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Application of RNA Interference (RNAi) against Viral Infections in Shrimp: A Review

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Abstract

Shrimp culture has long been done in Asia and America to provide high quality food to people. Modern aquaculture uses advanced techniques to increase shrimp production but it also has enhanced the occurrence of infectious diseases. Disease is the main pitfall for the development and sustainability of shrimp aquaculture worldwide. In the last decade several methods and strategies have been developed and evaluated under experimental conditions in order to curb the negative impact of viral infections. Among these, RNA interference is the most recent tool against viral diseases in shrimp and it is deemed as a promising biotechnology to boost shrimp production. This paper gives a broad overview of the RNAi methods used to fight viral diseases in shrimp aquaculture compared to the antiviral effect of methods previously evaluated against viruses. It also gives examples of the use of RNAi to learn more on mechanisms of the shrimp defense response. The application of RNAi to fight or treat viral infections in shrimp aquaculture has yet to come and it depends on the efficacy of RNAi against several viral diseases, evaluation of environment and food safety and the development of cheap, massive delivery methods of RNAi molecules to shrimp farming facilities.

Keywords: Shrimp Aquaculture; Penaeidae; Viral diseases; RNA interference; Preventative and therapeutic applications; WSSV.

Shrimp Aquaculture

Shrimp culture is an ancient practice done in some countries from Asia and America since the 15th century. In Asia, fish and shrimp larvae entered coastal lagoons with the high tide. These animals were enclosed through a traditional art called tambaks and were reared to maturity [1]. In pre-hispanic Mexico, shrimp postlarvae and fish larvae were enclosed during high tides in coastal lagoons using wooden barriers called "Tapos". Here, larvae were grown using the naturally available resources and were harvested when reached a suitable size [2].

Modern shrimp culture began in 1933 in Japan with the induced spawning and hatching of *Marsupenaeus japonicus* larvae. This method included the artificial rearing of larval stages up to postlarva using natural feed such as microalgae and *Artemia* nauplii [3]. This technology allowed the production of larvae in hatcheries instead of using larvae from the wild to stock grow-out ponds.

Shrimp aquaculture became a commercial activity since the 1950s in Asia [4] and the 1970s in America [5]. Semi-intensive shrimp farming developed thanks to a number of improved techniques for larval rearing and stocking ponds at higher densities, water fertilization and artificial diets [6,7]. As a consequence, shrimp aquaculture increased its production and economic importance. At present shrimp farming is done in several countries in Asia, America and Africa [5]. The importance of shrimp aquaculture lies in two main aspects: (a) it is a source of high quality protein for human consumption and (b) it provides employment and improves the economic status of people in low-income countries [8].

Despite the rapid growth of shrimp farming and its further expansion to other countries and regions, intensification of shrimp aquaculture has prompted stress factors that increased susceptibility to diseases [9,10]. Environmental stressors such as temperature or salinity fluctuations due to heavy rain or toxicity from industrial or agricultural pollutants have been related to development of disease and mortality [7,11]. Other factors involved in onset of disease include pond overcrowding, overfeeding, lack of nutritional requirements and poor water quality [12]. As a result, an increased appearance of infectious and/or opportunistic diseases caused by bacteria, viruses, fungi, parasitic protista and metazoa have been reported [7,13].

Infectious diseases

Infectious diseases currently represent the biggest threat to farmed shrimp production since they cause severe clinical signs and high mortalities. Viruses are the most damaging pathogens that affect farmed shrimp. Several viruses are considered a threat to the development of shrimp aquaculture because of their wide host range, pathogenicity and distribution [7,13-15]. Viruses that have caused severe epizootics and high mortalities in larvae, postlarvae and juvenile stages of shrimp include baculoviruses (monodon baculovirus [MBV], baculovirus penaei [BP], baculoviral midgut gland necrosis virus [BMNV]), parvo-like viruses (infectious hypodermal and hematopoietic necrosis virus [IHHNV], hepatopancreatic parvovirus [HPV]), a dicistrovirus (Taura syndrome virus [TSV]), a ronivirus (yellow-head virus [YHV]), and a nimavirus (white spot syndrome virus [WSSV]) [13,16]. Features of the main harmful viruses are described below (Table 1).

Infectious hypodermal and hematopoietic necrosis virus (IHHNV) - also known as *Penaeus stylirostris* densovirus (*Pst*DNV) [17-19] was first reported in batches of *Litopenaeus stylirostris* under super intensive culture [20] and also in batches of *L. vannamei* [21]. This pathogen rapidly spread to other countries in America (Mexico,

