RNAi Technology in Fish and Shellfish- Status and Prospects: A Review

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ABSTRACT

Ribonucleic acid interference (RNAi), a valuable tool for manipulating gene functionality in the laboratory, has also emerged as a powerful tool to suppress infection or replication of many pathogens that cause severe economic losses in fish farming. By taking advantage of the cell's endogenous RNAi apparatus, small interfering RNA of ~21-22 bp can be introduced into cells to induce target specific mRNA degradation. With the growing appreciation for the potential of RNAi technology, the diversity in vivo relevance to aquaculture is seemingly vast. Studies in the future should address the hurdles like delivery strategy stability and degradation of RNAi therapeutic molecule by nucleases in aquatic animals. In this article, we review the literature in the field of RNAi technology in aquaculture, summarize the status and prospects, which may open doors to its applicability potential as a therapeutic strategy to modulate host-pathogen interactions and inspire further trials.

Key words: Aquaculture, RNA interference, Therapeutic, Viral disease.

Ribonucleic acid interference (RNAi) is an ancient, naturally occurring intracellular sequence-specific post-transcriptional gene silencing mechanism. RNAi is widespread in eukaryotic cells from plants (Napoli et al., 1990) and invertebrates (Fire et al., 1998) to mammals, including human beings (Elbashir et al., 2001; Caplen and Mousses, 2003). It is essentially an endogenous cellular mechanism triggered by double-stranded RNA (dsRNA), leading to the degradation of homologous RNAs. The breakthrough necessarily happened, when Fire et al. (1998) described an exogenous application of double-stranded RNA (dsRNA) in the nematode worm *Caenorhabditis elegans* induced a more potent sequence-specific silencing response than the commonly used single-stranded antisense RNA alone.

An essential aspect of RNAi involves endogenous microRNAs (miRNAs). miRNAs are approximately 22 nucleotide RNAs that primarily negatively regulate post-transcriptional gene expression (Bagga et al., 2005). RNAi has undergone an information explosion in the last two decades and it is now established that as much as 5% of the human genome is dedicated to encoding and producing the >1,000 microRNAs (miRNAs) that regulate at least 30% of our genes (Jinek and Doudna, 2009). It is now understood that RNAi is charged with controlling vital processes of a biological organism, such as cell growth, tissue differentiation, heterochromatin formation and cell proliferation.

RNAi can, successfully, result in the knockdown of single or multiple genes, providing a quick and convenient method of analyzing gene function (Dykxhoorn et al., 2003). More specifically, small interfering RNAs (siRNA) of ~20-22 bp dsRNA molecules having a characteristic 2nt 3’ overhangs allow recognition and subsequent binding of RNAi machinery, ultimately leading to a homology-dependent degradation of the target indigenous mRNA. These siRNAs can be synthesized in large quantities with the current nucleotide synthesis technology provided the availability of cDNA sequence of a gene and the commercially available chemical reagents with which to perform the synthesis.

Principle of RNAi and its utility

Several strategies can be employed to produce synthetic siRNA, shRNA (small hairpin RNAs), small hairpin microRNAs (shmiRNAs) and long dsRNAs against the target gene that enter the RNAi pathway.

Synthetic siRNAs are small RNA duplexes composed of ~20-22 complementary base pairs (bps) with 2nt 3’ overhangs. Upon their entry in the cell, one strand of the duplex gets incorporated into the multi-subunit ribonucleoprotein complex (RISC) and leads to transient mRNA degradation. shRNA and shmiRNA-synthesizing vectors (containing sense as well as antisense strand) allow for the controlled or continuous expression of small transcripts in the cell. They are generally maintained as extra-chromosomal copies or stably integrated into the
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The RNAi pathway (Source: Milhavet et al., 2003).
In aquaculture species, especially shrimp, conventional vaccines based on adaptive immunity cannot be applied as they lack a specific immune response system. Shrimp, like other crustaceans, do not have adaptive immunity and rely on the innate immune response. Attempts have been made to develop prophylactic treatments, especially for commercially important shrimp species, based on RNA interference (RNAi). Fish Genetics and Biotechnology Division at ICAR-CIFE, Mumbai (INDIA) have developed an efficient DNA vaccine (based on RNAi) against the White spot syndrome virus (WSSV). Lab-scale challenge studies provided approximately 70% protection when administered by dip treatment to post-larvae of Peneaus monodon shrimp. The vaccine is currently in the pre-clinical development stage (own unpublished data).

