Journal of Entomology and Zoology Studies

Available online at www.entomoljournal.com

E-ISSN: 2320-7078
P-ISSN: 2349-6800
JEZS 2018; 6(3): 1751-1755

Received: 17-03-2018
Accepted: 18-04-2018

P Das
(1) ICAR-Central Institute of Freshwater Aquaculture, Bhubaneswar, Odisha, India
(2) Department of Pharmacology and Toxicology, College of Veterinary Science and Animal Husbandry, Bhubaneswar, Odisha, India

J Mohanty
ICAR-Central Institute of Freshwater Aquaculture, Bhubaneswar, Odisha, India

MR Badhe
ICAR-Central Institute of Freshwater Aquaculture, Bhubaneswar, Odisha, India

PK Sahoo
ICAR-Central Institute of Freshwater Aquaculture, Bhubaneswar, Odisha, India

KK Sardar
Department of Pharmacology and Toxicology, College of Veterinary Science and Animal Husbandry, Bhubaneswar, Odisha, India

SC Parija
Department of Pharmacology and Toxicology, College of Veterinary Science and Animal Husbandry, Bhubaneswar, Odisha, India

Correspondence
J Mohanty
Fish Genetics and Biotechnology Division, ICAR-Central Institute of Freshwater Aquaculture, Kausalyaganga, Bhubaneswar, Odisha, India

Assessment of protective response induced by whole antigens of fish ectoparasite, *Argulus siamensis* in rohu, *Labeo rohita*

P Das, J Mohanty, MR Badhe, PK Sahoo, KK Sardar and SC Parija

Abstract
Vaccination against fish ectoparasite, *Argulus siamensis* seems to be a better alternative to the harmful chemicals used for its control. A preliminary study was undertaken to assess the protective response induced by whole antigens of *Argulus* parasite in host fish, rohu (*Labeo rohita*). The whole antigens of *A. siamensis* (50 µg protein/fish) along with adjuvant was injected intraperitoneally three times at 14 days interval to rohu and 14 days after last booster, the fishes were challenged with 250 numbers of metanauplii of *Argulus* to record the load of parasites on host up to 12-days post-challenge. The immunized fish showed 35.42% of low grade infection (1–15 parasites/fish) and 22.92% high grade infection (>30 parasites/fish) as compared to 14.58% and 41.67%, respectively, in control fish. Further, the immunized fish showed reduced haemorrhages on body surface along with higher antibody response (measured through indirect ELISA) as compared to controls. The results indicated possibility of vaccine development against this parasite as method of long term protection.

Keywords: *Argulus siamensis*, antibody response, *Labeo rohita*, protection, whole antigens

Introduction
Freshwater aquaculture has emerged as one of the fastest growing food sectors in the global food industry. It includes varieties of fishes which have different market qualities based on their nutritive value. Like the animal sector, fish provide protein rich food for human consumption. To meet this demand, more intensive fish farming is growing rapidly wherein fish are cultured in a restricted area resulting in poor water quality as well as improper management. This further culminates in emergence of different infectious diseases. Among different diseases in freshwater aquaculture in India, parasitic diseases are more prevalent in the cultured fish causing heavy economic loss to the fish farmers [1]. Ectoparasites in general possess a major threat to the aquaculture industry among which *Argulus* spp. particularly *A. siamensis* is the most important one [2]. The economic loss due to argulosis was calculated to be ~Rs. 30,000/- per hectare per year in carp culture farms in India taking into account the factors like mortality, reduced growth rate, and costs associated with drug application [3]. *Argulus* parasites cause dermal ulceration, physiological stress, immunosuppression and reduced fish growth. It also leads to secondary infections, which has been linked to the transmission of infectious and other parasitic diseases [4]. This disease is usually controlled by chemicals or anti-parasitic drugs which possess concern over human health due to the residual effects of drugs and also emergence of resistance against the parasite [5]. Thus, control of argulosis has to be done preferably by prophylactic measures like vaccination which has already been proven to be safe and efficacious. Teleosts have elements of both innate and adaptive immune systems [6] and thus vaccination has been successful against many bacterial and viral diseases of cultured fish species. However, there is no commercial vaccine available for any parasitic diseases in fish. It is particularly difficult to develop a vaccine against any ectoparasite. However, the successful development of a vaccine against cattle tick, *Boophilus microplus* [7] showed the possibility of controlling ectoparasites through vaccination. Thus, the present study was carried out with an objective to know the protective efficacy of whole antigens of *Argulus* in a host fish, rohu (*Labeo rohita*) against argulosis, which would pave way towards development of vaccine against this parasite.

