Introduction

Aquaculture faces many challenges including combating infectious diseases, epizootics, and maintaining health standards. Diversification and intensification of aquacultural practices across the globe have resulted in an increase in the occurrence of infectious diseases. Argulosis is one such emerging disease caused by branchiuran ectoparasites of the genus *Argulus*, which is a major obstacle in the development of Indian carp culture industry. These arthropods parasitize freshwater fish and feed on mucus, epidermal cells, and blood, which causes dermal ulceration, physiological stress, immune suppression, and secondary infections, resulting in reduced growth and mortality. *Argulus siamensis* is the most prevalent species in the Indian aquaculture systems [1] and a loss to the tune of INR 300 crores (~ 44662830 US$) per annum is being incurred due to this disease only in Indian carp farming [2].

Carp culture is the mainstay of Indian aquaculture and amongst the important carp species cultured, *Labeo rohita* (rohu) is the most important because of its high growth potential and consumer preference (http://www.fao.org/fishery/culturedspecies/Labeo_rohita/en). It is inopportune for the Indian carp industry that rohu also happens to be the most susceptible species to *A. siamensis* infection in comparison to other cultured carps [3]. A wide variety of chemicals and drugs have been used traditionally to control this ectoparasite. Pesticides like organophosphates, pyrethroids, and other moult inhibiting drugs are the most abundantly used chemicals to control these parasites in culture conditions. The new drugs on the block which have been adopted by fish farmers of India are avermectins [4]. However, the repeated and intensive use of such chemicals and drugs leads to environmental contamination and resistance development by the parasite [5].

One of the environmentally safe and economically sustainable alternatives for control of ectoparasite infestations is possibly the use of vaccines. Identification of novel protective antigens is the limiting step in...
the development of vaccines against ectoparasites. Development of a vaccine with appreciable efficacy against any ectoparasite still remains the holy grail of parasitology researchers worldwide. Nevertheless, the development of Bm86-based commercial vaccines against *Rhipicephalus* (*Boophilus*) *microplus* tick (TickGARD, Hoechst Animal Health, Australia, and Gavac marketed by Heber Biotec, Cuba), whose efficacies have been proven by field studies in cattle [6,7,8], is an important milestone in parasite vaccinology. Thus, identification of protective antigens is a crucial step in development of effective vaccines against any ectoparasite.

The ribosome is universal to all organisms as a component of the protein translation machinery. In eukaryotic cells, ribosomes consist of individual molecules of ribosomal RNA and of a vast number of (more than 80) ribosomal proteins (RPs) which are organized into the major and minor subunits. Amongst these RPs, there is a group of acidic protein (pI = 3.0–5.0) known as the P proteins (P0, P1 and P2) due to their ability to be phosphorylated. These P proteins form a pentameric complex P0 (P1-P2), which constitutes the stalk region of the GTPase center of the large ribosomal subunit [9,10,11] and is an important structural unit in the translocation step of protein synthesis [12]. The ribosomal protein P0 is a highly conserved neutral protein in eukaryotes [13] having a molecular mass between 34 to 38 kDa [14]. P0 is a multifunctional protein in many cellular processes. It is involved in protein synthesis by formation of the complex with P1, P2, 28S rRNA, and the factor eEF2, and its absence leads to the generation of defective 60S ribosomal subunits, loss of protein synthesis, and cell death [11,15,16]. In addition, dephosphorylated P0 migrates to the nucleus where it shows apurinic/apyrimidinic endonuclease activity and is involved in DNA repair [17,18,19]. The regulatory role of P0 has also been documented in apoptosis, cell development, and carcinogenesis [20,21].

P0 has proved to be immunogenic as an antigen against several protozoa and bacteria like *Toxoplasma gondii*, *Neospora caninum* [22], *Trypanosoma cruzi* [23], *Leishmania infantum* [24,25], several species of *Babesia* [26,27,28], and *Plasmodium* [29,30]. P0 protein has been documented to be important for ingestion of blood and viability in the *Haemaphysalis longicornis* ticks, whose disruption by RNA silencing showed a mortality of 96% in ticks [31]. Recently, the efficacy of a 20 amino acids synthetic peptide of P0 was tested against the dog tick *Rhipicephalus sanguineus* by immunization [32]. This peptide derived from the immunogenic region of the tick P0, not very conserved in comparison to its host P0, showed an overall efficacy of 90% by significantly reducing the number of hatchings as well as adults.

