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Passive Immunization of Crayfish (*Procambius clarkiaii*) with Chicken Egg Yolk Immunoglobulin (IgY) Against White Spot Syndrome Virus (WSSV)

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Abstract White spot syndrome virus (WSSV) is a major cause of mortality in shrimp lacking a true adaptive immune response. In this study, high activity egg yolk immunoglobulin (IgY) against WSSV for passive immunization of crustaceans was already prepared as crude and purified product, while an indirect enzyme-linked immunosorbent assay test was used for quality control of IgY activity. The effectiveness of IgY of intramuscular injection, oral administration, and immersion was investigated in crayfish (*Procambius clarkiaii*) against WSSV. The result showed that the groups treated with IgY from inactivated WSSV and DNA vaccine were, respectively, 20% and 80% mortality, which were significant difference in survival rates ($P<0.05$) from the positive control groups. The groups in diet added 10% egg yolk powder and 1% IgY power showed 53.3% and 67.7% mortality, respectively, and the immersion showed 46.7% mortality, which have significantly different compared to the positive groups ($P<0.05$). These results indicated passive immunization of specific IgY antibodies through intramuscular injection, oral administration, and immersion have effective to protect crayfish against WSSV. It is noteworthy that IgY as feed additive and immersion solution is useful and feasible methods in practical work. Thus, our results suggest that the passive immunization of crayfish with IgY against WSSV will have potential development to prevent and control WSSV in practical culture.

Keywords WSSV · IgY · Inactivated vaccine · DNA vaccine · Passive immunization · Crayfish

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Introduction

White spot syndrome virus (WSSV) is one of the most virulent pathogen, causing high mortality and large economic losses in cultured shrimp [1–4]. The virus has a wide host range among crustaceans [3, 4]. Vaccination is commonly used to prevent disease outbreaks and is effective, but this kind of method does not seem to have effect to crustaceans because crustaceans lack a true adaptive immune response [5]. However, passive immunization using pathogen-specific antibodies raised in hens is a potential method against diseases, which especially make crustaceans lacking a true adaptive immune response obtain specific antibody. The beneficial effects of IgY have been reported in aquatic animals. Orally administrated anti-*Yersinia ruckeri* IgY marginally reduced mortality and intestinal infection caused by *Y. ruckeri* in rainbow trout (*Oncorhynchus mykiss*) [6]. Intraperitoneal (IP)-injected or/and orally administered Anti-*Vibrio anguillarum* IgY enhanced disease resistance of juvenile rainbow trout [7]. However, only one study described the effect of IgY in crustaceans. Kim et al. showed that IgY has a potential for immunotherapeutic application to prevent WSSV infection in shrimp (*P. chinensis*) [8].

Previous studies indicated that inactivated vaccine is conventional and more hopeful to be successfully developed in IgY production [9, 10]. DNA-designed IgY is a new method, which is via immunization with a gene vector that expresses and produces a corresponding antibody. Therefore, this approach allows direct generation of antibodies from plasmid DNA and avoids the costly and tedious preparation of purified antigens required for conventional antibody [11–13]. Cova found that DNA-designed IgY specific to human *H. pylori* Urease B can be of particular interest for passive immunotherapy of gastrointestinal tract infections and resist to the gastric barrier [13].

The major envelope protein VP28 and VP19 and nucleocapsid protein VP15 were significant structural proteins involved in WSSV infection [14–17]. Therefore, DNA vaccine, which contains fusion gene encoding VP28, VP19, and VP15 may be have valuable effect for immunization in hens.

In the present study, hens were immunized with inactivated WSSV and DNA vaccine, recombinant plasmid (pCI-VP28/VP19/VP15). Specific IgY against WSSV were obtained and purified. The effectiveness of passive immunization of crayfish (*Procambius clarkiaii*) challenged WSSV with anti-WSSV IgY has been examined by intramuscular injection, oral administration, and immersion of IgY.