Materials and methods

The experiments conducted using animals in this study were approved by the Institute Animal Ethics Committee of ICAR-CIFA, Bhubaneswar.

1. Maintenance of *Argulus* parasite

Live *A. siamensis* parasites from infected fish were collected from the ponds of CIFA farm and were maintained in the Institute wet laboratory along with host fish, rohu juveniles in 500 l fibre reinforced plastic tanks. The fish were fed with commercial pellet feed. Water quality parameters like pH, temperature and dissolved oxygen were checked intermittently and maintained at optimum. Regular water exchanges were carried out taking care of not losing the parasites from the tank.

2. Preparation of whole homogenate of *Argulus* parasites

The whole homogenate of *Argulus* parasites was prepared for immunization of rohu. Adult *Argulus* parasites were homogenized by Super FastPrep-1 homogenizer (MP Biomedicals, OH, USA) using lysing matrix C at a speed setting of 25 (4000 cycles per min) for 10 s in TBS (20mM Tris HCl buffer, pH-7.4 with 0.15M NaCl) buffer along with protease inhibitor cocktail (Promega, WI, USA) was used to prepare the homogenate. The homogenate was centrifuged at 10,000 rpm for 30 min and the supernatant collected. The protein concentration in the supernatant was quantified by BCA method using Pierce BCA Protein Assay Kit (Cat. No. 23225, Thermo Scientific, IL, USA) following manufacturer's instructions.

3. SDS-PAGE

Argulus antigens prepared as above was run in SDS-PAGE using 12% separating gel and 5% stacking gel [8]. The samples and molecular weight standards (Genei, India) were mixed with equal volume of sample buffer and heated in boiling water bath for 2 min prior to loading. The gel was run in tris-glycine-SDS buffer system at 200V for approximately 45 min and stained with Coomassie brilliant blue R250.

4. Immunization of rohu with *Argulus* antigens

Twenty four numbers of apparently healthy rohu of 50-100 g size obtained from the Institute farm were divided equally in two tanks to serve as immunized and control groups, and left for acclimatization in wet laboratory for 7 days.

The whole homogenate of *Argulus* parasites was diluted to a protein content of 0.5 mg/ml and emulsified with equal amount of Freund's Complete Adjuvant (FCA) (Sigma, USA). The fishes were immunized intraperitoneally with 0.2 ml of antigenic mixture that contained a dose of 50 µg of protein/fish. The fishes were booster injected two times on 14th and 28th days of primary injection with the same dose of antigen emulsified with equal volume of Freund's Incomplete Adjuvant (FIA) (Sigma, USA). Control animals similarly received injections with TBS emulsified with adjuvants. After 14 days of last booster dose, the fishes were challenged with *Argulus* parasites to assess the protective response as detailed later.

A similar experimental set up and immunization schedule was followed for another two groups consisting 6 numbers of fish each as control and immunized. The fishes were bled after 14 days of last booster injection. The serum was separated by centrifugation at 8000 rpm for 20 min and preserved at -20 ^{o}C. The serum samples were used to test the antibody level against the parasite antigens.

5. Challenge of fish against parasite infection

The immunized and control group fish of 12 numbers of each were challenged with *Argulus* parasites in the challenge facility laboratory of the Institute. Both the groups of fish were challenged with 250 numbers of metanauplii per fish following the method of Kar *et al*. [6]. In short, the eggs of *Argulus* parasites were collected and allowed to hatch in the laboratory. These metanauplii were counted and used for the challenge method in our experiment. The fishes were initially left with the metanauplii in a lesser volume of water to allow better opportunity for the parasites to attach to fish and then released into the tanks along with the water. The load of parasites attached to fish was recorded on 3rd, 6th, 9th and 12th days post-challenge by carefully counting the attached parasites on the body surface. The average numbers of parasites attached were calculated and a graph was plotted. Further, depending on the number of parasites attached, three different grades of infection was defined such as low (1–15 parasites/fish), moderate (16–30 parasites /fish) and high (>30 parasites/fish). The fishes were also looked into the extent of haemorrhages on the body surface between both groups of fish.