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Panama, Colombia, Ecuador and Argentina) [22-25], Asia (Indonesia, Malaysia, Philippines, Singapore and Thailand) [22,26] and French Polynesia [18]. Its genome is organized into three open reading frames (ORFs) encoding a non-structural protein, an unknown protein and a capsid protein, respectively [27]. IHHNV causes infection to several shrimp species [22,25,26] (Table 1). Clinical signs of IHHNV infection depend on the species age and size, being the early juvenile stages more susceptible to the disease. In *L. stylisrostris*, acute IHHNV infection displays reduced feeding and locomotion, behavioral changes during swimming and at the end animals sink into pond bottom and die [22]. In *L. vannamei*, acute IHHNV infection shows reduced growth rate and marked size differences within a pond population and causes deformity of the rostrum, antennae and/or cuticle which is known as the "runt deformity syndrome" (RDS) [28]. In *P. monodon* IHHNV infection apparently causes no clinical signs as no differences in size, weight or fertility was found between IHHNV-positive animals (determined by PCR and/or *in situ* hybridization) compared to healthy ones. No histological lesions (Cowdry-type A inclusion bodies) were observed in IHHNV-positive farmed animals [29,30]. This virus became the main pathogen both in shrimp fisheries and aquaculture in the 1980s in Mexico. It was estimated that its economical impact was between 0.5 and 1.0 billion US dollars [21]. This virus is still present in wild and cultured shrimp in Mexico and other countries.

Taura syndrome virus (TSV) - This virus was first reported in shrimp farms near Taura river, Ecuador in 1992 and from this location its name was given. The TSV genome consists of a single, positive-sense RNA strand of around 10 kilobases long [31]. It is composed of two ORFs. The first one is 6740 nucleotides long and encodes a putative non-structural polyprotein with several domains such as a helicase, a protease and a RNA-dependent RNA polymerase. The second ORF encodes three structural proteins VP2, VP1 and VP3 and spans 3036 bases from nucleotides 6947 to 9982. These ORFs are separated by a non-coding intergenic region of 210 bases [31]. Clinical signs were at first thought to be caused by chemicals used against banana pests in nearby plantations but in 1994 the viral etiology was confirmed [32]. Soon after its appearance, TSV spread to several countries in South, Central and North America as well as to Hawaii [33]. Since 1999 TSV was also detected in Asian countries such as Taiwan, Thailand and Korea which imported stocks of *L. vannamei* from South America [34-37].

Several shrimp species are susceptible to TSV infection [22,35,37,38] (Table 1). It appears that shrimp from the genus *Farfantepenaeus* (*F. aztecus* and *F duorarum*) are resistant to TSV infection since no clinical signs or histopathological lesions were detected upon experimental infection [39]. Size and age are factors for increased susceptibility to TSV infection. In specific pathogen-free *L. vannamei* larger animals are more susceptible to infection and mortality than early juveniles [6].

TSV infection has three clinical stages: acute, transition and chronic [40]. In the acute stage (3-5 d after the onset of infection) animals display soft exoskeleton, melanized multifocal necrosis and expanded chromatophores in uropods and pleopods. This stage is related to late premolt or early postmolt. Here, animals become weak, with empty digestive tract and often die during molting (cumulative mortality = 75-95%) [22,40]. Cellular lesions include pyknosis, karyorrhexis and necrosis in epithelia of cuticle, digestive tract, gills, antennal gland and haematopoietic tissues [41]. Animals in the transition stage (4-8 d after onset of infection) showed a reduction in severity and number of cellular lesions and melanization is observed. These features indicate the onset of the chronic phase [40]. Here, surviving shrimp (8 d after infection) showed wound repair leading to regeneration of epithelial tissues in affected organs. Mortality ceased and surviving shrimp molt shedding the necrotized cuticle [40,41]. The economic impact of TSV during 1992 to 1996 was estimated between 1.2 to 2 billion US dollars [40].

Yellow-head virus (YHV) - This pathogen has up to six different genotypes which produce the yellow head disease and include the gill-associated virus (GAV) from Australia [42-44]. Due to its morphology YHV was first thought to belong to the granulosis-like virus (Baculoviridae). Later, its genome was determined to be a RNA molecule [45,46]. The complete genome is a single linear (positive strand) RNA molecule of 26652 nucleotides [46,47]. The genome is organized into four distinct ORFs. ORF1a has a 3C-like protease motif, whereas ORF1b has a "SDD" polymerase metal ion binding domain helicase. ORF2 encodes putative nucleocapsid proteins (g7 and g2) and ORF3 encodes putative surface glycoproteins (p18/20, p33 and g2.1). ORF4 is very small and it is located at the end of the genome with no known product [46].

Yellow head virus first appeared in Thailand and later it spread to other countries in Asia (Taiwan, Indonesia, Malaysia, China,

Virus	Year first Recorded	Location	Shape	Size (nm)	Genome type	Genome size	Transmission type	Susceptible species
IHHNV	1981	Hawaii, USA	Icosaedral	20 – 22	ss DNA	4100 - 4700 nt	Horizontal / vertical	*Litopenaeus stylirostris, L. vannamei, L. occidentalis,* vertical *Farfantepenaeus californiensis, Fenneropenaeus chinensis, Penaeus monodon, P. semisulcatus, Marsupenaeus japonicus, Artemesia longinaris*
TSV	1992	Ecuador	Icosaedral	31 – 32	ss (+) RNA	10205 nt	Horizontal	*L. vannamei, L. stylirostris, L. setiferus, P. monodon, Metapenaeus ensis, F. chinensis, L. schmitti*
YHV	1990	Thailand	Bacilliform	150 – 200 x 40 – 50	ss (+) RNA	26652 nt	Horizontal / Vertical	*P. monodon, P. merguiensis, M. ensis, L. vannamei, L. stylirostris, L. setiferus, F. duorarum, F. aztecus, Palaemon styliferus, Palaemonetes pugio, Acetes sp.*
WSSV	1992	Taiwan	Bacilliform	210 – 380 x 70 – 67	ds DNA	292 - 307 kbp	Horizontal / Vertical	*Farfantepenaeus aztecus, F. duorarum, L. stylirostris, L. vannamei, L. setiferus, F. chinensis, F. indicus, F. Marsupenaeus japonicus, M. ensis, M. dobsonii, M. monoceros, P. monodon, P. penicillatus, P. semisulcatus, Parapenaeopsis stylifera, Solenocera indica, Trachypenaeus curvirostris*