**RNAi in fish and shellfish**

Diseases in aquaculture are one of the significant constraints in the way of aquaculture development and management globally. The culture of aquatic animals is an established industry now and is the source of substantial income to several countries worldwide. The rapid pace with which aquaculture is growing is poised to help in bridging the gap between global demand and supply of aquatic animal food products, management of health and reducing losses due to disease in aquaculture.

RNAi is a natural mechanism for post-transcriptional gene silencing. It is not only used as a potential tool in investigating the functionality of genes of interest but also to repress diseases that cause severe ecological and economic losses. RNAi is one of the possible alternatives to avoid the development of antibiotic resistance and reduce toxicity associated with drug application in aquaculture. Fishes were the first vertebrate species in which knockdown of gene expression using RNAi using long dsRNA with one strand designed complementary to the target gene (Wargelius et al., 1999). However, a tiny fraction of the embryos developed gene-specific defects; a large proportion of embryos developed with non-specific abnormalities. The first study using siRNAs in fishes was conducted in rainbow trout Oncorhynchus mykiss embryos carrying a vector expressing the green fluorescent protein (GFP). Boonanuntanasarn et al. (2003) were able to reduce the number of strongly fluorescent embryos by 60% compared to the negative control embryos, thus providing the first evidence of an effective siRNA-mediated gene silencing in fish.

**RNAi as an anti-bacterial tool**

Aquaculture, specifically the shrimp industry, is often faced with outbreaks of disease caused by bacteria and viruses. The discovery of RNAi has enabled studies on immune mechanisms and the function of genes involved in the process of fighting bacteria in shrimp species: prophenoloxidase (Fagutao et al., 2009; Liu et al., 2014), p38 mitogen-activated protein kinases (Yan et al., 2013), MjGal (galectin of Marsupenaeus japonicas) (Shi et al., 2014), L. vannamei Crustin (Shockey et al., 2009), M. japonicas Crustin (Hipolito et al., 2014), L321_RS19110 virulent gene of Pseudomonas plecglossicida (Zhang et al., 2018) and Macrobrachium nipponense Transmembrane C-type lectins (Huang et al., 2019). These studies using RNAi acted as a powerful tool in identifying an array of cellular processes that participate in bacterial infection as well as anti-bacterial defense mechanisms in aquaculture organisms.

**RNAi as an anti-parasitic tool**

An important arm of RNAi application, apart from being a gene validation tool, is its considerable potential for the development of pest control technologies. Being very sequence-specific in action, it may be possible to develop species-specific dsRNA pesticides. RNAi has been used to suppress gene expression in the intra-mammalian life stages (adults and schistosomula) of Schistosoma mansoni, which affects more than 200 million people worldwide and is responsible for 300,000 deaths annually (Da’dara and Skelly, 2015). The first evidence of gene silencing mediated by dsRNA in a fish parasite was reported by Ohashi et al. (2007). Since then, the RNAi technology has been mostly applied to study gene function of various pests and parasites responsible for disease outbreaks in aquaculture. Despite being considered a suitable and capable tool for functional genomics, very little research has been done on its use in the manipulation of the gene function of fish parasites.

Saleh et al. (2016) investigated the efficiency of using siRNAs to knockdown the expression of ATP/ADP antipporter-1 and methionine aminopeptidase-II genes of Heterosporis saurida (a microsporidian parasite of lizardfish, Saurida undosquamous) using previously developed in vitro cultivation model. The silencing of ATP/ADP antipporter-1 and methionine aminopeptidase-II genes reduced H. saurida infection levels in EK-1 cells, 4%0 and 60%, respectively. qRT-PCR and spore counts suggested that RNAi could be applied to explore gene function beyond microsporidia lines for various parasite and host combinations. In one study, siRNA-induced RNAi was used to silence the MyxSP-1 gene in the invertebrate host (Tubifex tubifex) to annul the Myxobolus cerebralis life cycle and prohibit whirling disease infection in the salmonid host (Sarker et al., 2017). This study revealed no disease development in salmonids and observation of a long-term RNAi in T. tubifex suggested a therapeutic proof of RNAi in vivo against salmonid whirling disease. RNAi appears promising in enlightening better about the gene functions in parasites for targeted drug delivery, silencing gene expression and reducing the role of vectors to transmit disease by parasites.