6. ELISA for the detection of anti-*Argulus* antibody

The antibody produced in the rohu serum against *Argulus* antigens was measured by an indirect ELISA. The 96-well ELISA plate (Griener, Germany) was coated with the *Argulus* whole antigens at a concentration of 5 µg/ml in 0.05M carbonate-bicarbonate buffer, pH 9.6 and incubated at 4 ^{o}C overnight. The plate was washed with TBST (TBS containing 0.1% tween 20) for three times and blocked with 5% skim milk powder for 2 h at room temperature (25 ^{o}C). The plate was then washed again with TBST and incubated with rohu serum in serial two fold dilutions starting with 1:25 to 1:25600. Six individual serum samples from both immunized and control fish were used in the test. After one hour, the plate was washed as previously and added with guinea pig anti-rohu serum (prepared earlier and available in the laboratory) at a dilution of 1:1500. After one hour of incubation, the plate was washed again as described earlier and goat anti-rabbit HRPo conjugate (GeNei, India) was added at 1:500 dilution. Finally, the plate was washed three times with TBST, added with substrate TMB/H_2O_2 (GeNei, India) and left in dark for 10 min. The reaction was stopped by adding 1N H_2SO_4 solution and reading was taken in a microplate reader (Metertech, Taiwan) at 450 nm. Mean and standard error of vaccinated and control groups at each dilution were calculated and the graph was plotted.The difference between both control and immunized groups at each dilution was calculated at 95% confidence interval and significance at $p<0.05$ with help of unpaired t-test using online GraphPad software.

Results

There was no mortality either in immunized or control group. The whole homogenate sample of *Argulus* prepared in FastPrep homogenizer was found to be efficient in grinding the parasite and the sample recovery was better compared to pestle and mortar homogenization. A protein concentration of approximately 250 µg proteins could be recovered from whole homogenates of 10-15 numbers of adult parasites. In SDS-PAGE, the protein bands were found intact without degradation (Fig. 1). The entire proteins in the sample ranged between 132.2 to 11.7 kDa and majority of the bands were found within a range of 132.2 to 29kDa.

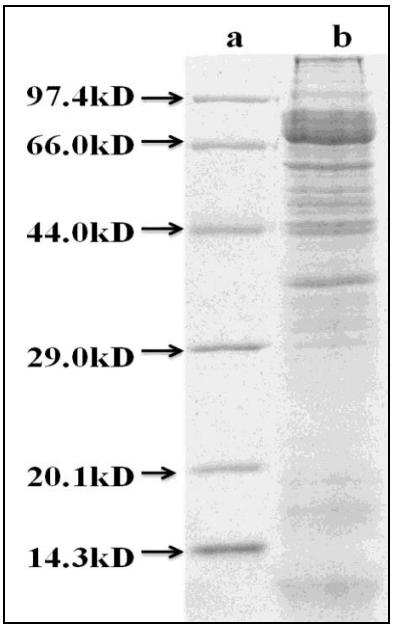

Fig 1: SDS-PAGE analysis of *A. siamensis* antigens in 12% acrylamide gel. a. molecular weight protein standards b. whole antigens of *Argulus*

The number of parasites attached to the fish was considered as a measure of the efficiency of the antigens against the infection. The 1st parasitic load was counted on 3rd day post-challenge which showed less numbers of parasites in immunized fish (avg. 14.92) compared to control group (avg. 22.92). The parasitic load was subsequently counted on 6th, 9th and 12th days post-challenge. A low load in parasite numbers on the immunized fish (28.5 vs 33.08 on 6th day, 22.92 vs 23.5 on 9th day and 29.58 vs 31.08 on 12thday in immunized vs control group of fish) was recorded (Fig. 2). Further, the numbers of fishes having different grades of infection were recorded and are shown in Table 1. Taking into account all 4 days of observation, 41.67% of fish in control group were highly infected having more than 30 parasites attached to their body surface compared to 22.92% of fish in immunized group of fish. Whereas, low grade of infection was observed higher in immunized group than the control group (14.58% vs 35.42% - control vs immunized). The moderate grade of infection was almost similar in both the groups (43.75% vs 41.67% - control vs immunized). Besides, body hemorrhages were marked more severe in the control group of fish (Fig. 3) compared to immunized fish.

