Fish & Shell sh Immunolog 26 (2009) 685 690









Protection of *Procambarus clarkii* against white spot s ndrome virus using inactivated WSSV

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ARTICLE INFO

Article histor : Received 1 Januar 2009 Received in revised form 5 Februar 2009 Accepted 21 Februar 2009 Available online 4 March 2009

Ke ords: White spot s ndrome virus Procambarus clarkii Vaccination BEI Inactivate

ABSTRACT

White spot s_ndrome virus (WSSV) is a highl_pathogenic and prevalent virus infecting shrimp and other, crustaceans. The potentialit_of binar_eth_lenimine (BEI)-inactivated WSSV against WSSV in cral_sh, *Procambarus clarkii*, was investigated in thit_stud__Eff.cac__of BEI-inactivated WSSV was tested b vaccination trials followed b_challenge of cral_sh with WSSV. The cral_sh injected with BEI-inactivated WSSV showed a better survival (P < 0.05) to WSSV on the 7th and 21st da_post-vaccination (dpv) compared to the control. Calculated relative percent survival (RPS) values were 7% and 60% on the 7th and 21st dpv for 2 mM BEI-inactivated WSSV, and 63%, 30% on 7th and 21st dpv for 3 mM BEI-inactivated WSSV. However, heat-inactivated WSSV did not provide protection from WSSV even on 7th dpv. In the inactivated WSSV particles. These results indicate the protective effect_of BEI-inactivated WSSV to protect *P. clarkii* from WSSV.

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1. I c

Electron microscop. (EM) showed that white spot s. ndrome virus (WSSV) is enveloped, bacilliform in shape and have a tail-like appendage at one end [1]. The virus contains double stranded DNA with an estimated st e of 292967 bp, with 181 open reading frames consisting of 39 structural proteins [2 4]. However, different genome st es have been reported from diverse virus isolates and large genome sequences have been reported from virus isolates from China and Thailand [5]. White spot s. ndrome virus has a broad host range within Decapoda crustaceans, including penaeid shrimp and craftsh [6 8]. White spot s. ndrome virus (WSSV) belongs to the new virus famil. *Nima iridae*, genus *Whispo irus* [9]. In China, production losses of 80% of farmed shrimp were attributed to WSSV [10,11].

Crustaceans do not possess an adaptive immune s stem, but now it is doubted for some investigation [12,13]. A recent stud, in vaccinated craftsh surviving from e perimental WSSV infections showed that it possess a resistance against WSSV [14–18]. Of the viral structural proteins, envelope proteins often pla_vital roles in virus entr. and assembl [19 21]. Vaccination using viral proteins, especiall VP28, has been reported to offer shrimp protection against WSSV infection [22 24]. However, a neutral ation assa with the combination of antibodies against different envelope proteins showed that a combination of VP36B and VP31 antibodies could strongl inhibit WSSV infection in craftsh. It revealed that multiple envelope proteins are involved in WSSV infection in craftsh during this process [25].

Furthermore, immunostimulation of shrimp with inactivated vibrio have been reported to provide some protection [26]. The shrimp intramuscularl, vaccinated with formalin-inactivated WSSV can induce a resistance to the virus of intramuscular (IM) injection on the 10th da post-vaccination [27]. These reports suggest that some of envelope proteins can induce an immune response and protect shrimp against WSSV. Thus inactivated WSSV will be a good vaccine to shrimp but inactivating with formalin and heat are not good method. Binar, eth lenimine (BEI) is a kind of a iridines and formed b, the c ch ation of 2-chloroeth lamine h drochloride (BEA). BEI is known to alk late nucleic acids but do not damage the protein of inactivated virus in the concentration of 1 mM [28,29]. In veterinar, medicine BEI is the preferred inactivating agent for producing vaccines containing animal viruses with DNA or RNA genomes [30 32]. Although chemical agents and ph sical methods have been studied on the inactivation of WSSV

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^{1050-4648/\$} see front matter \odot 2009 Elsevier Ltd. All rights reserved. doi:10.1016/j.fsi.2009.02.022

when WSSV was inactivated. Our stud, was carried out to e plore the possibilit, of protecting *Procambarus clarkii* from WSSV infec-tion b, vaccination with BEI-inactivated WSSV.