In this experiment, we provide the ribosomal P0 protein sequence characterization of *A. siamensis* and the host, one of the important Indian major carp species, rohu (*L. rohita*). Herein, we used a peptide of 21 amino acids from the ribosomal P0 protein of *A. siamensis* as a candidate antigen in a vaccination and challenge trial using *L. rohita*. This peptide was chosen from a highly immunogenic region within the P0 protein, which also showed low sequence similarity with host P0 protein, to avoid induction of tolerance in parasite or production of auto-antibodies in the host. This is a preliminary study that showed limited protection of immunized host against parasite challenge suggesting the P0 protein epitopes could be targeted as protective antigens for vaccine development.

2  Materials and Methods

2.1 Parasite

Populations of *A. siamensis* (bred from gravid parasites that were collected from naturally infected *L. rohita* cultured in the farm of the Central Institute of Freshwater Aquaculture, Bhubaneswar, India) were maintained on *L. rohita* (approximately weighing 500 g, n = 5) in circular 1000 L fibre reinforced plastic tanks in wet laboratory. Fish were fed commercial pellet diet at 2.5% of body weight. About 10% of water was removed daily along with the left-over feed and fecal matter. The parasites were allowed to develop through all stages, mature and mate on the host to produce egg strings. Thus, a stock of parasite population was maintained in laboratory that provided material for all subsequent experiments.

In order to collect metanauplii for establishing new infection, the eggs deposited on the sides of the tank were collected in beakers containing tap water and incubated in the laboratory for hatching in a previously standardized way [33]. Briefly, egg-strings were collected either by picking egg-bearing females directly from fish in a tank and allowing them to lay eggs in a beaker or by carefully scraping egg strings laid on the sides of the tank by gravid females or by hanging glass slides in aquaria. These egg strings were washed gently to remove debris and transferred to beakers containing non-chlorinated tap water. The eggs were incubated at 28°C with a daily refreshment of water until hatching. The hatched out metanaupliar larvae were subsequently used for experiments involving exposure of fishes to the larvae for infection, within 6–8 h of hatching.
2.2 Fish

Rohu (L. rohita) juveniles (50–70 g) showing no signs of disease (under gross and microscopic examination of skin, gill, intestine and kidney tissues of representative samples) and no previous history of parasitic infections were obtained from the Institute farm. Fishes were acclimatized in tanks of 1000 L capacity with tap water, for 15 days before conducting the experiment. They were fed with commercial pellet diet at 2.5% of body weight. About 10% of water was removed daily along with the left-over feed and fecal matter. The basic physico-chemical water parameters were measured systematically at seven-day intervals to maintain its optimal level throughout the experiment (temperature: 25–28°C, dissolved oxygen: 5.68 ± 0.69 mg L⁻¹, pH: 7.8 ± 0.21, nitrates: 0.016 ± 0.007 mg L⁻¹, ammonia: 0.108± 0.015 mg L⁻¹).

**Ethical approval:** The research related to animals use has been complied with all the relevant national regulations and institutional policies for the care and use of animals. The experiments conducted using animals in this study were approved by the Institute Animal Ethics and Biosafety Committees.

2.3 P0 sequences

2.3.1 cDNA synthesis

Complementary DNA (cDNA) samples were obtained by the reverse transcription of total RNA obtained from A. siamensis adult parasite and RNA obtained from kidney tissue of L. rohita by using Thermo Scientific RevertAid First Strand cDNA synthesis kit (Thermo Fischer Scientific Inc.) following the manufacturer’s instructions. Briefly, template RNA (1 µg) was added to random hexamer primer (1 µL) and nuclease-free water to make the final volume of 12 µL. Subsequently, 5X reaction buffer (4 µL), Ribolock RNase inhibitor (1 µL), 10 mM dNTP mix (2 µL) and RevertAid M-MuLV RT (200 U/µL) (1 µL) were added to make the final volume of the reaction mixture to 20 µL. The mixture was incubated at 25°C for 5 min followed by 60 min at 42°C. The reaction was terminated by heating at 70°C for 5 min. The reverse transcription reaction product was stored at -20°C for further use.

2.3.2 Amplification and cloning of P0 sequences

Nucleotide sequences that encode P0 ribosomal proteins of A. siamensis and L. rohita were obtained by polymerase chain reaction using self-designed primer pairs. The primers were designed by Primer premier software based on heterologous sequence information and from the transcriptome sequences of A. siamensis [34], as well as L. rohita [35] generated earlier in our laboratory.