Legends: nm - nanometers; nt - nucleotides; ss - single-stranded; ds - double-stranded; (+) positive strand; kbp - kilo base pairs

Table 1: Features of the four main viral pathogens in shrimp aquaculture.

Philippines, India), Australia and America (USA and México) [22,42,47]. Many shrimp species are susceptible to YHV both by natural infections and experimental challenges [22,47,48] (Table 1). Clinical signs include pale yellow body coloration, especially in hepatopancreas and gills in *P. monodon*. Other clinical signs include erratic swimming near pond shores and cumulative mortality up to 100% within 3 - 5 d after onset of clinical signs [44]. YHV causes systemic infection and replicates in tissues and organs of ectodermic and mesodermic origin such as gills, digestive tract, lymphoid organ, connective tissues of nerves, eyestalk, hepatopancreas and muscle [49]. Cellular lesions include pyknosis and kariorrhexis in epithelial cells in gills, connective tissues and hematopoietic tissues [48]. The estimated losses caused by YHV from 1990 to 2007 are 500 million US dollars [47].

White spot syndrome virus (WSSV) - This is an enveloped, non-occluded, bacilliform virus with a tail-like appendage at one end [50,51]. WSSV is one of the largest viruses infecting animals [52-54]. Its genome is also one of the largest recorded for viruses [55-57] (Table 1). It contains between 531 and 683 ORFs encoding peptides from 51 to 6077 aminoacids which represent 92% of the genetic information contained in the genome [55,56].

This pathogen was first recorded in Taiwan and soon after it spread to several countries in Asia and America. WSSV has a broad host range including several penaeid shrimp species, caridean shrimp, lobsters, crayfish, crabs and other decapod crustaceans [58].

Clinical signs include white spots in the inner surface of cuticle probably formed by accumulation of calcium carbonate due to dysfunction of epithelial cells [59,60]; reddish discoloration of the body, pleopods and uropods due to expansion of chromatophores [48,61]; reduced feeding, lethargy [52] and delayed clotting of hemolymph [62]. Cumulative mortality reaches 100% within 3-10 d after onset of clinical signs [63]. Histopathology shows hypertrophied nuclei of WSSV-infected tissues with intranuclear amphophilic inclusions and marginated chromatin [64]. Since it first appeared in 1992 the economic impact of WSSV on shrimp aquaculture is well over 8 billion US dollars [41] and currently remains the most damaging viral pathogen for the shrimp aquaculture industry worldwide.

Methods used to reduce impact of viral diseases

In the last 13 years several strategies have been developed and evaluated under experimental conditions to tackle the negative impact of viral diseases (particularly WSSV) in shrimp aquaculture. Evaluated products include:

Immunostimulants: These are products derived from bacteria (*Bacillus* sp. [65]), fungi (*Saccharomyces cerevisiae, Schizophyllum commune* see [65,66]), algae (*Sargassum polycystum* [67]) and herbs [68]. These organisms have cell walls containing substances such as peptidoglycans, β-glucans and/or lipopolysaccharides which activate both humoral (antibacterial activity, agglutinins, cytokine-like factors, modulators and clotting factors) and cellular (prophenoloxidase system, encapsulation, nodule formation and phagocytosis) defense responses in shrimp [65,69]. Immunostimulants are fed to experimental animals before and during WSSV challenge. Results showed that animals treated with these substances had reduced mortality compared to untreated controls [67,68,70,71]. Nonetheless, continuous use of immunostimulants may induce immunological fatigue to the shrimp [71,72] rendering this strategy ineffective and even damaging.

Natural or synthetic antiviral compounds: Plants and algae are known to have substances with antiviral properties and have been tested both *in vitro* and *in vivo* against human viral pathogens [73]. Some natural antivirals have been evaluated against WSSV in shrimp. Extracts with antiviral activity have been orally administered to shrimp before a WSSV challenge. Results of a diet supplemented with an extract of *Spirulina platensis* showed no antiviral effect but only a slight delay in mortality using a standardized oral inoculation procedure [74]. In contrast, an Indian plant extract from *Cynodon dactylon* supplemented to feed at 2% (w/w) showed 100% protection upon a *per os* WSSV infection [75]. The oral administration of a substance (bis[2-methylheptyl]phthalate) extracted from the Indian plant *Pongamia pinnata* before and during a WSSV challenge *per os* showed that treated animals had between 60 and 20% mortality depending on the concentration used (200-300 µg/g body weight, respectively) [76]. The only report on the use of a synthetic antiviral (cidofovir) against WSSV infection showed to be more effective than the *Spirulina*-supplemented diet to reduce and delay mortality of treated shrimp upon an intramuscular WSSV challenge. Nonetheless, cidofovir did not prevent WSSV infection [74].