2. Ma ′a.a

2.1. Cra sh

Cra rsh^{i} *P. clarkii*, appro imatel 20 g and 8 cm each, were reared at 25 ± 1 °C. The were kept in tanks with sand-intered, $\vec{0}$ one-treated and ow through freshwater and fed with commercial pellet feed at 5% of bod, weight per da, Walking legs from randoml selected individuals were subjected to RCR assa s to ensure that the cra rsh were WSSV-free before e perimental challenge challenge.

2.2. Measurement of iral infecti it (LD_{50})

The infectivit (lethal dose 50%: LD_{50}) of WSSV was used as the criterion for the virus inactivation tests. White spot s ndrome virus-infected shrimp, Fennerpenaeus chinensis, were collected from shrimp farms located near Ningbo, China. Ten grams of infected tissues (gills and tail muscle) were homogenie ed in 500 ml TNE buffer (50 mM Tris HCl, 400 mM NaCl, 5 mM EDTA, pH 7.5) containing a combination of protease inhibitors (1 mM phen l-meth lsulphon L uoride (PMSF), 1 mM beñ amidine, and 1 mM Na₂S₂O₅), and then centrifuged at 10,000 × g for 10 min at 4 °C. After intering b n lon net (400 mesh), this homogenate was centrifuged at 6000 × g for 25 min at 4 °C and intrated using a Millipore inter (pore si e 0.45 µm). This intrate was the original viral uid that was used for the inactivation tests afterwards. To measure the infectivit of this virus uid, 10-fold serial dilutions of the , uid were made from 10^4 to 10^9 and \mathbf{M} trated using a Millipore Ther (pore st e 0.45 μ m). These diluted uids were injected into each of 30 health, craitish (average bod, weight 20.0 g) at the dose of 0.1 mL/craitish. Mortalit, and clinical signs were observed dail for two weeks.

2.3. Inacti ated WSSV

0.2 M binar, eth lenimine (BEI) was prepared b, c cH ation of 0.2 M 2-bromoeth lanine-HBr in 0.2 M NaOH at 37 °C for 1 h. The β -naphthol violet (a pH indicator) was added to the solution to check the formation of BEI which causes a change in colour from violet to orange. This solution was added to 10¹ WSSV dilution as 1:100 (v/v) to a concentration of 2 mM. The solution was incubated at 37 $^{\circ}\text{C}$ with continuous stirring for 6 h, 12 h, 18 h, 24 h and the reaction was stopped b, addition of sodium thiosulphate. And 0.3 M BEI solution prepared b, the same way was added to 10 diluted WSSV solution to a concentration of 3 m. Mand incubated at 37 °C with continuous stirring for 24 h and the reaction was stopped b_addition of sodium thiosulphate.

For preparation of heat-inactivated WSSV, the viral suspension was diluted 10-fold in TNE and inactivated for 15 min at 65 °C.

For in vivo injection e periment the health crait sh collected from stock were divided into nine groups (30 craitsh per group per tank). For test the safet, of BEI and heat-inactivated WSSV si groups of 30 craitsh were intramuscularl. (IM) injected with 0.1 mL inactivated WSSV solution. A group of 30 craitsh was IM injected with 0.1 mL of WSSV (10 dilution) which was the sted at injected with 0.1 mL of WSSV (10 dilution) which were heated at 37 °C for 24 h to test its effects on WSSV. The positive control groups were injected with 0.1 mL of WSSV (10 dilution) and the negative control was injected with 0.1 mL of TNE solution (Table 1).

B 490/test	Effect of BEI and			921
with BEI-inactivated WSSV.	Group	Treatment	Dead/tested	Mortalit (%)
	2 mM BEI 1	3		;
<i>kii</i> , appro imatel 20 g and 8 cm each, were C. The were kept in tanks with sand-intered, d ow through freshwater and fed with feed at 5% of bod, weight per da Walking legs ected individuals were subjected to RCR assa s to				

2.4. Electron microscop and SDS-PAGE

BEI-inactivated WSSV and heat-inactivated WSSV particles were negativel_stained with 2% sodium phosphotungstate (PTA, pH 7.0) on collodion-carbon coated grids. All observations were made with a JEOL 1230' transmission electron microscope (JEOL, Japan) operating at 70 kV. The two kinds of inactivated WSSV were anal zed b sodium dodec L sulfate-pol acr Lamide gel electrophoresis (SDS-PAGE) according to Laemmin [35]. The gels were stained with Coomassie brilliant blue (0.1% Coomassie Blue R-250 in 1% acetic acid and 40% methanol). A premi ed protein molecular weight marker (Fermentas), with proteins ranging from 14.4 to 116 kDa, was co-electrophoresed to determine the molecular weights of the WSSV proteins.