PCR amplification reactions consisted of an initial denaturation at 95°C for 3 min followed by 35 cycles of denaturation at 95°C for 30 s, annealing at respective temperatures of each gene (54.9°C for P0 of rohu and 52.2°C for P0 of A. siamensis) for 1 min, and 72°C extension for 1 min, followed by a final extension at 72°C for 10 min using 1.5 units of Taq DNA polymerase (Bangalore Genei, Merck Specialities Pvt. Ltd., India). The PCR products (8 mL) were visualized on a 1% agarose gel stained with ethidium bromide. The amplified PCR products were purified and cloned into commercial pTZ57R/T (Fermentas, Thermo Scientific, USA) vector for sequencing. Three clones of each sequence were sequenced (Xcelris, India) for confirmation.

2.3.3 Bioinformatic analysis of P0 sequences

Sequence alignment was done in ClustalW multiple alignments implemented in BioEdit version 7.0.9.0 [36] and amino acid sequences were deduced. Analysis of amino acid sequence identity was performed using BlastX (www.ncbi.nlm.nih.gov/blast/) and ClustalW programs Bioedit version 7.0.9.0. Complete coding sequence was derived using ORF Finder (http://www.ncbi.nlm.nih.gov/ gorf/gorf.html) and amino acid sequence was deduced. The isoelectric point and molecular weight of the protein were calculated using Compute PI/Mw tool implemented in ExPASyBioinformatics Resource Portal (http://web.expasy.org/compute_pi/). The functional domains were identified using SMART domain architecture analysis (http://smart.embl-heidelberg.de/).

Other protein analyses such as hydrophobicity, prediction of the accessibility degree of amino acid residues in the protein, and prediction of potential B epitopes inside the protein were carried out for parasite selected peptide sequence using bioinformatic programs [37,38].

2.4 Synthesis of the P0 peptide and conjugation to the Keyhole Limpet Hemocyanin (KLH)

The peptide pP0 corresponding to 21 amino acids of the region with lowest similarity of the parasite P0 protein to...
the rohu P0 protein was obtained by chemical synthesis (Sigma Aldrich Chemicals Pvt. Ltd, USA). The purification was performed by reverse phase chromatography, and the purity and molecular mass of the peptide was verified by mass spectroscopy at the synthesis point.

The synthetic peptide was conjugated to Keyhole Limpet Hemocyanin (KLH) of *Megathura crenulata* by the soluble carbodiimide method [39]. Briefly the conjugation was done using succinic anhydride spacer agent. The carboxyl groups of the carrier protein were activated with 1-ethyl-3-(dimethylaminopropyl)-carbodiimide (DIEC) followed by addition of the synthetic peptide. The conjugates were extracted out by gel filtration chromatography and the final concentration of the conjugate was estimated using the bicinchoninic acid method [40] at the synthesis level.

### 2.5 Immunization and challenge of *L. rohita*

The conjugated peptide in phosphate buffered saline (PBS) was adjuvanted in Freund’s complete/incomplete adjuvant (Bangalore Genei, India) in 1:1 proportion to make the immunogen. Only PBS with respective adjuvant in similar proportion formed the control injection. Rohu juveniles were allocated at random into two experimental groups as follows:

In group 1, forty rohu juveniles were injected with pP0-KLH conjugate at a dose of 1.5 µg/g of body weight (200 µL of immunogen-adjuvant to each animal). The fish were divided into four tanks with ten fish in each. These fish received a single intraperitoneal dose of the immunogen with Freund’s complete adjuvant. The two subsequent boosters were given on the 14th day and 28th day using Freund’s incomplete adjuvant in a similar fashion.

In group 2, forty rohu juveniles (10 fish each in 4 tanks) were injected with PBS and Freund’s complete adjuvant in a 1:1 proportion (i.e. control injections) at a dose of 200 µL/animal that served as the control group. Subsequently, PBS and Freund’s incomplete adjuvant were used as boosters in the control group.

All the animals of groups 1 and 2 were challenged with 200 metanauplii per animal 7 days post booster dose following the standardized protocols as described by Kar et al. [41]. Briefly, each fish was brought to 50 L water in plastic buckets provided with aeration and 200 metanauplii emptied into the bucket. The fish was held in the bucket for 1 h until most of the parasites attached to it and then carefully transferred to tanks along with water used in challenge. Twenty fish (10 each from control and immunized group) were maintained together in the same tank (replicated in 4 tanks). The fish of the control group were fin clipped for identification. The fish were inspected visually to observe the parasite load in each group. The progress of the infection and mortality was recorded for individuals of each group.

To check the immune response in the serum, 10 animals from each group were bled after 7 days post-second booster. The fish were bled through the caudal vein after being anaesthetized with MS222 (Argent chemical, Redmond, USA). Blood samples were allowed to clot at room temperature for 30 min and then kept at 4°C for 3 h. The clotted samples were centrifuged at 5,000 g for 5 min at 4°C to collect the serum, which was stored immediately at -70°C until further analysis.

