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ISSN: 0975-7538
Research Article

Studies on Passive Protection of Shrimps against WSSV using Chicken Egg Yolk Antibodies (IgY)

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ABSTRACT

White spot syndrome virus (WSSV), is one of the most virulent pathogens, causing high mortality and dramatic economic losses in cultured shrimp worldwide. In India, commercial shrimp farming started gaining roots only during the mid-eighties particularly in Kerala and West Bengal, for several decades. Up to date no effective measure is available to prevent the spreading of WSSV. Therefore, it is urgent to find an effective method to control this pathogen. Chicken egg yolk immunoglobulin (IgY) provides an inexpensive and effective source of antibodies for the passive immunization of animals suffering from WSSV. In the present study, infected animals were collected from coastal areas and WSSV antigen was prepared and immunized in white leghorn chickens to generate egg yolk antibodies. In conclusion; the specific IgY preparation in this study can provide a novel approach to the prevention and treatment of WSSV infection, which will have an industrial prospect.

Keywords: Shrimp; Chicken Egg Yolk Antibody; WSSV; Passive therapy

INTRODUCTION

Shrimp are swimming, decapod crustaceans classified in the order Caridea, found widely around the world in both fresh and salt water. India's brackish-water and marine aquaculture sector produces only marine shrimp, mainly black tiger shrimp, *Penaeus monodon,* and smaller proportions of Indian white shrimp, *Fenneropenaeus indicus,* and Pacific white shrimp, *Litopenaeus vannamei*. *Penaeus vannamei* is the most important shrimp species in terms of aquaculture production. The other important species are *P. monodon*, *P. chinensis*, *P. merguiensis*, *P. japonicas, P. indicus* and *P. vannamei*. White spot syndrome virus (WSSV) is the most pathogenic among the penaeid shrimp viruses. It has a wide host range including all cultured shrimp species as well as other crustaceans like crabs and crayfish (Wang *et al*., 1998). White spot syndrome virus (WSSV) was first discovered in Taiwan in 1992 (Inouye *et al.,* 1994). It is an enveloped, rod shaped, double-stranded circular DNA virus with sizes ranging from 290 to 305 kb depending on the origin of the isolate causing high mortality in cultured shrimp. The WSSV epizootic in Asia has been the cause of serious losses for shrimp farmers and is largely responsible for lost production in the range of 3 billion dollars per year (Lundin, 1997). Due to the wide range of potential hosts of WSSV (Flegel, 1997), control of the disease is extremely difficult. While no specific treatment for WSSV infection exists, development of new strategies like antibody mediated neutralization (Hulten, 2001) offers a potential solution to overcome WSSV infection.

Egg yolk immunoglobulin (IgY) antibodies are the predominant serum immunoglobulin in birds, reptiles and amphibians, and are transferred in the female from serum to egg yolk to confer passive immunity. IgY harvesting does not involve bleeding of the animal, and maintaining high levels of pathogen-specific antibodies in eggs through vaccination of hens is easily accomplished. IgY offers the possibility of using specific IgY for passive immunization, which is a particularly useful method to invertebrates lacking a true adaptive immune response system. In this study, we report an alternative measures used to overcome these infections by the generation of chicken egg yolk antibodies (IgY) against WSSV antigen.

MATERIALS AND METHODS

Animals

White Leghorn Laying chickens, 21weeks old, were bought from Suguna Poultry Farm, Udumalpet. The hens were kept in isolated cages at the animal house and provided regular food and water. They were used in the study for the production of anti-wssv antibodies. Efficacy studies were carried out in shrimp. Infected animals were collected from Sona Shrimps, Pondicherry. The crustaceans were collected in sterile bags and

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Received on: 07-01-2014
Revised on: 17-02-2014
Accepted on: 21-02-2014

transported to the laboratory using dry ice box and the samples were processed.

Virus Purification

Shrimps were injected intramuscularly with a lethal dose of WSSV using a 30-gauge needle. Dead shrimps were stored at -80°C Deep-freezed WSSV infected shrimps were thawed and muscle tissue was homogenized using a homogenizer in 10-fold volumes of phosphate buffered saline (PBS) at 4°C and centrifuged at 12,000 × rpm for 10 min at 4°C. The supernatant was filtered through a 0.45 µm membrane and used for WSSV challenge experiments and tested for the presence of WSSV by PCR. Virus samples were stored in aliquots at -80°C (Xie *et al*., 2004).