Inactive viral particles, recombinant viral proteins and virus neutralization: These strategies have been widely evaluated against WSSV in shrimp. The rationale lies in the fact that some shrimp surviving a WSSV outbreak may become resistant to a subsequent WSSV infection. This phenomenon was described as a "quasi-immune response" [77]. Later, several studies evaluated the protective effect of inactive viral particles [78,79] or recombinant viral envelope proteins to prime the innate shrimp defense system [80-83]. In addition, monoclonal and polyclonal antibodies directed against WSSV envelope proteins have been used to inactivate WSSV particles through virus neutralization assays [84-86]. Neutralized virions were then used to inoculate animals in order to induce protection upon a subsequent WSSV challenge. A virus neutralization assay evaluated three tenfold dilutions (10^{-1}, 10^{-2} and 10^{-3}) of a WSSV stock. Each dilution was mixed with an equal volume of a purified monoclonal antibody against WSSV VP28 and incubated at 28˚C for 2 h. A positive (WSSV) and a negative (saline buffer) controls were included. The WSSV-antibody mix (100µl) was intramuscularly injected in the second pleonite of shrimp (n=20 per treatment). Control animals inoculated only with WSSV showed 100% mortality at 7 d post inoculation (dpi). Shrimp given virus concentrations 10^{-1} and 10^{-2} neutralized with WSSV-antibody had a slight delay in mortality reaching 100% mortality at 11 dpi. Animals given the 10^{-3} WSSV-antibody concentration showed 20% mortality at 25 dpi. All surviving animals were WSSV-negative by PCR. These results indicated that antibody neutralization of WSSV was dose-dependent [86]. *In vivo* neutralization assays had an efficacy of 50 - 85% shrimp mortality [84]. Recombinant subunit peptides displayed 20 - 40% shrimp mortality depending whether WSSV challenge occurred between 3 and 21 d post treatment [80]. In other experiments mortality was 48% with VP292 (0.1 mg/g shrimp in 20 µl) [82] and between 30-5% with VP28 (1mg/g shrimp in 20µl) as the recombinant peptides were administered twice during the experiment [78].

DNA vaccines: The strategy was first applied to shrimp by introducing foreign DNA into *P. monodon* eggs and embryos through electroporation. The rate of success was between 37 - 19% and survival of transgenic eggs into juvenile shrimp was 0.6% [87]. Another study done in *M. japonicus* delivered DNA to embryos using microinjection, electroporation and particle bombardment. Of these, microinjection was the most effective as high amounts of foreign DNA was delivered with this method [88]. The protective efficacy against WSSV using

plasmids encoding WSSV envelope proteins such as VP15, VP28, VP35 and VP281 was evaluated in recent studies [89,90]. Delivery routes were intramuscular [89,90] and oral. The latter used an attenuated *Salmonella typhimurium* bacterium as a delivery vehicle and it was adsorbed into commercial feed [91]. Shrimp *P. monodon* treated with a vp28 DNA vaccine delivered intramuscularly showed 10% mortality when WSSV challenge occurred 7 d post vaccination (dpv). Mortality increased to 20, 80 and 95% when WSSV challenge was done at 14, 25 and 50 dpv, respectively [90]. Another study used a plasmid containing the WSSV gene vp28 and it was injected to *P. monodon* at 7, 14, 21 and 30 dpv. Mortality of treated shrimp at those time points was: 10, 24, 33 and 44%, respectively [89]. Oral delivery of a plasmid containing the WSSV gene vp28 expressed in *S. typhimurium* showed protection against WSSV challenge in vaccinated crayfish *Cambarus clarkii*. Crayfish mortality at 7, 15 and 25 dpv was 17, 33 and 43%, respectively [91].

Invertebrates do not possess an adaptive defense system like vertebrates. Nonetheless some studies have indicated the presence of a specific defense response against viral infections. One work showed that some shrimp that survived a natural or experimental WSSV infection were more tolerant to a subsequent infection with WSSV. This result suggested the existence of a "viral neutralizing factor" [77] which could make possible the specific recognition of viral molecules and hence the ability to counteract infection.

Another hypothesis on the virus-specific defense response of shrimp was formulated in 1998 and since then it has been documented with experimental and field data. This hypothesis is called the viral accommodation concept [92] which states that many invertebrates including crustaceans can adapt to new viral pathogens to become asymptomatic carriers without displaying disease a few years after these viruses first appeared. The reason why viruses remain infectious and virulent but does not cause disease to tolerant animals is unknown. It is proposed that such a tolerance involves some sort of specific memory to prevent viral triggered apoptosis and it can occur in all life stages of a host species [92]. These concepts have opened the way to evaluate different strategies to protect animals using inactivated virus and/or recombinant envelope proteins that may block host cell receptors for viruses. Moreover, DNA vaccines encoding such viral proteins have also been developed and proved effective against viral challenges. These approaches have inappropriately been termed "vaccines" rather than antiviral treatments. More studies on this issue are needed to understand the defense mechanisms that induce viral tolerance.