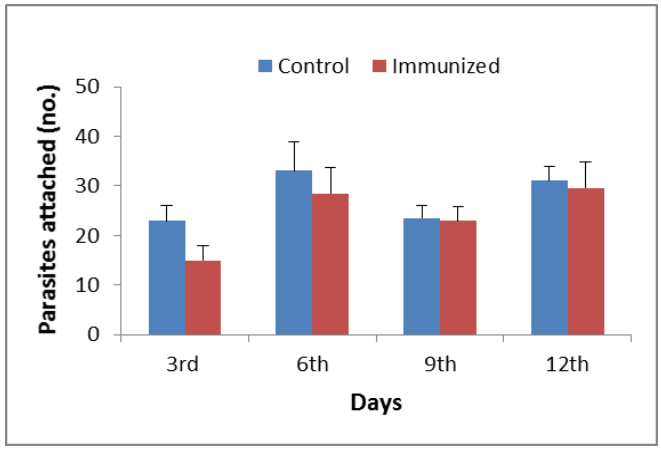

Fig. 2. Parasite load on rohu immunized with whole antigens of *Argulus* and challenged with the parasites.

Table 1: Numbers of rohu affected with different grades of *Argulus* infection.

Grades of infection (no. of parasites)	3rd day post-challenge		6th day post-challenge		9th day post-challenge		12th day post-challenge	
	Control	Immunized	Control	Immunized	Control	Immunized	Control	Immunized
Low (1-15)	2	6	3	4	1	2	1	5
Moderate (16-30)	6	5	2	6	9	8	4	1
High (>30)	4	1	7	2	2	2	7	6

Fig 3: *Argulus* infected rohu from control group showing hemorrhages near pectoral fin.

Level of antibody production was assessed by an indirect ELISA method comparing control and *Argulus*-immunized rohu serum samples. The absorbance values of both control and immunized groups plotted in the graph showed the significantly enhanced antibody level in the rohu serum of the immunized group from a dilution of 1:25 to 1:3200 (Fig. 4). At 1:25 dilution, the maximum absorbance value was recorded in immunized serum as 1.14 against 0.53 in control serum.

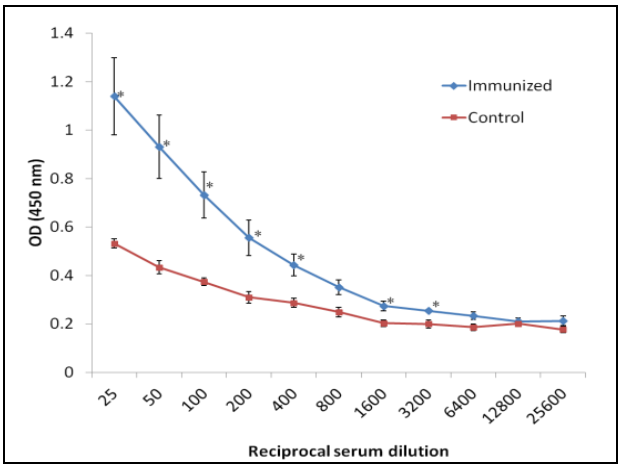

Fig 4: Anti-*Argulus* antibody level detected by an indirect ELISA in serum of rohu immunized with whole antigens of *Argulus*. *indicates statistically significant from respective control.

Discussion

Fish ectoparasite, *Argulus siamensis* is the most important parasite of Indian carp aquaculture system. The present preliminary attempt was found to prove the possibility of exploring immunological means for its control rather than

depending upon drugs or parasiticides that were having residual effects and other concerns.