WSSV Detection

DNA was isolated by proteinase method followed by polymerase chain reaction (Lo *et al*., 1996). Then agarose gel electrophoresis was performed, gel plates were cleaned and scaled with cello tape and selected comb was placed and 1g agarose melted in 100ml of 0.5 x TBE at 65°C and the melted agarose solution was cooled to 40-45°C procured into the gel plate and allowed to solidify for 15 minutes at room temperature. Once the gel solidified, the comb was removed and the gel was placed in the electrophoresis tank containing 0.5x TBE buffer. DNA samples were checked by loading 20 microlitre of each sample along with molecular DNA and running at 50V for 2.5 hours. After electrophoresis, the gel was placed in 0.5 microgram/ml ethidium bromide solution for 10 to 15 minutes and destained in sterile double distilled water for 10 minutes.

Antigen Preparation

Antigen was mixed and emulsified with Freund's complete adjuvant (FCA) in 1:1 ratio of WSSV antigen and adjuvant taken separately in 2ml sterile syringes connected by a small plastic tube. Care was taken to mix the FCA thoroughly to disperse the Mycobacterial cells in the oil acracel mixture. The process was repeated several times through the syringe and tube in order to increase the dispersion of antigen phase. The final emulsion was tested by dropping on to cold water, so that the initial drop spreads over the surface, but the subsequent drops remained discrete when extruded from the syringe and were taken for immunization. Freund's Incomplete Adjuvant (FIA) was also prepared in a similar way as that of complete adjuvant. Antigen was mixed and emulsified with Freund's Incomplete Adjuvant (FCA) in 1:1 ratio of WSSV antigen and adjuvant.

Immunization of chickens

21 weeks old white leghorn chicken was intramuscularly injected at multiple sites of the breast muscles with 0.5ml of inactivated WSSV along with equal volume of Freund's complete adjuvant. After two weeks interval the chicken was immunized with same quantity of antigen with Freund's incomplete adjuvant. Further booster doses were carried out by injecting the same amount of antigen, whereby the chicken was hyper immunized. Test bleedings were made and checked for anti-WSSV serum antibodies. Further, eggs laid by the chicken under the test were collected regularly, marked for identification and stored at 4°C.

Isolation and Purification of antibodies from egg yolk

The antibodies were extracted from egg yolk using polyethylene glycol (PEG) (Polson *et al.,* 1980) and precipitated by ammonium sulfate. The partially purified antibody suspension was subjected to dialysis. The IgY was further purified using DEAE cellulose ion-exchange column chromatography. The IgY fraction was then concentrated with polyvinylpyrrolidone (PVP) at room temperature. The concentration of total protein was estimated using Lowry's method (Lowry *et al.,* 1951) and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Laemmli *et al.,* 1970).

Determination of antibody titer by indirect ELISA

ELISA plates were coated with antigen at a concentration of 1 µg/100 µl/well using coating buffer (0.05 M carbonate bicarbonate buffer; pH 9.6) and incubated overnight at 4°C for binding of antigens. After coating, unbound antigens in the wells were removed by washing thrice with PBS containing 0.05% Tween-20 (PBST). The empty sites were blocked by adding 200 µl per well of 1% BSA in PBS and the plates were incubated at 37°C for 1 h. Plates were subsequently washed with PBST and incubated with 100 µl of either polyclonal chicken antibodies or egg yolk antibodies (IgY) at appropriate dilutions. Control wells had PBST and preimmune sera served as respective controls. Plates were incubated for 1 h at 37°C and subsequently washed with PBST.

For the chicken antibodies, 100 µl of diluted (1:1000) rabbit anti-chicken immunoglobulin coupled to horseradish peroxidase (Genei Pvt. Ltd, Bangalore) was added, and the plates were incubated for 1 h at 37°C. After incubation the plates were washed with PBST and enzyme activity was determined by adding 100 µl of TMB solution with H_2O_2 (Genei Pvt. Ltd, Bangalore). The plates were allowed to stand at room temperature in the dark for 20 min. The reaction was stopped by adding 50 µl of 4N H_2SO_4 and the plates were read at 490 nm in an ELISA reader. All samples were tested in triplicate (Voller *et al.,* 1976).

Stability of IgY

Heat Stability

IgY solution were incubated at 4°C, 10°C, 25°C, 37°C, 60°C, 70°C, 80°C and 90°C for 30 minutes. The heat treated IgY cooled in a water-ice bath. The remaining antibody activity was measured by ELISA and tube ag-

glutination test. Antibody activity was represented as a percentage of the untreated control.

pH Stability

The pH of IgY was modulated to the desired pH 2, 4, 6, 8, 10 and 12 with NaOH or HCl. Then the solutions were incubated at 37°C for 2 hours. After incubation each IgY solutions was neutralized. The remaining antibody activity was measured by ELISA and tube agglutination test. Antibody activity was represented as a percentage of the untreated control.

Stability of IgY in Liquid Yolk

The yolks were separated from immune eggs and pooled together. 15ml of yolk was sampled from the pooled yolk and subjected for purification of IgY by PEG extraction method (Polson *et al.,* 1980); the purified IgY was used as untreated control. Then the pooled yolk were equally dispersed in to different containers and exposed to various temperature and pH separately for the desired incubation time. After incubation, the IgY from each sample was purified and the stability of IgY was determined by its agglutinating capacity and Native-PAGE (Poly Acrylamide Gel Electrophoresis) analysis.