Materials and Methods

WSSV Preparation

WSSV was purchased from Yellow Fisheries Research Institute Chinese Academy of Fishery Sciences (Qingdao, China). Tissue from purchased shrimps infected WSSV were harvested, homogenized in TNE buffer (0.05 M Tris, 0.1 M NaCl, 0.001 M EDTA, pH 7.4) using a sterile mortar and pestle and centrifuged at 4,000×*g* for 15 min at 4 °C. The supernatant was filtered through a 0.45-μm PVDF membrane (Millipore), and 100 μl of the filtrate was injected intramuscularly into healthy crayfish. After several days, gill was withdrawn from dead crayfish and centrifuged and filtered as described above. The filtrate containing the virus was then labeled as WSSV stock solution and stored at −70 °C. Prior to each experiment, the stock was diluted with PBS to required concentrations for enzyme-linked immunosorbent assay, challenge or neutralization tests.

Crayfish

Healthy crayfish (approximately 50 g body weight) were collected from Changxing market in Dalian, China. Crayfish (n=15) were stocked in 200-L aquaria, each fitted with an individual recirculation filter system and heating at 26±1 °C. The crayfish were acclimatized for 4–5 days prior to each experiment then they were fed three times daily at 4% of body weight with a commercial feed (GuangDong Evergreen Group Co., LTD. China).

Preparation of Immune Vaccine

Inactivated WSSV vaccine: WSSV stored at −70 °C was inactivated by 0.5% formalin.

DNA-designed vaccine: The fusion gene encoding the three genes of major envelope protein VP28 and VP19 and the nucleocapsid protein VP15 was inserted pCI vector to construct the recombination plasmid (pCI-VP28/VP19/VP15) for immunization as DNA-designed vaccine. The recombination plasmid was purified by kit (Purelink™ Hipure Plasmid Maxiprep Kit, Invitrogen, USA) and quantified by spectrophotometry (JASCO, UV-560, Japan) to use in immunizations.

Immunizations

Lohmann laying hens (120-day-old) were divided into four treatment groups (n=6). The control group was injected with PBS. Group one was immunized with 100 μg of pCI vector. Group two was initially co-immunized with 50-μg/hen CpG ODNs and 100-μg DNA vaccine. Group three was initially injected with inactivated WSSV emulsified with an equal volume of complete Freund's adjuvant in first immunization and then was immunized with incomplete Freund's adjuvant in boosting immunizations. Each hen was injected at five different sites (200 μl per site) including breast muscles (two sites per left or right) and neck subcuticle (one site). The second and third injection, were given at 2-week intervals following the first injection using the same route and double dosages. After immunization 20 days, the eggs laid were collected daily and stored at 4 °C. The egg yolk was separated and diluted (1:6, v/v) with double-distilled water that was acidified with concentrated HCL to obtain a final pH 5.0. The suspension, water-soluble fraction (WSF), was used in ELISA.

Enzyme-linked Immunosorbent Assay

The activity of IgY in samples was monitored by indirect ELISA. 96-well ELISA plates (BIOFIL, Canada) were coated by adding 10× dilution WSSV solution, and incubated overnight at 4°C. The plate was washed three times with PBST (pH 7.4, 0.01 M PBS containing 0.05% Tween 20) and blocked with 100 μl/well of PBS containing 1% (w/v) BSA, at 37 °C for 2 h. After three times rinses with PBST, samples (100 μl) of IgY were added to each well, and incubated at 37 °C for 2 h. The plate was washed again, and 100 μl/well of rabbit anti-chicken IgG (Sigma, USA) conjugated with HRP (1:30,000) were added and incubated at 37 °C for 2 h. The plate was washed five times with PBST and 100 μl of TMB (Sigma, USA) substrate solution (2 mg/ml TMB in buffer, pH 5.0) was added to each well. The plate was incubated at room temperature to allow chromophore development, after which the reaction was stopped by the addition of 50 μl of 2 M H_2SO_4 to each well. The optical density of the wells was determined at 450/630 nm with a plate reader (SUNRISE, Tecan, Austria).