SDS-PAGE was carried out to monitor the integrity of proteins in the sample. Addition of protease inhibitor particularly helped in maintaining the integrity as is evident in the gel (Fig. 1); unlike without addition, where the sample possibly gets degraded and forms smear in the gel (results not shown). The polypeptides of *Argulus* antigens were ranging within molecular weights of 132.2 to 11.7kDa, which was similar to Saurabh *et al*. [9] who noticed polypeptides from the same species ranging from 130.55 to 16.22kDa. A similar polypeptide range of 100 to 15kDa has also been reported in *A. foliaceous* [10].

In our experiment, the protective response in rohu against *Argulus* parasite was assessed by immunization-challenge experiment. The immunization was done with the whole parasitic antigen to check any kind of protective response manifested by host, rohu against the parasitic challenge. The whole antigens have also been tried as vaccines with several other parasites [11-12] including attempts against another similar ectoparasite of marine fish species, salmon lice (*Lepeophtheirus salmonis*) [13]. Loukas and Good [14] stressed upon the better efficiency of whole parasitic vaccines compared to subunit vaccines presently researched for many parasitic diseases. In the present study, the whole homogenate of the parasitic antigens was mixed with equal proportion of adjuvant to enhance the immune response in rohu. Adjuvants are known to enhance the magnitude of antibody production in the host body as well as increase the longevity of specific immune responses against the antigens [15]. Upon *Argulus* challenge, the parasite load attached on the body surface of the immunized and control fish was considered as the criterion of immunization efficiency. There was a comparatively lower parasitic load on the immunized fish indicating some degree of protection to the host conferred by the injected antigen. This was further strengthened by the observation on the number of parasites attached on each individual fish accounting to different grades of infection. The high grade of infection was comparatively lower in immunized fish than the control and the reverse was with low grade infection. The higher percentage of low grade infection in immunized group indicated further partial efficacy of this whole antigens. Also, the severity of hemorrhagic patches on the skin was found relatively higher in control group than the immunized group confirming to a protective response conferred by the immunized antigens. Kar *et al*. [16] experimented with ribosomal P0 peptide from *A. siamensis* in host *L. rohita* and they observed only a delayed mortality with vaccinated group. Grayson *et al*. [13] also reported a partial immunity in Atlantic salmon to *L. salmonis* using extracts derived from adult caligid copepods. However, similar trials with peptide/recombinant proteins of my32 protein against the ectoparasite, sea lice showed a 57% inhibition of infestation in vaccinated group in challenge experiment [17].

Production of antibody against *Argulus* antigens in rohu serum was confirmed by an indirect ELISA. Enzyme-linked immunosorbent assays (ELISAs) are among the most commonly employed laboratory techniques often used in vaccine research to detect the level of antibodies and to establish correlates of immune protection [18]. In our experiment, the ELISA was conducted taking the whole antigens of the *Argulus* parasites that determined the antibody production in rohu sera. The significant differences observed between immunized and control sera depicted the production of antibody in immunized fish against the *Argulus* antigens, which is possibly responsible for the level of protection observed in our immunization-challenge experiment.

Thus, the results of this preliminary experiment showed some degree of protection conferred by the whole parasite antigens to fish host and strengthens the possibility of a vaccine strategy against *Argulus* parasite in rohu, *L. rohita*.

Conclusion

The study depicts that immunization with whole antigens of *Argulus* parasites induced protective capability in rohu, *L. rohita* against the parasite as evident from lower parasite burden and less severe body hemorrhages as well as higher specific antibody against *Argulus* antigens in immunized fish. Thus, vaccination with a suitable antigen from the parasite may protect the fish on long term basis and can be a suitable alternative to the harmful chemicals currently in use for its control.

Acknowledgements

The authors are thankful to the Director, ICAR-Central Institute of Freshwater Aquaculture, Bhubaneswar for providing necessary facilities during the study. The financial support received from ICAR, New Delhi under CRP on Vaccines and Diagnostics project is also acknowledged.