**RNAi as an anti-viral tool**

Among the multiple problems and diseases in aquaculture, viruses are recognized as the most numerous organisms in the aquatic (mainly marine) environment. Besides the natural fish stocks, these viruses are devastating and cause debilitating losses in aquaculture where fish are confined
and intensively reared (Leong and Fryer, 1993). The crucial viral diseases in aquaculture globally are caused by different genera, mostly within families like Birnaviridae, Iridoviridae, and Ixoderae (Mahy and Regenmortel, 2008). Among the many potential applications of RNAi, its best attraction is due to its use as a powerful molecular tool to screen host genes involved in pathogenicity and other critical biological processes, as well as to validate potential drug targets (Hong-Geller and Micheva-Viteva, 2010). Also, the silencing of viral genes stands out as a promising therapeutic approach for the development of antiviral strategies. RNAi can help to inhibit virus replication inside the host cell by regulating viral replication genes, reducing virus spread by inhibiting the formation of new viral particles and, consequently, maintaining the control of a possible outbreak of the disease (Wise et al., 2008). Many successful studies have demonstrated the potential value of RNAi technology to interrupt viral contagion in fish involving varied virus families. For example, red-spotted grouper nervous necrosis virus (RGNNV) of the genus Betanodavirus (Wu et al., 2008, Su et al., 2009a), Rhabdovirus family member viral hemorrhagic septicemia virus (Schyth et al., 2007; Bohle et al., 2011), Red sea bream iridovirus (Dang et al., 2008), Grass carp reovirus (Su et al., 2009b, Zenke et al., 2010).

The first description of RNAi technology as a potential approach to the therapy of aquaculture viral diseases was reported by Xie et al. (2005). The study provided evidence of siRNA mediated inhibition of virally encoded genes in cell lines. Using in vitro transcribed siRNA to block the expression of the major capsid protein (MCP) encoded by the tiger frog virus (TFV), the results of the study suggested that siRNA effectively inhibited TFV replication in fathead minnow (FHM) cell lines. RNAi, mediated by siRNA, has been demonstrated to have activity against an extensive range of viruses and is now considered a potential antiviral tool, especially in organisms that have evolved the RNAi mediated viral immunity (Stram and Kuzntzova, 2006).

Although, it is unclear whether RNA silencing naturally restricts viral infection among vertebrates, there are signs that this is undoubtedly the case. At present, it is well known that while the vertebrates, as their primary innate immune response to virus infection, rely on the Interferon pathway. In contrast, the invertebrates mostly lack such a system and depend on the anti-viral RNAi defense system (Sidahmed and Wilkie, 2010). Indirect evidence for the persistence of RNAi-mediated anti-viral immunity in fish has been reported. The demonstration of the binding and protection of dsRNA from Dicer cleavage by a small fish Betanodavirus protein named B2 and subsequent suppression of the RNAi silencing pathway provided a shred of reliable evidence that fish Betanodavirus have evolved a strategy to sustain replication by blocking the RNAi pathway (Fenner et al., 2006; Fenner et al., 2007). This suggests that such mechanisms also act as an anti-viral immune system in fish. Later, a study by Su et al. (2009b) revealed significant up-regulation of Dicer in the liver during the first 24h post Grass carp reovirus (GCRV) injection in Gobiocypris rarus, suggesting that viral-dsRNA can activate the RNAi pathway in fish. They also hypothesized the inhibition of the anti-viral function of RNAi mechanism by virus inclusion bodies, throwing more light on the anti-viral mechanism of RNAi pathway in fish.

During the initial phase, the crucial hurdles towards the advancement of RNAi were the problem with obtaining successful delivery and both siRNA and transfection agent-related Mx-interferon pathway induction. Ruiz et al. (2009) constitutively expressed siRNA designed against VHSV-L (Viral hemorrhagic septicemia virus) polymerase gene to increase the number of anti-viral siRNAs per cell in epitheloma papulosum cyprinid cell line. The resulted in a sequence-specific interference upon the transformation of clones with a mixture of three shDNAs (corresponding to three selected siRNAs), rather than with individual shDNAs. Additionally, the interference was not specific for VHSV, as infection with a heterologous fish rhabdovirus virus Infectious hematopoietic necrosis virus (IHNV) was also reduced akin and this was not due to Mx response of the cells. A possible interaction of the shDNAs with the viral double-stranded replicative intermediate might explicate the lack of specificity reported in the study (Lima et al., 2013).

Shrimp aquaculture is plagued with viral disease outbreaks and that remains a significant concern in the development of the shrimp aquaculture industry. Prevention and control of these outbreaks in shrimp primarily rely on the strengthening the innate immune response. Innate anti-viral immunity in shrimps was depicted by in vivo RNAi knockdown experiments for the first time in shrimps by employing a non-specific and sequence-independent dsRNA to inhibit TSV or WSSV infections in L. vannamei (Robalino, 2004). Westenberg et al. (2005) suggest the poor uptake of dsRNA/siRNA by the cells as a possible explanation for the sequence-independent reaction. Circulation of this RNA in the hemolymph may induce a general defense response by signaling through a cellular receptor.