Preparation of IgY

Egg yolk powder After the egg yolk was separated from eggs, yellow egg yolk powder was obtained directly by freeze-drying.

Purified IgY powder IgY was purified from the water-soluble fraction by a combination of several purification techniques including two-step salt precipitation (precipitation with 50% saturated ammonium sulfate followed by precipitating with 14% (*w*/*v*) sodium sulfate) and ultrafiltration using a Vivaflow 50 tangential flow ultrafilter (Vivascience, Hannover, Germany) with a 100-kDa cutoff membrane. Finally, white floccular IgY powder was obtained with ultrafiltration followed by freeze-drying.

Feed Preparation

The basal diet containing all necessary nourishment was purchased from feed market to prepare feed used in assay. Egg yolk powder (10% *w*/*w*) and IgY power (1% *w*/*w*) from inactivated WSSV as additives were added into feed powder. The powder was mixed well and added commercial adhesive (Zhejiang Jingbao Feed Co., LTD. China) to produce 2-mm diameter granular feed. The feed added IgY dried under a stream of ambient temperature air. The stability of feed in water was evaluated detect by sense method.

Intramuscular Injection of IgY

In this experiment, 250 mg/ml IgY powder was incubated with 100× dilution of crude WSSV stock for 1 h at room temperature before injection. Then 100 μl the mixture of antibody and WSSV was intramuscularly injected into crayfish in the third abdominal segment. At the same time, a positive control (WSSV only) and a negative control (0.01 M PBS, pH 7.4) were included in injection. The crayfish were checked for mortality each day.

Oral Administration of IgY

Crayfish were fed three times daily at 4% of body weight during experiments. The control group was given feed added nonspecific IgY. The treatments were given prepared feed containing specific IgY antibody against WSSV. After 10 days of continuous feeding, the crayfish were challenged with 100 μl WSSV solution and the mortality was monitored each day.

Immersion of IgY

The crayfish were immersed with final concentration 250 mg/ml specific IgY antibody power for 15 min prior to this experiment. Specific IgY antibody power was added into water to reach 100 mg/L final concentration. The control group was treated by nonspecific IgY. After 10 days of continuous treatment, the crayfish were challenged with 100 μl WSSV solution, and the mortality was monitored each day.

Statistical Analysis

SPSS statistical software version 11.5 (SPSS, Chicago, IL, USA) analyzed data of the survival rates among the groups was performed using the χ^2 test at a 5% confidence level. The protection against WSSV after vaccination was calculated as the relative percent

survival (RPS; (1 − vaccinated group mortality/control group mortality) ×100) according to Amend described [18].

Results

Monitoring of Activity of IgY

The activity of IgY in WSF was monitored every 10 days by indirect ELISA. As shown in Fig. 1, the level of specific activities of IgY increased 10 days after the initial immunization and then rose constantly. The activity of IgY from inactivated WSSV against WSSV reached a peak at 40 days and declined thereafter. The activity of IgY from DNA vaccine also showed the same pattern as that of IgY from inactivated WSSV, but the peak was delayed 10 days. Anti-WSSV IgY still maintained high antibody activity on day 110.

Protection Induced by IM Injection of IgY

Using high titre anti-WSSV IgY powder from inactivated WSSV (higher than 1:51,200) and DNA vaccine (higher than 1:3,200), mortality of crayfish is shown in Fig. 2. The mortality of positive control was 100% while that for the negative control achieved mortality of 6.7% at day 7. The groups treated with IgY from inactivated WSSV and DNA vaccine showed 20% and 80% mortality, respectively. The RPS values were obtained for the inactivated WSSV (80%) and DNA vaccine groups (20%). The χ^2 test showed a significant difference in survival rates ($P<0.05$) between treatment groups and the positive control group.