RESULTS

Confirmation of WSSV infection by polymerase Chain Reaction

The primers used for the WSSV confirmation from the sample was taken from WSSV PMS 146F1/R1 and F2/R2. The DNA from suspected shrimp samples was taken and two nested primers for the gene are used for amplification.

Total protein estimation of IgY

Protein content of the water soluble fraction obtained by the PEG method, ammonium sulfate-precipitated fraction, dialyzed fraction and column purified antibody fraction were estimated Y using Folin – Ciocalteau reagent Lowry *et al.,* (1951). The IgY concentration in egg yolk was increased during the immunization period and reached 8.5 mg/ml after immunization.

Protein profile of purified IgY by SDS PAGE

The molecular weight of chicken egg yolk antibodies was determined by SDS- PAGE using 10% polyacrylamide gel according to the method of Laemmli (1970). The high molecular weight protein 180KDa showed the purity of IgY. Protein profile of IgY antibodies from different steps of purification were analyzed and visualized by Coomassie Brilliant Blue staining. A standard molecular protein marker was also run in parallel along with IgY fraction.

Estimation of antibody titre by ELISA

The antibody titrations were carried out using Nunc polyvinyl microtitre plates by the method of Voller *et al*., (1976). Indirect antibody capture assay (IACA) showed that the antibodies generated in chickens against WSSV viral antigens. The antibody level in column purified fraction was checked in ELISA by diluting fractions of different dilutions with PBS. Increasing antibody titre was observed up to 1:100000 dilutions after 60 days immunization.

Stability of IgY

Heat Stability

Specific reactivity of IgY after treated with different temperatures such as 4°C, 10°C, 25°C, 37°C, 60°C, 70°C, 80°C and 90°C for 30 minutes was estimated by ELISA. The study revealed that the IgY solution was stable at 4°C, 10°C, 25°C and 37°C. Approximately 25% of the reactivity has lost at 60°C when compared to the reactivity of untreated control. At 70°C there was a significant decrease in the antibody activity and the complete lose of activity was observed at 80°C and 90°C.

pH Stability

The stability of IgY when incubated at different pH such as 2, 4, 6, 8, 10 and 12 for 2 hours at 37°C was assessed by determining the difference between the specific reactivity of treated IgY and untreated control IgY using ELISA. The study results showed that the IgY was stable at pH ranges between 4 and 10. There was significant decrease in the activity of IgY at pH 2 it has retained only 20% of its specific reactivity. The complete lose of antibody activity was observed at pH12.

Stability of IgY in Liquid Yolk

The stability of IgY at different temperature and pH treatments was determined when it was in its original form such as Liquid Yolk. After incubating the Yolk for desired time intervals at different temperatures and pH ranges (as studied previously for purified IgY-Extract), the IgY was extracted from the treated egg yolk by Polson *et al.,* (1980) method and then the residual activity of IgY was determined by ELISA. IgY with liquid yolk was relatively stable to high temperature, at 80^{o}C and complete loss of activity at 90^{o}C. The pH stability of IgY with liquid yolk was found to be pH 3.0 to pH11.

Discussion

The present investigation relates to a yolk antibody against the shrimp infected by the shrimp white spot virus. Such spots are due to the accumulation of calcium salts. They begin at the abdominal 5, 6 shell segments and spread to whole body thereby the shell and muscle to be easily separated owing to their loosened binding. The infected shrimp are tinged with pink or reddish brown due to the diffusion of chromatophores, and almost 100% of them are killed within 3-10 days from the day the symptom was first observed.

Crustaceans do not possess an adaptive immune system, but now it is doubted for some investigation (Arla *et al.,* 2000) that it possess a resistance against WSSV