Manipulating water temperature: Increasing water temperature at 32˚C before, just after or even until 18 hr post WSSV inoculation reduced virus replication and shrimp mortality (0-30%) compared to animals maintained at 27˚C (100%) [93,94]. The route of WSSV inoculation did not influence the protective effect of hyperthermia [95]. The beneficial effect of hyperthermia was significant even in periods of 18 h at 33˚C (0 - 40% mortality) [96]. Studies done in shrimp and crayfish have shown that although hyperthermia reduced virus replication, animals remain infected due to some replication as determined by competitive and real-time PCR [97,98]. Low water temperature is also effective to inhibit virus replication in species living in temperate or cold water regions. In shrimp *M. japonicus*, water temperature at 15˚C showed better inhibition of WSSV replication than 33˚C [99]. Likewise, crayfish species such as *Pacifactacus leniusculus Astacus astacus* and *P. clarkii* maintained at temperatures of 4,10 or 12˚C showed 0% mortality upon WSSV infection. In contrast, WSSV-infected animals maintained at 22-24˚C had 100% mortality [100,101]. Although the mechanism for inhibition of virus replication is not known, it has been suggested that hyperthermia may induce apoptosis of infected cells [98] thus aborting virus replication. It has also been suggested that hyperthermia may impair the biochemical properties of enzymes essential for virus replication, thus inhibiting replication but the animals remain infected [93].

RNA interference (RNAi): This mechanism was first described in the nematode *Caenorhabditis elegans* [102]. Later it was found in several other organisms such as fungi, plants and animals. The first biological function established for RNAi was as antiviral in plants [103]. RNAi can become a useful biotechnological tool against viral infections both in humans and animals.

RNAi starts with the presence of RNA molecules such as double-stranded RNA [103]. Upon entry, an enzyme called Dicer (a type III endonuclease) cleaves long dsRNA into double-stranded short interfering RNAs (siRNAs) [104]. The siRNA molecules are taken up by the RNA-inducing silencing complex (RISC) comprised by various proteins which unwinds siRNAs into single stranded molecules. The antisense strand remains attached to RISC and it is coupled to its homologous target mRNA to induce endonucleolytic cleavage. Long dsRNA molecules make it possible to produce various siRNA molecules targeting a single mRNA thus increasing effective gene silencing [104].

Evaluation of RNAi to control viral infections in shrimp

Several studies with RNAi have been done to fight viral diseases in shrimp. These have shown the existence of two pathways of antiviral immunity in shrimp: a sequence-independent (innate) and a sequence-specific (RNAi-mediated) [105]. It has been recently shown that long sequence-independent dsRNA molecules activate the mRNA expression of the RNAi molecules Lv Sid-1 and Lv Ago-2 just like sequence-specific dsRNA molecules.

Lv Sid-1 is a transmembrane protein thought to serve as a channel for the systemic spread of siRNAs molecules throughout the animal [106]. Lv Ago-2 is an isoform of the Argonaute protein family. This is a core protein component of the RNAi silencing complex (RISC) which has two domains: PAZ and PIWI. PAZ has nucleic acid binding capability whereas PIWI has a RNAse H-like structure probably involved in the enzymatic mRNA cleavage function [106]. In shimp as in many other higher organisms the mechanisms involved in both the innate antiviral defense and RNAi activity are activated by the same molecular pathway to produce an efficient antiviral response [106].

Innate antiviral immunity: The first *in vivo* RNAi experiments done in shrimp (*Litopenaeus vannamei*) used non-specific dsRNA to inhibit TSV or WSSV infections. A sequence-independent, dose-dependent antiviral state was induced against TSV or WSSV using dsRNA from immunoglobulin [Ig] heavy chain from duck or pig. Shrimp treated with unrelated dsRNA sharply reduced mortality (50-75%) compared to untreated controls [107]. This result indicated that shrimp possesses an innate antiviral immunity. Other studies showed that using sequence-independent dsRNA induced a non-specific antiviral effect. A LacZ dsRNA molecule was intramuscularly injected to determine the antiviral efficacy of sequence-independent dsRNA against WSSV using a high infectious dose. Results showed an innate antiviral response which delayed shrimp mortality for up to 24 h but did not prevent infection or reduced shrimp mortality [108]. An *in vitro* study used a green fluorescent protein (GFP) dsRNA against YHV infection of lymphoid organ primary culture cells. Results showed that

cells treated with GFP dsRNA allowed virus replication but at lower levels than mock-treated cells [109]. Another work *in vivo* showed that treatment with GFP dsRNA reduced shrimp mortality to 50% whereas control animals showed > 90% mortality at 8 dpi [105]. Other study showed mortality between 13 and 33% of shrimp treated with GFP dsRNA and challenged with 1 or 2 LD_{50} of WSSV. In contrast control animals challenged with 1 LD_{50} had 45% mortality at 6 dpi whereas control shrimp challenged with 2 LD_{50} had 90% mortality by 3 dpi [110].