Protection Induced by Oral Administration of IgY

The result of passive immunization on oral feed is shown in Fig. 3. The mortality of positive control was 100% at day 10, while the negative control achieved mortality of 6.7%. The groups in diet added 10% egg yolk powder and 1% IgY power showed 53.3% and 67.7% mortality, respectively, and have significantly different compared to the positive

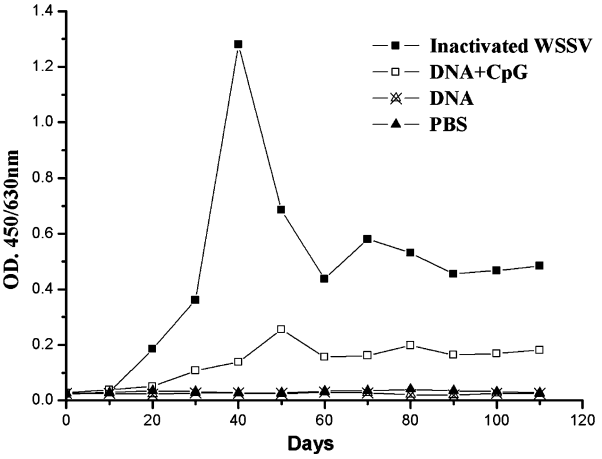

Fig. 1 The change of specific activity of IgY in egg yolk from hens immunized with during the immunization period. The level of specific activity in 1:3,200 dilution of the water–soluble fraction was measured by ELISA using crude WSSV as an antigen

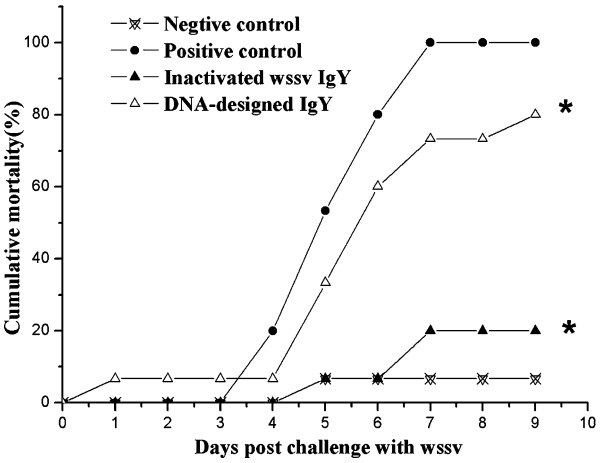

Fig. 2 Time–mortality relationship of IM injection vaccination experiment using purified IgY. Cumulative mortality rates of crayfish from the experimental groups injected with the mixture of WSSV and IgY antibodies from inactivated WSSV and DNA vaccine are plotted against the time. The negative control is treated with PBS alone, while the positive control is injected with WSSV alone. *Lines marked with an asterisk* are significantly different from the positive control group ($p<0.05$)

group ($P<0.05$). This indicated that oral administration with IgY from inactivated WSSV shows a low initial mortality.

Protection Induced by Immersion Administration of IgY

Figure 3 also shows the time–mortality relationship of immersion experiment of anti-WSSV IgY. The mortality of positive control was 100% at day 10, while the negative control achieved mortality of 6.7%. The immersion group showed 46.7% mortality. It is significantly different compared to the positive group ($P<0.05$).