References

1. Lakra WS, Abidi R, Singh AK, Sood N, Rathore G, Swaminathan TR. Fish introductions and quarantine: Indian perspective. National Bureau of Fish Genetic Resources, Lucknow, India, 2006, 198.
2. Sahoo PK, Mohanty J, Garnayak SK, Mohanty BR, Kar B, Jena JK *et al*. Genetic diversity and species identification of *Argulus* parasites collected from major aquaculture regions of India using RAPD-PCR. Aquaculture Research. 2013; 44:220-230.
3. Sahoo PK, Mohanty J, Garnayak SK, Mohanty BR, Kar B, Prasanth H. Estimation of loss due to argulosis in carp culture ponds in India. Indian Journal of Fisheries. 2013; 60:99-102.
4. Walker PD, Flik G, Wendelaar Bonga SE. The biology of parasites from the genus *Argulus* and a review of the interactions with its host. Host-Parasite Interactions. Garland/Bios Scientific publishers, Abingdon, UK, 2004, 107-129.
5. Hemaprasanth KP, Raghavendra A, Singh R, Sridhar N, Raghunath MR. Efficacy of doramectin against natural and experimental infections of *Lernaea cyprinacea* in carps. Veterinary Parasitology. 2008; 156:261-269.
6. Kar B, Mohanty J, Hemaprasanth KP, Sahoo PK. The immune response in rohu, *Labeo rohita* (Actinopterygii: Cyprinidae) to *Argulus siamensis* (Branchiura: Argulidae) infection: kinetics of immune gene expression and innate immune response. Aquaculture Research. 2015; 46:1292-1308.
7. Willadsen P, Bird PE, Cobon GS, Hungerford J. Commercialization of a recombinant vaccine against *Boophilus microplus*. Parasitology. 1995; 110(Suppl.):S43–S50.
8. Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature. 1970; 227:680-685.
9. Saurabh S, Mohanty J, Garnayak SK, Sahoo PK. Identification of immunodominant polypeptides of the freshwater fish lice *Argulus siamensis* (Wilson) - preliminary findings. Indian Journal of Fisheries. 2012; 59(1):103-106.

10. Ruane N, Mccarthy TK, Reilly P. Antibody response to crustacean ectoparasites in rainbow trout, *Oncorhynchus mykiss* (Walbaum), immunized with *Argulus foliaceus* L. antigen extract. Journal of Fish Diseases. 1995; 18:529-537.
11. Giunchetti RC, Reis AB, da Silveira-Lemos D, Martins-Filho OA, Corrêa-Oliveira R, Bethony J *et al*. Antigenicity of a whole parasite vaccine as promising candidate against canine leishmaniasis. Research in Veterinary Science. 2008; 85:106-112.
12. Coban C, Igari Y, Yagi M, Reimer T, Koyama S, Aoshi T *et al*. Immunogenicity of whole-parasite vaccines against *Plasmodium falciparum* involves malarial hemozoin and host TLR9. Cell Host Microbe. 2010; 7:50-61.
13. Grayson TH, John RJ, Wadsworth S, Greaves K, Cox D, Roper J *et al*. Immunization of Atlantic salmon against the salmon louse: identification of antigens and effects on louse fecundity. Journal of Fish Biology. 1995; 47(Suppa):85-94.
14. Loukas A, Good MF. Back to the future for antiparasite vaccines? Expert Review of Vaccines. 2013; 12:1-4.
15. Carter D, Reed SG. Role of adjuvants in modeling the immune response. Current Opinion in HIV and AIDS. 2010; 5:409-413.
16. Kar B, Mohapatra A, Mohanty J. Sahoo PK. Evaluation of ribosomal P0 peptide as a vaccine candidate against *Argulus siamensis* in *Labeo rohita.* Open Life Sciences. 2017; 12:99-108.
17. Carpio Y, Castillo CG, González JML, Acosta J, Tuero LB, Morales A *et al*. Development of a vaccine against sea lice. The 10th International Sea Lice Conference, Portland, Maine, USA, 2014, 115.
18. Barrette RW, Urbonas J, Silbart LK. Quantifying specific antibody concentrations by Enzyme-Linked Immunosorbent Assay using slope correction. Clinical and Vaccine Immunology. 2006; 13:802-805.