Vaccination approach to prevent *Argulus siamensis* infection-success, challenges and preparedness

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**Introduction**

The aquaculture industry constitutes a major source of food fish production, which largely contributes to the nutritional security, foreign exchange, and livelihood support in the form of employment to around 14 million people in India sub-continent. India is the second-largest fish producing country in the world with a total production of 13.7 million metric tonnes in 2018–19 out of which 65% comes from the inland sector. Looking from an economical point of view, fish and fish products have currently emerged as the predominant sector in the agricultural export of India, with 6953 million US$ (INR 47,620 crore) in value in 2018–19. However, to match the ever-growing demands of increasing populations, fish farmers and industries are employing poor aquaculture practices that compromise fish health by suppressing its immune response and making them vulnerable to diseases. This leads to repeated disease outbreaks and subsequent economic loss in Indian major carp industry, including both farmed and wild fish populations. Amongst many such infectious agents, parasites pose a severe threat to the Indian major carp species resulting in huge loss, particularly in Rohu (*Labeo rohita*), which is one of the widely cultured species [1].

The frontrunner of such problematic fish parasites is crustacean ectoparasite *Argulus* spp. (*fish lice*), which currently poses a big challenge to the Indian as well as global fish farming. This parasite has the potential to affect almost all scaly fishes. Argulosis outbreaks have been reported from all continents, except Antarctica. However, from India, as of today, there is a single report which estimated the loss due to *Argulus* infestation in aquaculture amounting to approximately 29,524/- INR/ha/year corresponding to ~US $615/ha/year. Thus, only argulosis amounts to INR 300 crore (~US$62.5 million) loss to the Indian carp farming sector. Further, at any particular point of time, around 48% of aquaculture ponds are found to be infected with this parasite [2]. Though argulosis infection is widely prevalent, studies reporting loss due to this ectoparasite are relatively less compared to other similar ectoparasites affecting marine scaly fishes. For instance, damage due to sea lice infestation has accounted for US $436 million in losses to the Norwegian salmon industry as estimated in 2011 [3]. Moreover, the percent of loss in total biomass growth per production cycle due to average infestations varied from 3.62 to 16.55% [3]. Similarly, *Lepophtheirus salmonis* (sea lice), is an economically important fish ectoparasite in other European countries and America, and its infestations in salmonid culture are worrisome in nature that demand effective management strategies [4].

**Biology of ectoparasite *Argulus***

This devastating ectoparasite contains about 129 valid species and occurs in marine, estuarine and freshwater habitats [5]. They get attached to their host’s integument by maxillary suckers and hooks, and feed on its blood and external tissues. Most of the damage is caused by feeding activities with their modified mouth parts like pre-oral stylet and labial spines which are capable of secreting toxins or digestive enzymes, thus contributing to fish loss by its direct effects like dermal ulceration, physiological stress, immune suppression and secondary infections, and also by its indirect effects like reduced fish growth and mortality [6]. A mature gravid female *Argulus* after taking a meal, leaves the host and lays eggs in typical rows on any hard substratum. As many as 1200 eggs can be laid at any one time and cemented to the substrate.
The hatching of the eggs is majorly temperature dependent. The naupliar larvae released from eggs are immediately parasitic which frequently undergo moulting and mature on host body surface till the mature male and female mate for reproduction.

**Current scenario on management of argulosis**

*Argulus* seems to be widely-studied freshwater ectoparasites due to its ubiquitous presence in trout, carp culture, ornamental fish industry, and the associated economic loss. Systematic efforts for its control are in progress owing to its presence in diverse aquatic culture systems, however, the war against argulosis is still continuing. The current strategies of prevention or control of fish lice infections largely depend on veterinary medicines including organophosphates, benzoyl phenyl ureas, pyrethrins compounds, and avermectins [7-9]. Such anti-parasitic chemicals are either supplemented in fish-feeds or as oral medications or applied as topical bath treatments at regular intervals. Alternatively, a variety of non-medicinal control strategies are practiced to control parasitic infestations in aquaculture; which are currently at different stages of commercial implementation. Such alternative approaches include the use of bamboo mats to remove egg strings of the parasite [10], cow urine with diverse antimicrobial property [11,12], and herbal anti-parasitic agents [11]. It has been noticed that the ectoparasite develop resistance to most of the drugs used, thereby requiring frequent application of medications during a single culture operation [13], thus creating a potential for resistance development [14]. For example, frequent treatment with emamectin benzoate (SILC™) against control of *A. coregoni* leading to tolerance development [14], insecticide lindane resistance by this species of parasite [13] and our own observations from field level applications of wide array of drugs by carp farmers with poor effectiveness prove the tolerance or resistance development towards available parasiticides by this group of parasites [15]. Similar resistance pattern was also noticed in sea louse to azamethiphos and teflubenzuron [16], and in terrestrial animals to wide array of drugs [17]. On the other hand, certain advanced approaches aiming at lowering the susceptibility of host to sea louse infection through the administration of immunostimulants, vaccination, or selective breeding are also being investigated [18].