Though the molecular mechanisms involved in anti-viral defense remain unknown for most crustacean species, understanding these mechanisms in combination with various effective strategies can contribute to improving disease management in shrimp aquaculture and fisheries. For that reason, a decent understanding of viral entry and propagation in shrimp, as well as the host-pathogen interaction at the cellular and molecular levels is crucial.

RNAi also has been widely used as a powerful tool in identifying the genes involved viral infection (Li et al., 2007), host immune responsive genes (Wang et al., 2013), ovarian maturation (Xie et al., 2017) in shrimps. RNAi has been used to determine the function of various genes from shrimp that are involved in virus propagation and infection by knocking down virus-specific genes or downregulating host genes that are related to viral replication mechanisms. Numerous studies with RNAi have been done to combat viral diseases
in shrimp by silencing specific genes of interest like Toll-like receptor (Yang et al., 2007, Labreuche et al., 2009), rab7-like proteins (Wu and Zhang, 2007), a caspase-3 protein (Rijjaravanchich et al., 2008) and the proPO system (Charoenasapsri et al., 2009). Sequence-specific dsRNA has been used to inhibit virus replication in shrimp against Taura syndrome virus (TSV), infectious hypodermal and hematopoietic necrosis virus (IHHNV), yellow head virus (YHV) and WSSV (Robalino et al., 2005; Attasart et al., 2010; Ho et al., 2011).

Several studies, with varied results, have been carried out to trigger an RNAi anti-viral response, using siRNA, against WSSV since this is one of the most dangerous infectious pathogens due to the current intensity of shrimp aquaculture practices. Different target genes were considered in these studies. Injection of 19bp siRNA against the vp19 gene did not protect L. vannamei against WSSV. Still, innate anti-viral immunity, sequence-dependent anti-viral protection and gene silencing could be induced by injection of long dsRNA molecules (Robalino et al., 2005). Another study used short siRNA (21bp) against WSSV vp28 or vp15 genes and reported a significant reduction in shrimp mortality compared to controls (Westenberg et al., 2005). Administration of a sequence-specific vp28-siRNA into infected shrimp every day for three days induce gene silencing in vivo in Penaeus japonicus shrimp with no WSSV DNA found at the end of the experiment, suggesting that vp28-siRNA completely eradicated the virus from infected shrimp (Xu et al., 2007). Ufaz et al. (2017) evaluated the capacity of nanoparticulate RNA interference (RNAi) to down-regulate genes in Penaeus vannamei shrimp and protected shrimp against White Spot Syndrome Virus. Apart from showing that the length of the administered dsRNA correlates with gene knockdown, they also proved that RNAi targeting viral-protein 28 (vp28) protected the shrimp against WSSV infection with 95% more survival of animals treated with RNAi compared to no survival in the untreated controls. All these studies on the effect of dsRNA and siRNA on the response of shrimp infection suggest that the use of RNAi is not a straightforward approach in shrimp. Few reports suggest that only sequence-specific siRNAs can inhibit virus infection in shrimp (Wu et al., 2007) with evidence that siRNA molecules longer than 50bp might be more efficient to silence target mRNAs (Labreuche, 2010).

While most of these studies on the applicability of RNAi to inhibit disease occurrence or virus were mostly conducted on a small scale, the successful results clearly showed that RNAi has immense potential in improving the survival of shrimp in aquaculture. On similar lines, Thailand researchers succeeded in using RNAi by oral delivery (using Artemia as a dsRNA-delivery system) against WSSV infection in P. monodon leading to reduced percentages in cumulative mortality and delayed average time of death (Thammasorn et al., 2013). Researchers in India used a different delivery approach by conjugating a DNA expression system (expressing antisense RNA against a specific viral gene) with an appropriate delivery vehicle (a cationic polymer) for sustained intracellular release. The expression of the antisense RNA knocked down the target gene and offered >70% protection from the virus as compared to the control group in lab challenge studies (own unpublished data).

**Delivery strategies**

One of the most critical concerns in RNAi-based therapy is the mode of delivery of the therapeutic molecule into the research subject. Any attempt to understand a gene’s function or control disease in diverse aquatic organisms necessitates the optimization of efficient delivery protocols of RNA molecules into the cells or organisms. RNAi has a theoretically broad therapeutic potential against many diseases. However, one of the main obstacles to supplement RNAi’s success in translation from lab to field is the delivery of RNA molecules to the cytoplasm of specific cells of therapeutic interest. Numerous extra- and intra-cellular biological barriers to RNAi delivery exist in an organism necessitating the smart designing of delivery strategies. A variety of delivery strategies have been developed for the successful delivery of siRNA molecules, both in vivo and in vitro. Some of the delivery strategies include electroporation, microinjection, oral pathway, lipid nanoparticles, polymer-based systems and protein-based systems. Each delivery method has its benefits and drawbacks.