2.5. PCR anal sis for WSSV

Total DNA was e tracted from walking legs of craush with an animal tissue genomic DNA mini-prep kit (Sangon, Shanghai). The samples were tested with one primer set VP28-FW (5'-CGCACA GACAATATCGAGAC-3') and VP28-RV (5'-GTCTCAGTGCCAGAGTAG GT-3'), amplifing part of WSSV VP28 gene, was used to screen for WSSC-positive animals. PCR was performed with the VP28 primer pair using the following protocol: 5 min at 94 °C followed b_35 c_cles at 94 °C for 1 min, 55 °C for 1 min and 72 °C for 1 min. The PCR products were anal² ed b, electrophoresis on 1% agarose gels stained with ethidium bromide and visual ed b, ultraviolet transilluminator.

2.6. Vaccination e periments

For vaccination e periment 12 groups of 30 inter-molt cra with an average weight of 20 g were selected. Before starting the vaccination e periment the crash were tested for the presence of WSSV b. one step PCR. Si groups of craits hwere vaccinated b. JM injection with 0.1 mL of 2mM BEI, 3mM BEI and heat-inactivated WSSV and the controls were IM injected with 0.1 mL of 2mM BEI, 3mM BEI and TNE solution. Seven da safter the initial vaccination, three groups and their control groups were IM injected with 0.1 mL of WSSV dilution (1×10^7) for the challenge test. Another three groups and their control groups were challenged b_the same wa at 21st da_post-vaccination (dpv) (Table 2).

2.7. Statistical anal sis

The mortalities of the tested and control groups were compared statisticall using the chi-square test (χ^2) at a significance level of 5%. The relative percent survival (RPS) values were calculated

Tab 2

Resistance against e perimental WSSV infection in *Procambarus clarkii* vaccinated with BEI or heat-inactivated WSSV.

Time of challenge	Dead/tested	Mortalit (%)	RPS (%)	P-value
7 dpv				
2 mM BEI-inactivated WSSV	7/30	23	77	0.000*
Control	30/30	100		
3 mM BEI-inactivated WSSV	11/30	37	63	0.000*
Control	30/30	100		
Heat-inactivated WSSV	27/30	90	10	0.609
Control	30/30	100		
21 dpv				
2 mM BEI-inactivated WSSV	12/30	40	60	0.000*
Control	30/30	100		
3 mM BEI-inactivated WSSV	21/30	70	30	0.021*
Control	30/30	100		
Heat-inactivated WSSV	29/30	97	3	0.831
Control	30/30	100		

Control group craftsh were injected with TNE solution mi ed with BEI or not. Significant difference (5% level) compared with the corresponding unvaccinated group in indicated b \pm .

according to Amend [36]. Cumulative mortalities, RPS values and *P*-values were determined at the termination (24th da.) of challenge test made seven da s after the last vaccination.

3. R . . .

3.1. Measurement of iral infecti it (LD₅₀)

The results of viral infectivit (LD_{50}) as measured b, the mortalit of injected crash showed 100% in all test groups until 10^7 of the serial dilutions, but showed 55% for the 10^8 dilution group. Therefore, the LD_{50} value of the original viral uid was estimated to be 10^8 dilution. So we used 1×10^7 dilution of WSSV as the challenge dose in the challenge test.