**Roadblocks in antiparasitic treatment and control of Argulida: challenges faced**

It is pertinent to mention here that there is currently no FDA-approved drug available for the treatment and control of *Argulus*. The past and present antiparasitic treatment options have been found to be unsuccessful in controlling mortality caused by this ectoparasite. In addition, drug resistance to many medicines is rising due to improper drug usage; besides, poor aquaculture practices and bad hygiene become the limiting factors for the treatment of parasitic diseases in fish. Moreover, these chemicals and their applications have significantly been found to be associated with health hazards to other aquatic animals and the surrounding environment, disrupting the ecological balance [19]. Although developing a resistant variety of fish would render protection over generations, the success achieved in the sea louse selection program in salmon breeding is not so encouraging. Again, running a selection program for *Argulus* will be a big challenge considering the multiple carp species being cultured in same pond and the difficulty in developing a challenge model as the parasite is too small and parasitic load on a single fish would be uncountable. Concurrent to these limitations, antiparasitic treatment for effective control of *Argulus* has also received negligible attention, perhaps due to lack of knowledge about the immune response of the host to the parasite. During its early larval stage, *Argulus* appears invisible to the naked eye, hence has the potential to cause severe damage to fish before the fish farmer could identify the parasite and recognize the problem.

Among the various options available for control of parasites discussed in general, vaccination seems to be more appropriate, if available, as it would provide long term, eco-friendly protection in a cost-effective manner, even for a low-valued fish species [19]. For this, reverse vaccinology approach can be adapted where one can identify a candidate antigen or concealed antigen of the parasite that would render long term protection, and utilize this antigen to generate specific immune responses in the host species. For example, peritrophins have been identified as potential vaccine candidate in sea louse based on the reverse vaccinology approach, i.e. looking from transcriptome data to screen the gene of interest, validating the expression of the gene isoforms at developmental stages of the parasite and finally in silico epitope analysis to production of recombinant or subunit or peptide-based vaccine [20]. For looking for candidate antigens involved either in parasite development or its multiplication remains a big obstacle considering the lack of whole-genome sequence information of this parasite. In many cases, adjuvants are added to the candidate antigens to facilitate protection towards injected vaccines. While vaccinating fishes of low weight or smaller in size, booster immunization is applied to enhance the immune response. In addition, *Argulus* being an ectoparasite are phylogenetically diverged species even amongst species level [11] and studies related to understanding the host immune response towards the parasite are presently lacking or at its infancy [21]. To add on to the existing problems, issues are also faced in getting enough parasites at a similar stage of growth by culture for developing a challenge model. Moreover, these parasites suck very little amount of blood from the host, and the vaccine candidate should be strong enough to protect, even with little blood meal by the parasite. Again unlike mammalian secretory antibody lgA, the counterpart secretory antibody molecules like IgZ or IgD in fish mucus or skin needs further in-depth investigation [22,23] and currently, there is no detection system available to measure the antibody levels in the skin or mucus using antibody-based assay system.

Though oral vaccination would be relatively easy to administer by mixing in the feed as well as cost-effective from handler’s and fish health perspective; but mass oral vaccination of fish is not routinely practiced. The problems with the development of oral vaccines are diverse including the need of using higher doses to be effective, the necessity to protect the antigen against intestinal degradation, and the challenges in finding optimal conditions to overcome oral tolerance. Nonetheless, an oral booster vaccination of rainbow trout against *Yersinia ruckeri* that cause Enteric Red Mouth disease was found to be effective and largely dependent on the immune response [24]. Hence, the real challenge of vaccine development against ectoparasites arises from our need to understand the complex host-ectoparasite interactions and identification of such molecular pathways involved. Last but not the least, the effective immune memory and persistence of protective antibody response in fish are also equally crucial, and need to be well-understood before developing a commercial vaccine in carp model.