Specific RNAi antiviral immunity: Sequence-specific dsRNA has been used to inhibit virus replication in shrimp against TSV, IHHNV, YHV and WSSV. Studies done with dsRNA against a putative protease from TSV showed that sequence-specific dsRNA strongly inhibited TSV replication (11% mortality) in shrimp infected *per os*, while controls showed 100% mortality at 5 dpi [111]. Replication of IHHNV or HPV has successfully been inhibited with specific dsRNA. An amount of 1.5µg specific dsRNA against genes encoding structural or non-structural proteins showed a transient reduction of virus replication at 8 dpi [112]. The preventative and therapeutic effect of two dsRNA against IHHNV were evaluated. One dsRNA (433 bp) was directed against an ORF1/2 sequence whereas the other (436bp) was directed against ORF3 encoding a structural protein. Animals were treated with 2.5µg/g body weight 12h before intramuscular IHHNV challenge. Additional treatments were done at 3 and 6 d post virus inoculation. PCR analyses showed a high inhibition of IHHNV DNA in treated animals at 5, 8 and 10 d post challenge. In contrast, control animals showed high IHHNV DNA levels by 5 d post challenge [113]. The therapeutic effect of ORF1-2 dsRNA was evaluated at 12, 24 and 48 h post IHHNV challenge and it showed a high inhibition of IHHNV DNA in animals treated 12 or 24 h post challenge and the antiviral effect lasted for 5 d [113]. The therapeutic effect of two combined dsRNAs directed against a non-structural and a structural genes of HPV was shown in animals naturally-infected with HPV. Upon four consecutive injections (0.8µg each) at intervals of 5 d it was shown that treated animals were cleared of HPV infection [114].

The efficacy of dsRNA to inhibit YHV infection was determined *in vitro* against sequences of genes encoding a helicase, polymerase, protease and the structural proteins gp116 and gp64. Results showed that higher YHV inbition was achieved using sequences targeting non-structural genes [109]. An *in vivo* experiment using intramuscular injection of dsRNA against YHV protease showed 0% mortality of treated shrimp compared to > 90% mortality in controls at 10 d post challenge [105]. Another study presented the therapeutic effect of treating shrimp with 25 µg dsRNA against YHV protease at 3, 6, 12 or 24 h post YHV challenge. Animals treated up to 3 h post challenge showed high survival (60%) in contrast to 100% mortality in untreated animals at 2 dpi. Detection of YHV cDNA by RT-PCR assay showed that animals treated 3, 6 or 12 h post YHV challenge had reduced levels of viral cDNA compared to untreated controls. This result indicated that YHV infection can be reversed if treated early with dsRNA [115].

Several studies on dsRNA have been done against WSSV since this is the most lethal pathogen in shrimp aquaculture. Different efficacies in inhibition of WSSV replication have been achieved using sequence-specific dsRNA against various genes encoding structural and non-structural proteins (Table 2).

The duration of the antiviral effect of sequence-specific dsRNA against WSSV has been determined to be short-term (up to 10 d after treatment). As time between treatment and WSSV challenge increased the antiviral efficacy was gradually reduced [108]. Therefore, methods to increase the duration of antiviral effect are required. It was shown that the continuous re-infection of treated shrimp extends the antiviral effect up to 30 d after challenge and significantly reduced shrimp mortality [108]. Other strategy used to enhance the duration of the antiviral effect is the repeated administration of dsRNA [113-116]. Continuous administration of dsRNA through feed may be a suitable way to increase the duration of the antiviral effect in cultured shrimp.

Specific RNAi antiviral immunity by siRNA: Works using siRNA to trigger a RNAi antiviral response have shown controversial results. Injection of siRNA (19 bp) against vp19 did not protect shrimp against WSSV challenge [111]. Another study used short (21bp) siRNA against WSSV vp28 or vp15 and they induced a significant reduction in shrimp

Type	Gene	Administration route	Concentration (µg)	virus dose	Mortality (%)	duration (days)	Reference
	vp28	intramuscular injection	4	2500 SID_{50}	13	10	[108]
	vp28	intramuscular injection	5	$4x10^{-8}$	15	10	[111]
	vp28	intramuscular injection	6	1-2 LD_{50}	0	7	[110]
	vp28	oral (coated in feed)	n.d	n.d	63%	15	[139]
	vp28	oral (chitosan nanoparticles)	n.d	n.d	32%	15	[139]
Structural	vp281	intramuscular injection	6	1-2 LD_{50}	20 - 47%	7	[110]
	vp26	intramuscular injection	4	2500 SID_{50}	21%	10	[108]
	vp26	intramuscular injection	25	$1x10^{-5}$	100%	30	[140]
	vp24	intramuscular injection	25	$1x10^{-5}$	37%	30	[140]
	vp19	intramuscular injection	25	$1x10^{-5}$	66%	30	[140]
	vp15	intramuscular injection	25	$1x10^{-5}$	37%	30	[140]
	RR2	intramuscular injection	5	$4x10^{-8}$	22%	10	[111]
Non-structural	DNA pol	intramuscular injection	5	$4x10^{-8}$	56%	10	[111]
	PK	intramuscular injection	6	1-2 LD_{50}	7%	7	[110]

Legends: SID_{50} - shrimp infctious dose 50% endpoint, LD_{50} - lethal dose 50% endpoint. N.d. not determined. RR2 - ribonucleotide reductase small subunit. DNA pol - DNA polymerase. PK - protein kinase.