3.2. Inacti ation of WSSV

IM injection e periment showed that WSSV had been inactivated b. BEI completel, and the solution was safet, for oral vaccination (Table 1). 2 mM BEI resulted in mortalities of 100% in 6 and 12 h, 90% in 18 h and 100% in 24 h. The result reveals that BEI can inactivate WSSV in temperature of 37 °C and the proper concentration of 2 mM and 3 mM BEI also inactivated WSSV completel. No mortalit, was recorded in the negative control and the positive control resulted in mortalities of 100% on the 17th da. The cumulative mortalities in the 37 °C heated WSSV group indicated WSSV could keep the infectivit, even e posed to 37 °C over 24 h. So onl. BEI as the inactivant contributes to the completel, inactivation of WSSV. WSSV could be completel, inactivated b, either 20 min e posure to 60 °C or 10 min e posure to 50 °C [33]. Although aquatic viruses receive thermal protection from the natural aqueous environment as water temperature is seldom higher than 35 °C, WSSV showed a resistance to high temperature. In this stud, WSSV could be completel inactivated b, 65 °C for 15 min.

3.3. Electron microscop

The TEM microphotograph showed that the envelopes of 2 mM and 3 mM BEI-inactivated WSSV seem to have a little changed compared with the purified WSSV (Fig. 1), but the virus particles are intact basicall. But the envelope of WSSV was destro, ed badl, and the nucleocapsid was e posed to the outer in heat inactivated WSSV (Fig. 1).

3.4. SDS-PAGE anal sis

The SDS-PAGE anal_sis showed that more than ten bands in the purified WSSV and 2 mMBEI-inactivated WSSV, but onl_field WSSV and 2 mM BEI-inactivated WSSV (Fig. 2). In the purified WSSV and 2 mM BEI-inactivated WSSV, the bands of VP15, VP19, VP28 and VP281 obviousl_fe_ist. In 3 mM BEI-inactivated WSSV onl_the bands of VP26 and Vi28 were obvious. No protein was found in linear heat-inactivated WSSV (Fig. 2).

3.5. Vaccination e periment

The time mortalit relationships in the vaccination e periment are shown in Fig. 3. The cumulative mortalit percentage (CMP) of the groups vaccinated with 2 mM BEI, 3 mM BEI, and heatinactivated WSSV and challenged at 7 dpv was 23%, 37% and 90% respectivel. (Table 2, Fig. 3a). Calculated RPS values were 77%, 63% and 10% for the groups vaccinated with 2 mM BEI, 3 mM BEI, and heat-inactivated WSSV (Table 2). Significantly lower mortalit (P < 0.05) in the 2 mM BEI-inactivated WSSV (23%) and 3 mM BEIinactivated WSSV (37%) vaccinated group compared with its control group (100%). But no significant differences in cumulative mortalities were observed between the groups injected with heatinactivated WSSV and its control group.

The CMP of the groups vaccinated with 2 mM BEI, 3 mM BEI, and heat-inactivated WSSV vaccinated groups challenged at 21 dpv were 40%, 70% and 97% respectivel. (Table 2, Fig. 3b). Calculated RPS values were 60%, 30% and 3% for the groups vaccinated with 2 mM BEI, 3 mM BEI, and heat-inactivated WSSV (Table 2). Significantl, lower mortalities (P < 0.05) were observed in the 2 mM BEI-inactivated WSSV (40%) and 3 mM BEI-inactivated WSSV (70%) vaccinated group compared with its control group (100%).

4. D. c.

Recentl various agents, i.e. inactivated WSSV, antibacterial components, and subunit recombinant envelope proteins tried so far against WSSV have shown encouraging results [14 18,22 24,27,37,38]. Now more and more report indicated that envelope proteins, especiall VP28, could offer shrimp protection against WSSV infection [22 25]. In this investigation we found that the protection effect of inactivated WSSV is dependent on the integrit of envelope proteins as previous report in the vaccination trial of veterinar virus [30,32,39,40]. We also found that the protection will decrease along with da after last vaccination like previous reports [23,27].

The Electron microscop_showed that maybe the envelope of WSSV is changed a little in 2mM and 3 mM BEJ (Fig. 1). And the SDS-PAGE anal_sis indicated that higher than 2 mM concentration of BEI ma_change the envelope proteins of WSSV more (Fig. 2). The loss of envelope protein in 3 mM BEI-inactivated WSSV ma_e_plain the significant difference among the RPS of 2 mM BEI, and heat-inactivated WSSV. Because envelope proteins have been comfirmed that could protect the crustaceans against WSSV. In this stud_we think that 2 mM BEI can inactivate WSSV completel_and preserve the integrit_of envelope proteins in optimum. The significant differences (P < 0.05) between BEI and heat-inactivated WSSV ma_be e_plained b_the loss of envelope proteins, which provided the protection against WSSV.