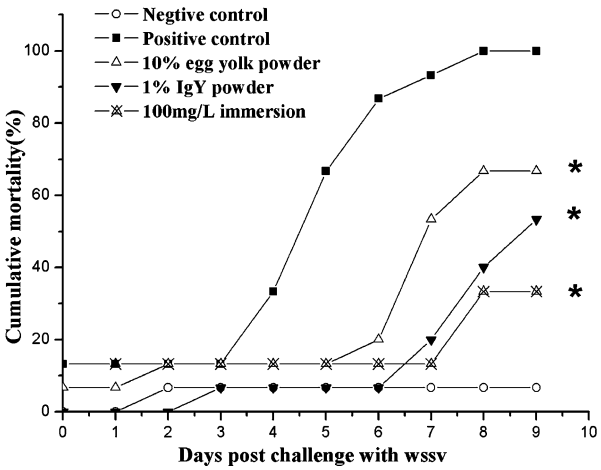

Fig. 3 Time–mortality relationship of oral administration and immersion vaccination experiment using purified IgY. Oral administration treatments are given prepared feed containing 10% egg yolk antibody powder and 1% purified IgY antibody powder against WSSV. Immersion experiment is treated with purified IgY reaching 100 mg/L final concentration. Before challenge, crayfish have a passive immunization period for 10 days. Cumulative mortality rates of crayfish are plotted against the time after challenge with WSSV. The negative control is treated with nonspecific IgY and challenged with PBS, while the positive control is challenged with WSSV. *Lines marked with an asterisk* are significantly different from the positive control group ($p<0.05$)

Discussion

In case of infected WSSV, crustaceans lacking a true adaptive immune response usually have high mortality. The initial treatment such as reducing densities and decreasing water carrying capacities is often uneconomical and has inferior effect. Therefore, some studies have focused on alternative methods of disease control such as the use of vaccines and immunostimulants [19–21]. Antibiotics, herbal extracts, amylose, curdlan, scleroglucan, et al. are used to determine if they impact viruses affecting shrimp, specifically WSSV [22–23]. These methods attempt to stimulate the immune system rather than specific therapy. However, some compounds, such as antibiotics, have serious disadvantages, which especially make medication tolerance and residual effect. In addition, it is a bit hard to develop an effective vaccine as only nonspecific innate immune response exists in crustaceans [24]. Passive immunization using pathogen-specific antibodies raised in hens can resolve above problems. This strategy makes crustaceans lacking a true adaptive immune response obtain specific antibody. On the other hand, IgY antibody is crude and has been shown to be effective, safe, abundant, and stable [25–27].

Kim et al. used protein vaccine, recombinant protein, to obtain IgY antibody, which could neutralize or show antiviral activity [8]. As we all know, the DNA vaccine has some advantages since it allows rapid design of a vaccine that contains immunologically reactive sequence without a procedure of protein expression and purification. We have successfully obtained IgY from the egg yolk of chickens vaccinated with a DNA vaccine, namely recombinant plasmid, comprising three genes encoding structural proteins (VP28, VP19, VP15) of WSSV [28]. In this paper, we used this kind of fusion DNA vaccine and inactivated vaccine to immunize hens, and easily obtain IgY.

The present study showed that the level of specific activities of IgY increased 10 days after the initial immunization. The lag time of 10 days can be explained by the time it takes for specific antibodies produced in chicken serum to be transferred and accumulated in egg yolk as reported by Li et al. [29]. To obtain high activity IgY antibodies against WSSV, it was the key to protect crayfish against WSSV. In this study, we found that IgY antibodies have high affinity for WSSV especially from traditional inactivated vaccine (Fig. 1). However, the further purification of WSSV may be needed to immunize hens, which must obtain higher activity anti-WSSV IgY. The overall activity of IgY from DNA vaccine was lower than that of from inactivated WSSV. This difference may be attributed to the complicated immune status of chickens and DNA vaccine, recombinant plasmid, as a weak immunogen. DNA immunization is complicated because the immune response to these vaccines depends on several parameters: the structure of the antigen, the type of adjuvant included in the vaccine, the vaccine vehicle and the route of vaccine injection [30]. Thus, it is necessary to enhance the activity of IgY antibody from DNA vaccine through improving and immunogenicity and immune strategy of DNA vaccine in further study. In addition, the higher absorption value in ELISA may be obtained if the coated antigen in well is the further purified WSSV or purified construction proteins.