Table 2: *In vivo* evaluation of dsRNA efficacy against different WSSV genes encoding structural and non-structural proteins.

mortality compared to controls. Nonetheless, the same mortality reduction was achieved by injecting GFP-siRNA [117]. Another study used 21bp siRNA against WSSV vp28 in *M. japonicus* and showed that shrimp treated with 100 µl siRNA (6µM/shrimp) significantly reduced shrimp mortality (70%). Administration of a consecutive siRNA injection to infected shrimp every day for three days inhibited virus replication. At the end of the experiment no WSSV DNA was found, suggesting that siRNA could eradicate WSSV infection [116]. An *in vivo* experiment using five sequence-specific siRNA against WSSV: DNA polymerase, ribonucleotide reductase small subunit (rr2), thymidine kinase-thymidylate kinase, vp24 and vp28 showed a significant reduction in mortality at 6 d post challenge (50, 50, 66, 33 and 33%, respectively). A vp28 siRNA sequence (21bp) with a 6bp mutation was used as a sequence-independent siRNA which failed to protect shrimp from WSSV challenge. This result indicates that only sequence-specific siRNAs are able to inhibit virus infection in shrimp [118]. It was recently demonstrated that siRNA molecules longer than 50bp might be more efficient to silence target mRNAs [106]. The study done previously [111] failed to induce an antiviral response probably because the siRNA used was too short (19bp) and it may not be recognized by the mechanisms that take up dsRNA [106].

RNA silencing of shrimp endogenous genes involved in virus infection

RNAi has also been used to determine the function of various genes from shrimp that are involved in virus infection. This is done by silencing genes encoding certain proteins or enzymes of interest [119]. In shrimp a number of proteins involved in antiviral immunity have been studied by RNAi silencing, including: a toll-like receptor [120,121], rab7-like proteins which are involved in virus entry [122], a caspase-3 protein, involved in apoptosis [123] and the proPO system [124].

The innate system is the first defense line against pathogens which is activated by a number of molecules that recognize different pathogen-associated molecular patterns (PAMPs) [125] which include peptidoglycans, lipopolysaccharides, beta-glucans and foreign dsRNA. These in turn activate different defense responses [126]. In vertebrates, toll-like receptors (TLR) are involved in recognition of PAMPs and activation of defense responses against pathogens. Foreign dsRNA is recognized by TLRs involved in the activation of the RNAi antiviral response [125]. Recently a TLR has been found in *P. monodon* [127], *L. vannamei* [120] and *F. chinensis* [126]. The function of such a molecule in shrimp was investigated. Expression of Toll in shrimp challenged with *Vibrio anguillarum* was upregulated at 8 h post challenge [126]. Another study followed expression of Toll in *L. vannamei* and found that upon challenge with *V. harveyi* it was upregulated to a maximum at 24 h post challenge [128]. Toll silencing was done in animals using dsRNA (1 µg/g shrimp). Three days later shrimp were challenged with 8000 CFU *V. harveyi* or WSSV and mortality was followed. Significant increase in mortality was found in animals challenged with *Vibrio*. Shrimp challenged with WSSV showed no difference in mortality compared to untreated controls [128]. These results agree with the findings of [127] where WSSV-challenged animals showed no Toll upregulation indicating that the TLR found in shrimp is not involved in antiviral defense response. Another work silenced Toll in shrimp *L. vannamei* and other animals were given sequence-independent dsRNA. Later (48h) both groups were challenged with WSSV. No differences in mortality were observed between these treatments.

A small GTP-binding protein was found in *P. monodon* [129] and later also in *M. japonicus* [122] and *L. vannamei* [130]. Rab proteins are involved in endocytic trafficking, phagosome formation, maduration and lysosomal degradation [122,131]. A Rab7-like protein has been found in shrimp to bind to WSSV VP28 thus it is involved in WSSV infection [129]. Silencing shrimp Rab7 has shown efficacy to inhibit viral infections. Animals treated with dsRNA against shrimp Rab7 and later challenged with either WSSV or YHV showed very low levels of WSSV mRNA or YHV mRNA, respectively [132]. Silencing shrimp Rab7 has been determined *in vivo* against various shrimp viruses. Silencing Rab7 effectively inhibited a Laem-Singh virus infection in *P. monodon* when treated before virus challenge or up to 24h after challenge [133]. Likewise, silencing Rab7 in *L. vannamei* (2.5µg/shrimp) 48 h before TSV challenge greatly reduced TSV replication (90%) compared to untreated shrimp [130]. Moreover, silencing of a WSSV gene (rr2) and Rab7 endogenous gene from shrimp reported no enhanced inhibition of virus replication. Mortality values (≈ 95%) were similar between shrimp treated against WSSV rr2 and animals treated with combined dsRNA against WSSV rr2 and shrimp Rab7