The first experiment attempting to introduce foreign DNA into P. monodon shrimps by electroporation delivery method was conducted in 1999 (Tseng et al., 2000). Afterward, electroporation was used several times for the introduction of foreign DNA into the L. schmitti, Artemia (Amicar et al., 2000; Chang et al., 2011) etc. Electroporation technique was also employed to deliver siRNA molecule into embryos of model shrimp (Artemia sinica) to knockdown the As-sumo-1 gene (Chu et al., 2014). These studies support the electroporation technique to deliver nucleic acid into embryos of fish and shellfish at earlier life stages. Additionally, electroporation can be performed with significantly large numbers of zygotes or embryos at the same time.

The microinjection method has also been used widely to introduce nucleic acids, including dsRNA, into fishes and shellfish, at different stages of development. Some of the studies where siRNA was delivered to aquatic animals with mixed success using microinjection include Daphnia magna (Kato et al., 2011), Macrobrachium rosenbergii (Sharabi et al., 2016), P. vannamei (Cha et al., 2015), P. monodon (Chimwai et al., 2016).

Several transfection reagents have been employed for transfecting siRNA into different cell lines. Lipofectamine 2000 and Oligofectamine (Invitrogen) are being routinely used for siRNA delivery. So far, there is no chemical transfection method for dsRNA delivery reported in shrimp. However, a DNA transformation experiment in L. vannamei was successfully performed with fertilized eggs at the one-cell stage using microinjection, electroporation and the jetPEI transfection reagent method providing evidence that jetPEI could potentially be used to introduce dsRNA into...
embryos of the shrimp species, at least (Lu and Sun, 2005; Sun et al., 2005).

Vector-based delivery methods include those derived from viruses and plasmids. Viral vectors are recognized as efficient delivery systems for RNAi technology, but due to the induction of toxic immune response and the risk of genome integrations, their use in aquaculture is limited (Kay et al., 2001; Thomas et al., 2003).

Oral delivery of RNAi therapeutic molecule, either naked or conjugated with a polymer or in the form of bacteria that contain the specific dsRNA/siRNA or in the form of fish/shellfish feed (Attasart et al., 2013; Treerattrakool et al., 2013; Thammasorn et al., 2013) has been successfully applied to many arthropod species (Whyard et al., 2009; Wuriyanghan et al., 2011). Ulfaz et al. (2017) reported the successful anti-viral activity of nanoparticulate RNAi for targeting the WSSV vp28 gene. The study demonstrated the gene knockdown in healthy and disease models and at the same time, achieved significant protection against a viral challenge.

Inference and prospects

The progress of RNAi’s technology from initial discovery to clinical applications in vivo has been astounding. RNAi has, lately, emerged as a robust tool to inhibit gene function and a promising approach to disease management in aquaculture. Existing hype and excitement surrounding RNAi are justified; nonetheless, multiple hurdles and concerns for the practical field application of RNAi in aquaculture are currently existing. An efficient and careful designing of RNA construct, dose optimization, delivery strategy, chemical modifications to increase stability, increasing cell uptake are some of the rational and practical issues to be addressed for RNAi application in fish and shellfish. The selection and use of an appropriate delivery strategy suitable for each aquaculture system are one of the main impediments to make RNAi a practical tool on the industrial scale. To further hasten the pace at which RNAi can be used in aquaculture, a more capable cellular delivery scheme and stabilization tailored to these animals is vital.

The aspects of RNAi presented in this review are encouraging for use in aquacultured animals and bring novel translational ideas and elucidations. Within a short time, RNAi has become a critical method to explore cellular processes involved in anti-bacterial, anti-parasitic and especially anti-viral defense mechanisms in non-model fish and shellfish of commercial value. An increase in the viral outbreaks in the global aquaculture (especially in shrimps) demands more effective therapeutic agents for disease management and control. RNAi-based therapies might prove to be effective against the same if the issue mentioned above are addressed rationally. Despite these hurdles, however, RNAi is the best prospect for developing new powerful therapeutic approaches against the viral pathogens of aquaculture. In the future, we should see continued advancements in the application of this remarkable technology and it can only be but anticipated that the future of RNAi technology in aquaculture is going to be a bright one.

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