmed by Nested PCR for WSSV infection, centrifuged at low speed (at ×1500*g* for 10 min at 4 °C) and stored in −70 °C as the viral stock (Van-Hulten et al., 2001; Du et al., 2006; Witteveldt et al., 2004a, 2004b).

2.2. Dosimetry and virus irradiation

The viral stock was *aliquoted* in the samples at 5 mL and irradiated by 10-Mev electron accelerator (IBA Company, Model Rodotron TT200) with a beam current of 2 mA. Different doses of electron beam, i.e. 1, 3, 5, 10, 20, 25 and 30 kGy, were used for irradiation of the frozen viral samples held on dry ice, with three repeats for each dose of electron beam. The uncertainty of the measured doses was calculated as the standard deviation of the measured values: 0.93 ± 0.030, 2.53 ± 0.284, 5.05 ± 0.040, 9.87 ± 0.252, 18.75 ± 0.627, 24.71 ± 0.431, 29.90 ± 0.476. Dosimetry was carried out briefly; two containers with the same shape was used, the first container included dry ice and a vial of viral sample and the second container included a vial of water and a dosimeter on it (FWT dosimeters were used for 1–5 kGy and CTA dosimeters for up to 10 kGy). As the vial of viral sample was on dry ice with an exact thickness and density=1.4–1.6 g/cm^3, and the second vial (contain water and dosimeter) put on a kind of polymer (PET) with density=1.4 g/cm^3. Both of the containers were irradiated at the same time and the dose of irradiation which was read by dosimeter used for the viral sample (IAEA, 1967; Ziaie et al., 2008).

2.3. In vivo-irradiated and non-irradiated 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 g. Virus quantification involves counting the number of virus particles in a specific volume to determine the virus concentration (virus titration). Lethal dose$_{50}$ (LD$_{50}$) is the measure of lethal virus titer. This endpoint dilution assay quantifies the amount of live virus required to kill 50% of infected hosts. The irradiated and non-irradiated WSSV samples were diluted in steps from 10^{0} to 10^{-5} in sterile PBS and for each dilution

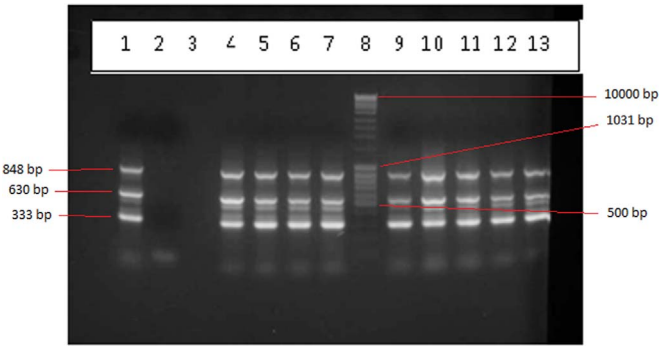

Fig. 1. The results of Nested PCR; Lane 1: positive control; Lanes 2 and 3: negative controls; Lanes 4, 5, 6 and 7: WSSV-infected shrimp samples; Lane 8: DNA ladder; Lanes 10–13: infected crayfish.

10 μL was injected intramuscularly into 12 shrimps. The samples in group 1 were injected with sterile PBS as the negative controls and the other groups were used for WSSV dilutions. All the shrimps in the negative control group survived, whereas mortality due to WSSV infection occurred in all the groups with a virus dilution in one week. All the dead shrimps were examined for WSSV by Nested PCR (Witteveldt et al., 2004b). Lethal dose$_{50}$ (LD$_{50}$) was calculated by Karber method (Karber, 2002) as the virus titration. The dose/survival curve was drawn using Origin software, and D$_{10}$ value (It referred to as the D value, is a measure of the treatment required to inactivate 90 per cent, i.e. one log reduction) of the organisms present or to reduce the microbial population to one-tenth its number) (IAEA, 1967) and optimum dose of electron beam for viral inactivation were determined according to the dose/survival curve. Finally, 100 mL of the viral stock was irradiated with the optimum dose of electron beam and formulated as the electron beam-irradiated (EBI-WSSV) vaccine (Motamedi-Sedeh et al., 2015).

2.4. Safety test

The infectivity of the irradiated inactivated virus sample by the optimum dose of electron beam was determined by inoculating *P. semiculcatus* post-larvae (weighing 1 g) via immersion method at 20 °C for 7 days, which were then sub-cultured on fresh post-larvae four times during 30 days.

2.5. Diet additive

Prebiotic Immunogen (International Commerce Corporation USA, INC) was used in order to improve immune responses in shrimp. It was incorporated into one diet (commercial crumbled feed) at a concentration of 0.2% (PID). Control diet (CD) was also prepared using the same composition of ingredients, except the Immunogen.

2.6. Administration of EBI vaccine by injection and immersion routes

Young shrimp, L. *vannamei*, weighing (10–15 g) were obtained from a farm that routinely implements biosecurity measures and has no history of WSSV outbreak. Some of the shrimp samples were analyzed by polymerase chain reaction (PCR) to confirm the absence of WSSV. Approximately 200 shrimps were selected in 20 groups (n=10) and kept in aquariums with flow-through seawater at 25–27 °C and fed commercial crumbled feed at 5% of body weight per day before and during the experiment. The samples in groups 1–8 were injected with four dilutions of EBI-WSSV vaccine (1, 1/2, 1/4, 1/8) intramuscularly in the 4th or 5th abdominal segments twice for each dilution with 50 μL; four groups were fed with PID every day and the other four were fed with CD. The samples in groups 9–16 were vaccinated by bath-immersion route with 1:20 V/W ratio (one volume of vaccine for 20 g