### 2.6 Analysis of immune response by Dot Blot assay

The dot blot assay was performed using a nitrocellulose membrane. The pP0-KLH (2 µL, 5.32 mg/mL), P0 (2 µL, 2 mg/ml dissolved in PBS) (Sigma), KLH (2 µL, 2 mg/ml dissolved in PBS) (MP Biomedicals, India), and PBS (2 µL) were directly applied as a spot on the membrane. The membrane was left at room temperature for one hour and submerged for 30 min in a blocking solution containing 5% skimmed milk powder. The membrane was incubated with the primary antibody (i.e. rohu serum from pP0-KLH injected rohu) at a ratio of 1:100. The membrane was later rinsed three times with TBS-T (20 mM TrisHCl, 150 mM NaCl, 0.05 % Tween 20, pH 7.4). The membrane was again incubated with secondary antibody (i.e. anti-rohu rabbit serum) at a concentration of 1:1000 (available in our laboratory). The membrane was rinsed and further incubated with goat anti-rabbit IgG-HRP conjugate (Bangalore Genei, India). The membrane was developed with 3,3’diaminobenzidine (DAB, Amresco, USA) and hydrogen peroxide (E.Merck, Germany). The reaction was terminated using tap water.

### 3 Results

#### 3.1 P0 sequence analysis

The amplified P0 sequence of rohu had an open reading frame of 950 bp from which a 316 amino acid sequence of P0 protein was deduced with a molecular mass of 34,246.42 Da and a theoretical isoelectric point of 5.91. It showed 96% identity to 60S acidic large ribosomal protein
Peptide-based vaccine against *A. siamensis*

The P0 sequence of *Cyprinus carpio* (AHB33743.1) and 97% with that of *Hypophthalmichthys molitrix* (ADF97607.1). In addition, putative conserved domains of ribosomal 60S superfamily and ribosomal L10 P0 superfamily were detected in the sequence. The domain architecture analysis predicted the presence of ribosomal L10 domain from amino acid position 5 to 106 and ribosomal 60S domain from position 231 to 315 in rohu.

The P0 sequence of *A. siamensis* had an ORF of 945 bp and a 315 amino acid sequence of P0 polypeptide was deduced. It had molecular mass of 34,548.82 Da and theoretical isoelectric point of 6.47. The sequence analysis showed 79% identity to 60S ribosomal acidic protein P0 of *Ixodes scapularis* (AAY66850.1) with the presence of a putative conserved domain of ribosomal L10 P0 superfamily. In *A. siamensis*, ribosomal L10 domain was predicted at amino acid position 5 to 106 and ribosomal 60S domain from position 231 to 314.

The sequences of P0 from both ectoparasite and host were compared to find out a region of least similarity, which could be targeted to be used as antigen for vaccine development (Fig. 1). Other protein analyses such as hydrophobicity, prediction of the accessibility degree of amino acid residues in the protein and prediction of potential B epitopes inside the protein were carried out using bioinformatic programs (Figs. 2 & 3). The region of the parasite P0 protein that has less sequence similarity with host P0 protein was found between amino acids 285 and 310 (Fig. 1). We also found that this region of the P0 protein coincides with an area of low hydrophobicity, and thus has a high probability of being exposed on the surface of the protein (Figs. 2 & 3). The synthetic peptide had a molecular mass of 2360 Da, as verified by mass spectrometry and purity of 95.5%, as checked by HPLC. The final concentration of the conjugated pP0-KLH was found to be 12 mg/mL.

![Fig. 1](image-url). The amino acid sequence alignment of the P0 protein of *A. siamensis* and *L. rohita*. The identical amino acids in the host sequence are represented by (□).
3.2 Immunization of rohu with pP0-KLH and challenge with A. siamensis

The immunogenicity of the peptide was evaluated by dot blot assay, to check if antibodies were produced against the injected pP0-KLH conjugate in rohu (Fig. 4). Positive reactions were noticed on the nitrocellulose membrane with serum of immunized rohu at antigen spots of pP0-KLH, P0 and KLH. No significant staining was observed in the negative control serum of rohu (results not shown). The staining appeared to be relatively weaker against the un-conjugated peptide P0 in comparison to pP0-KLH and KLH.

The utility of pP0-KLH as a vaccine antigen to control A. siamensis infection was evaluated by challenge of the immunized rohu with A. siamensis metanauplii. Both immunized and control rohu were infected with metanaupliar larvae and the development of disease was observed closely in both the groups. There was no significant difference between the parasite load in both the groups and the mean number of parasites remained comparable in both the groups. However, a mortality of 75% was observed in the control group. These mortalities were recorded within 7–15 days post-infection. A mortality of 59% was reported in the experimental group and these mortalities were recorded only after 15 days post-infection. No re-infection was allowed in both the groups and the infection cleared within 40–45 days in both the groups on the survivors.