**Vaccination success achieved in tackling ectoparasites**

Exposure with potential vaccines against ectoparasites would induce effective, long-time prevention against the disease. Over the years, humongous efforts have been made globally to develop vaccines against parasitic infections. To date, there are no commercially available vaccines for parasitic diseases in humans, possibly due to the challenges faced in vaccine development in pharmaceutical sectors. Instead, anti-parasitic drugs are more widely adopted, even though they possess detrimental environmental side-effects and drug resistance [25]. The first-ever vaccine against an ectoparasite was developed in the early 1990s, a recombinant vaccine against tropical cattle tick (*Boophilus microplus*), which was successfully applied for veterinary applications. The tick vaccine composed of recombinant BM86/BM95 antigens, which were a membrane-bound glycoprotein found in the gut digest cells of tick, was greatly appreciated for its cost-effective method and eco-friendly nature of vaccine development [26]. On this rationale, several other attempts were also made by taking antigenic targets of parasites...
based on their important biological functions - in generating protective immune responses. These include, but not limited to, gut membrane-associated protein, recombinant vitelligenin-like, house secretory/excretory, adhesion/cuticle binding, and akirin proteins [6]. However, these attempts have resulted in moderate effects and showed limited commercial applications owing to multiple factors; such as concealed antigens or challenges faced in the optimization of the expression of recombinant proteins. The first recombinant anti-sea lice vaccine prototype was developed against L. salmonis trypsin which provided only 20% protection in Atlantic salmon smolts whereas another recombinant vaccine was developed targeting vitelligenin of the parasite that reduced lesion development by the parasite and adult female numbers but did not appear to affect male lice [27]. A recent report showed promising effects of fish vaccination with recombinant proteins against adult female (P33) or chalimus II (P36) sea lice infestations [28]. In a similar manner, we had performed an initial trial of ribosomal P0 peptide as a potential vaccine candidate for control of fish ectoparasite infections, i.e., A. siamensis in L. rohita and noted delayed mortality with relative percent survival of 16% following experimental challenge [29]. The use of RNA interference (RNAi) presents yet another valuable tool for screening of potential targets and development of vaccines against arthropod ectoparasites [30]. These techniques were exploited to identify and confirm the presence of a novel akirin 2-like protein in L. salmonis and C. rogerescueyi, and the subsequent recombinant my32 protein was tested as a vaccine candidate in Atlantic salmon [31]. RNAi would also be a possible in vitro assay system for quickly narrowing down number of candidate genes to reach at the target gene.

Preparedness and progress made so far to tackle Argulids in fish

The ICAR-Central Institute of Freshwater Aquaculture (ICAR-CIFA), has been a frontrunner in the context of disease diagnostics, management and awareness of A. siamensis infection in freshwater fish. As on date 14 species of Argulus are being reported from India causing argulosis in freshwater and farmed fish populations. Sahoo & his co-workers in 2013 have first conducted a nationwide survey on the diversity and distribution of Argulus in India and reported that A. siamensis is the predominant species in Indian freshwater aquaculture [1]. Moreover, the same group in 2013 for the first time performed whole transcriptome analysis of A. siamensis by high throughput Illumina Sequencing that generated putative 46,352 contigs, additionally 19,290 CDS and 59,019 ORFs were identified from the assembly in that study. Further, the functional characterization of annotated CDS and transcripts by KEGG pathway analysis validated a group of genes involved in important pathways that could be tested as novel candidates for vaccine development against this freshwater fish louse [32]. In addition, we have processed both male and female parasites separately to generate reference transcriptome sequences by using single molecular real-time (SMRT) sequencing technique in the PacBio Sequel system. Total full length nonchimeric clustered consensus isoforms for female Argulus parasite was found to be 84,337 whereas for males it was 2,09,271. Total transcripts were mapped in the GO database depicting their functions in biological processes, molecular functions, and cellular components for both sexes. Such critical sequence information of A. siamensis male and female would be instrumental for development of efficient vaccine candidates to the parasites.

To facilitate a better understanding of the molecular basis of host-pathogen interactions, Kar et al. [33] for the first time studied the specific immune response of L. rohita infected with A. siamensis by measuring the transcriptional changes in various isotypes of immunoglobulins (e.g. IgM, IgG, and IgZ) in rohu carp tissue samples. Over the years, ICAR-CIFA has developed a challenge facility for year-round availability of Argulus parasite and standardized challenge protocol. In another attempt, a western blot method has also been developed and used to identify immunogenic proteins of Argulus that could be targeted as potential vaccine candidates [34]. The ICAR-CIFA has interdisciplinary research collaborations, proper amenities for wet lab maintenance, skilled manpower, basic instruments, and molecular biology laboratory facility with widely experienced researchers to address the challenges faced in fish louse Argulus management. Currently, the group is actively involved in screening as well as delivery of vaccine targets by a combination of genomics and immunoproteomics [35] approach through exploration of mucosal immunity in fish.

Declaration of Competing Interest
The authors declare that there is no conflict of interests involved in this manuscript.

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