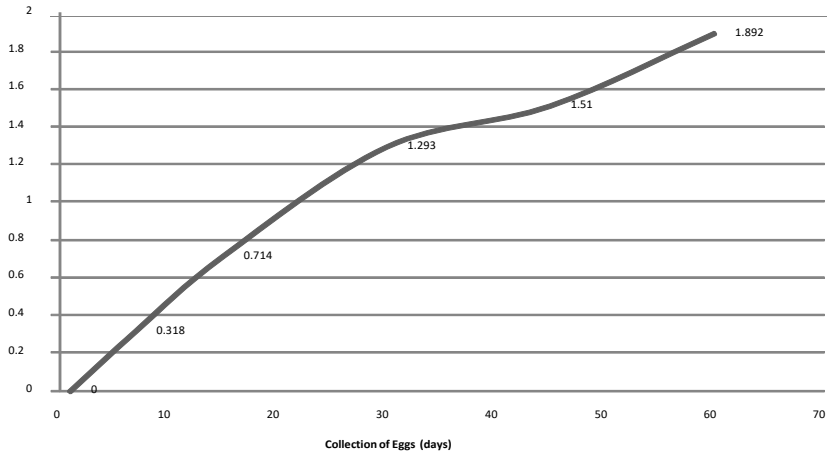

Figure 1: Dynamics of antibody production in hens immunized with WSSV

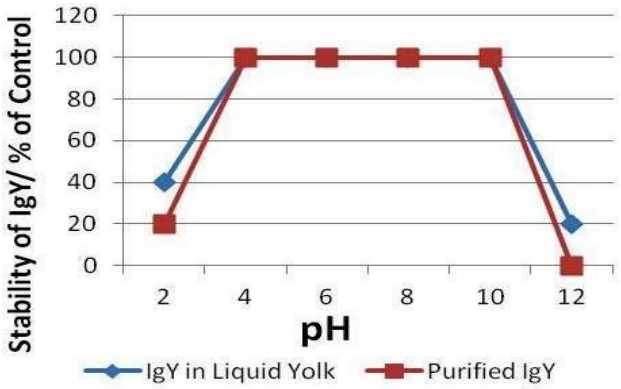

Figure 2: Stability of IgY at Various pH

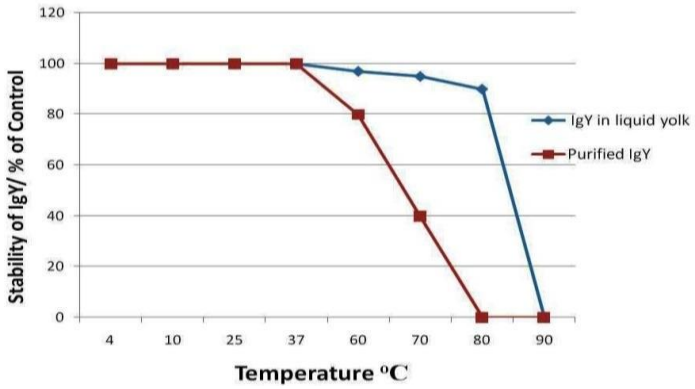

Figure 3: Thermal Stability of IgY

(Du *et al.,* 2006 and Fu *et al.,* 2008). In this study infected shrimps were collected and antigen was prepared by the method followed Xie *et al.,* (2006) DNA was isolated by proteinase K method and confirmation of the positive sample was done by PCR. PCR can detect light infections in brood stock, naupli, post larvae and juveniles (Lo *et al.,* 1996) and quantitative PCR can be used for the quantification of the viral load.

Egg yolk immunoglobulin (IgY) antibodies are the predominant serum immunoglobulin in birds, reptiles and amphibian, IgY harvesting does not involve bleeding of the animal, and maintaining high levels of pathogenic specific antibodies in eggs through vaccination of hens is easily accomplished. The study was carried out to raise specific polyclonal hyper-immune antibodies in chicken against the WSSV virus.

Twenty one weeks old white leghorn chickens were immunized with WSSV antigen along with Freund's Complete Adjuvant. The chicken received booster injections at 14 days interval. The eggs were obtained and the antibodies were purified from egg yolk using Polyethylene glycol method described by Polson *et al.,* (1980). The egg yolk antibodies were further purified by DEAE cellulose ion exchange column chromatography and the fractions were concentrated by polyvinyl pyrolidone (PVP) powder.

The antibodies were detected in egg yolk after a week. The concentration of antibodies increased in the egg

yolk with subsequent booster doses with an average yield of 56 mg per egg yolk. The purity of chicken egg yolk antibodies and its molecular weight were determined by sodium Dodecyl sulphate Poly acrylamide Gel Electrophoresis (SDS-PAGE) The lane shows the protein profile of purified chicken egg yolk antibody at 180 KDa. The activity of egg yolk antibodies were determined by ELISA, showed the presence of specific antibodies for WSSV antigen (Alday *et al.,* 1998). The antibody level in column purified fraction was checked in ELISA with different dilutions using PBS. Highest titre of 1:100000 was observed during 60^{th} day of observation. PCR screening of post larval and rejection of WSSV positive batches before stocking could greatly improve the chances of successful harvest. This gives an idea for the prevention of WSSV virus infection in post larval stage of the shrimp from the hatchery to main pond using chicken egg yolk antibody.

CONCLUSION

Recently, passive immunization has gained much attention, as compared with active immunization. It is known that the hen transfers serum IgG to the egg yolk and that this antibody gives immunity to its offspring. The antibodies present in egg yolk have been termed as IgY. Eggs may be a suitable source of antibody for passive immunization, which requires large amounts of antibodies. The present study clearly indicates that the chicken egg yolk antibodies raised 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 aquaculture industry.

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