4 Discussion

Targeted immunological management of the host is the way forward for the control of diseases in the aquaculture sector because vaccines and immunostimulants are cost-effective and environmentally-friendly alternatives to chemical compounds used as drugs and therapeutics.
Fig. 3. Antibody epitope prediction results of P0 protein sequence of *A. siamensis* (http://tools.immuneepitope.org/tools/bcell/).

Fig. 4. Dot blot immunoassay for identification of antibody production against pP0-KLH on nitrocellulose membrane.
Anti-argulus vaccine development has not yet received due attention from researchers, owing to our limited knowledge about its physiological and biological pathways at the molecular level. However, the transcriptomic analysis of *A. siamensis* (Bioproject ID: 167720; Accession: PRJNA167720) now provides the genomic information of the parasite for a better understanding of the proteins and metabolic pathways which are critical to the development and survival of the parasite [34]. Taking cue from experiences with vaccine development in sea lice and ticks, this study was designed to evaluate the ribosomal protein P0 as candidate vaccine antigen against *A. siamensis*.

The immunized rohu responded to the pP0 antigen by mounting antibody production, as noticed in dot blot. Specific antibodies were produced against the injected pP0-KLH conjugate. The antibody response also proved that in spite of the high level of amino acid similarity between the P0 of the host *L. rohita* and the parasite *A. siamensis*; the peptide used for immunization escapes induction of tolerance, which generally results in cases of proteins with high sequence similarity.

Generally, for a strong immunogenic response, it is essential to tag the peptide with a carrier protein. Although the peptide may be recognized by the B cells, the B cell must still receive help from a helper T cell recognizing a sequence within the same immunogen. Carrier proteins to which short peptides are covalently coupled generally provide a recognition site to the T cell for induction of the B cell response [42]. KLH has many foreign epitopes, a large molecular mass, and poor solubility, and as a result has been considered to be an effective carrier protein for conjugation with peptides by many previous researchers [43,44,45]. In the present study, we used KLH as the carrier protein based on the earlier studies. However, the carrier protein and adjuvants used in the study can be optimized for better immunogenic and protective responses.

Peptides are inherently less immunogenic because the peptide sequences used to present epitopes are short and thus contain insufficient information to fold into the correct shape necessary to mimic conformation-dependent epitopes [32]. Therefore, booster immunizations play an important role in development of a strong immunogenic response. In the present study, we evaluated the protective response in rohu after two boosters. Though an antibody response is detected following three immunizations, the weak protection provided by the peptide against the parasite challenge could be improved by modulating the frequency of immunizations or any suitable modifications in the sequence or adjuvant. Rodriguez-Mallon et al. [32] demonstrated a protective response in rabbits against the dog tick *Rhipicephalus sanguineus* after four immunizations with the P0 peptide derived from the tick. One of the interesting findings noticed here is delay in mortality in vaccinated fish with the use of this peptide as compared to control.

P0 protein is a structural component of ribosomes and is essential for cell viability in all organisms [11,15]. When transported to the nucleus it shows apurinic/apyrimidinic endonuclease (APE) activities that are involved in DNA repair and apoptosis [17,18]. Researchers have shown that gene silencing of the P0 gene led to failure of blood sucking and the resultant death of dog ticks, and hypothesized that this was due to a nonfunctional ribosomal large subunit in cell growth or an induction of apoptosis [31]. Such studies need to be designed to evaluate the effect of non-functional P0 in *A. siamensis*, if it is to be developed into a vaccine. Therefore, further studies are needed to elucidate the effects of host antibodies on the *A. siamensis* P0 protein.

In conclusion, this is a preliminary study done to evaluate if the P0 peptide can be developed into a vaccine candidate for control of *A. siamensis* infection. The results presented here suggest that the repetition of the experiments with optimization of the formulation and immunization schedule is needed to evaluate the peptide as a candidate antigen. It is also essential to understand the biological role of P0 in the parasite to evaluate its importance in the physiology and survival of the parasite. Nevertheless, the study opened up scope for vaccine development against argulosis in carp.

**Acknowledgements:** This work was supported by the ICAR National Fellow Grant conferred to the corresponding author. The authors also wish to thank Dr P. Jayasankar, Director, ICAR-CIFA, Bhubaneswar for providing necessary facilities during this study.

**Conflict of Interest:** Authors state no conflict of interest

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