In the present study, IgY was purified by two-step salt precipitation procedure, which improved and referred to the method of Akita [31]. Precipitation with 50% saturation ammonium sulfate followed by precipitating with 14% sodium sulfate gave 46% optimum IgY recovery and 77% purity (data from our study team). The two-step salt precipitation could provide a novel approach to produce high yields of active IgY with high purity and easily scaled up for large scale production of IgY. The purified IgY powder could be expediently used through different modes in experiments. In addition, crude egg yolk power and WSF were effective, simple, and convenient sources of specific antibodies.

It is well known that crayfish is a successful laboratory model for the study of crustacean defense mechanisms and susceptible to WSSV and act as an alternate host [32, 33]. When we tested the efficacy of IgY in crayfish after challenge with definite dose WSSV, the mortality of crayfish of the control always reached 100% within a week and that of treatment groups were not very satisfied survival rates. Moreover, the sign of reduced food intake after 2–3 days of challenge was observed, which was not in agreement with Rajeev et al. [33] results that the sign of lethargy and reduced food intake was observed during the experiment in moribund crayfish after 5–6 days of challenge. Therefore, it is likely that the lethal dose of challenge WSSV was high. It is assumed that results should have been more satisfied if the challenge dose is appropriate. Thus, the lethal dose of challenge WSSV should be explored in further study.

The present study showed that the application of IgY significantly resulted in increased survival rates compared with the control when against WSSV, supporting the beneficial effect of IgY. The results also demonstrated that crayfish injected with IgY can obtain higher survival rate than those of crayfish-fed and crayfish-immersed IgY. Without regard to dose, it may be due to more amount of IgY having been received when injected intramuscularly, whereas IgY in feed would be more susceptible to destruction [6, 7]. In our study, feed using oral administration possessed the acceptable stability of feed in water. The figure of feed was unchanged and separating particle from prepared feed was appreciably more than the commercial feed after 2 h immersion in water. To take production process into account, increasing pelleting pressure of feed which could generally weaken particle separation will be put in further practice. In complicated water environment, the efficiency of immersed IgY may be reduced. But both immersion and oral modes of passive immunization are more feasible and practical ways for crustaceans, which does not need manipulation of experimental animals and easily carry out on a large scale. Thus, feeding of IgY can be effective as a means of disease prevention. It is noteworthy that the immersion treatment method showed 46.7% mortality (Fig. 3), which demonstrated the passive immunization through the immersion of specific IgY was also feasible and practical in shrimp culture.

The hen is relatively small and could be fed and managed easily. Once immunized successfully by a relatively small quantity of antigens, a hen can lay eggs continuously which contain specific antibodies in the yolk [34]. The advantages of chicken IgY over antibodies from other mammals include a high concentration and freedom from specific pathogens, making use of IgY a broader prospect for development [35, 36]. To crustaceans living in water environment and popularization on a large scale in culture, it is important that IgY antibodies can be processed easily, which makes crustaceans lacking a true adaptive immune response obtain passive immunization by appropriate and effective modes. In our study, the frozen-dried powder have been proved efficacy of protection crayfish against WSSV. Thus, the use of spray powder and crude WSF shall further be taken into account.

In summary, high activity anti-WSSV IgY antibodies raised in hens could be obtained in our study. Whether IgY is from inactivated vaccine or DNA vaccine, specific IgY antibodies could significantly reduce the mortality of crayfish compared with the control by intramuscular injection. High titer IgY from inactivated vaccine could also significantly reduce the mortality of crayfish by oral administration and immersion. Although it was not completely protective, results from present study indicated that the application of IgY could partially control the prevalence of WSSV. Thus, it is thought that passive immunization of crustaceans with chicken IgY against WSSV have effects of immunotherapy and immune prevention against WSSV and can be developed as a potential strategy for preventing and treating WSSV in practical culture.

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