No mortalit, was recorded in vaccinated crassingly before the challenge test indicated that the inactivated WSSV vaccine was safet, for vaccine crassingly and a continued in the inactivation e periment, the challenge test made on the 7th and 21st dpv resulted in significantly lower mortalit, (P < 0.05) in the 2 mM and 3 mM BEI-inactivated WSSV vaccinated, group compared with its control



F . **1.** Electron microscopic view of negativel, stained, normal WSSV particle (A), 2 mM BEI- inactivated WSSV particle (B), 3 mM BEI-inactivated WSSV particle (C) and heat-inactivated WSSV (D). (A. bar = 200 nm; B D. bar = 100 nm).



F. **2.** Fifteen percent Coomassie brilliant blue-stained SDS-PAGE gel of puried WSSV and BEI-inactivated WSSV. Lane 1. Puried WSSV. Lane 2. Protein marker (14.4 kDa, 18.4 kDa, 25 kDa, 35 kDa, 45 kDa, 66.2 kDa and 116.0 kDa). Lane 3. 2 mM BEI-inactivated WSSV. Lane 4. 3 mM BEI-inactivated WSSV. Lane 5. Heat-inactivated WSSV.

group (100%). However, the RPS on 21st dpv showed a significant difference between 2 mM and 3 mM BEI-inactivated WSSV. The difference may be e amined by the damages in the envelope proteins of WSSV that showed in Electron microscop, and SDS-PAGE. Electron microscop, showed that a little damage happened to the envelope proteins of WSSV in 2 mM and 3 mM BEI-inactivated WSSV but heat at 65 °C for 15 min destro ed the envelope proteins completel. (Fig. 2). The anal sis of SDS-PAGE indicated that in the linear of 3 mM BEI-inactivated WSSV the band of VP28 was not obvious and the band of VP15, VP19 and VP281 could not be found compared within the linear of 2 mM BEI-inactivated WSSV. The envelope of heat-inactivated WSSV particle was destro, ed may e plained that no proteins was found in the linear of heat-inactivated WSSV (Fig. 1d, Fig. 2). The high cumulative mortalit, in the heat-inactivated WSSV vaccinated group indicated that heat-inactivated WSSV did not provide a protection from WSSV, and Namikoshi et al. also reported the same result [27]. So we think that the protection provided by inactivated WSSV is dependent on the integrit, of envelope proteins of WSSV.

dependent on the integrit, of envelope protein of WSSV. Our results showed that vaccinating craftsh with BEI-inactivated WSSV would protect the craftsh against WSSV seven da s after the last vaccination. Craftsh *P. clarkii* is susceptible to WSSV as penaeid, shrimp and WSSV caused more than 90% mortalit, in craftsh through oral challenge and IM injection [15]. Craftsh injected with 3 mM BEI-inactivated WSSV showed no bad signs and



 ${\bf F}$. 3. Time mortalit relationship of e periment 1. Juvenile craits have vaccinated inactivated WSSV followed by viral challenge at (A) 7 da s and (B) 21 da s post-vaccination. Cumulative mortalit, rates of shrimp from the e perimental groups, as indicated in Table 2 are plotted against time (18-da, period) after challenge.

be active like normal crash in this stud. We believe that vaccination with

BEI-inactivated WSSV was successful in protecting craush against WSSV, similar indings were also obtained b. Namikoshi et al. using formalin-inactivated WSSV [27]. In conclusion, cra ish can be induced a resistance to WSSV b_BEI-inactivated WSSV, but BEI in concentration of above 2 mM will damage the envelope proteins of WSSV and reduce the protective electer, of BEI-inactivated WSSV. These results comment that the protective electer, of vaccine made of BEI-inactivated WSSV lies on the integrit, of envelope proteins of WSSV and reveal the possibilit of vaccination of P. clarkii with BEIinactivated WSSV.

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The authors thank the management of Feed Science Institute, Zhejiang Universit, for providing the facilities to carr out this work. We also appreciate Jian Hong, Linlin Fu for their valuable advice. This research was supported b, the Planned Science and Technolog. Project of Zhejiang Province, China (No. 2008C32034).

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