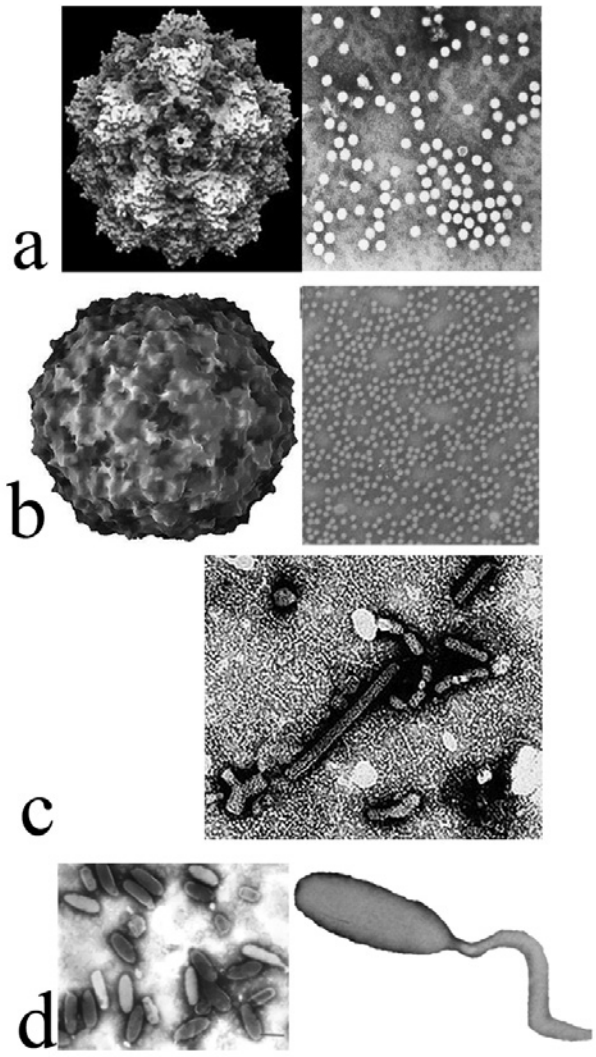

Figure 1: Shape, relative size and transmission electron microscopic view of virions of four major viruses infecting shrimp. (a) IHHNV; (b) TSV; (c) YHV; (d) WSSV. a, b and c were modified from [22] and d was modified from [58].

gene [134]. In contrast, the combined silencing of a YHV protease and shrimp Rab7 gene (YHV dsRNA = 1.5 µg / shrimp; Rab7 dsRNA = 0.63 µg / shrimp) by dsRNA enhanced virus inhibition and reduced shrimp mortality (10%) compared to animals treated only with YHV dsRNA (30% mortality) [135].

Another shrimp endogenous gene involved in WSSV infection is caspase-3. This gene encodes a protein that directs programmed cell death or apoptosis. Apoptosis may be a defense mechanism against viral infections by destroying infected cells thus limiting virus replication [136]. In crustaceans it has been hypothesized that virus-induced apoptosis provokes shrimp mortality [92]. Therefore, inhibiting virus-induced apoptosis through silencing shrimp caspase 3 would reduce shrimp mortality [123]. An experiment was done silencing shrimp caspase-3 by injecting dsRNA twice. Upon a WSSV challenge with a high dose, shrimp treated with caspase-3 dsRNA displayed 100% mortality at 7 d post challenge but it was observed a delay in time of mortality. In contrast, shrimp treated with dsRNA against WSSV vp19 had 5% mortality. Using a low WSSV dose, mortality was reduced in animals treated with caspase-3 dsRNA [123]. These results indicate that inhibiting caspase-3 failed to reduce shrimp mortality due to WSSV infection and it suggests that apoptosis may not be involved in shrimp mortality upon WSSV infection.

The proPO system is a major immune defense mechanism in shrimp. Upon activation, proPO triggers a cascade of reactions leading to phagocytosis, encapsulation, nodule formation and melanization. All these reactions are mainly directed against bacterial and fungal infections [137]. The effect of proPO on defense response against a bacterial challenge was determined by silencing a component of the proPO system in *P. monodon*. Shrimp were injected with 20 µg dsRNA each before challenge with 200,000 CFU *V. harveyi*. Mortality of proPO-silenced shrimp was 100% whereas untreated shrimp challenged with *Vibrio* had 50% mortality at 5 d post challenge. This result shows that proPO is essential for antibacterial defense in shrimp [124].

Advances in massive delivery of RNAi molecules

RNAi is a promising tool against infectious diseases in human medicine, veterinary medicine and aquatic animal health. In nematode worms and shrimp the experimental efficacy of RNAi molecules has been done through injection. This administration method is not suitable for the massive delivery and/or continuous distribution of RNAi molecules to large populations of animals like those existing in shrimp hatcheries or grow-out ponds.

A number of works have addressed methods to produce massive amounts of RNAi and its delivery to a large population. The first report on delivery of dsRNA by feeding was done in 1998. Nematode worms were fed bacteria expressing sequence-specific dsRNA against three nematode endogenous genes. Animals displayed transiently-induced gene interference observed as a distinct phenotype compared to controls. This result indicated that RNAi molecules can spread throughout the body of these animals and that RNAi molecules can cross the intestinal tissues and enter different cell types [138].

A few studies have evaluated the ability to produce RNAi molecules in large quantities and their massive delivery to shrimp by oral route. A work on RNAi synthesis in bacteria and its delivery to shrimp by oral

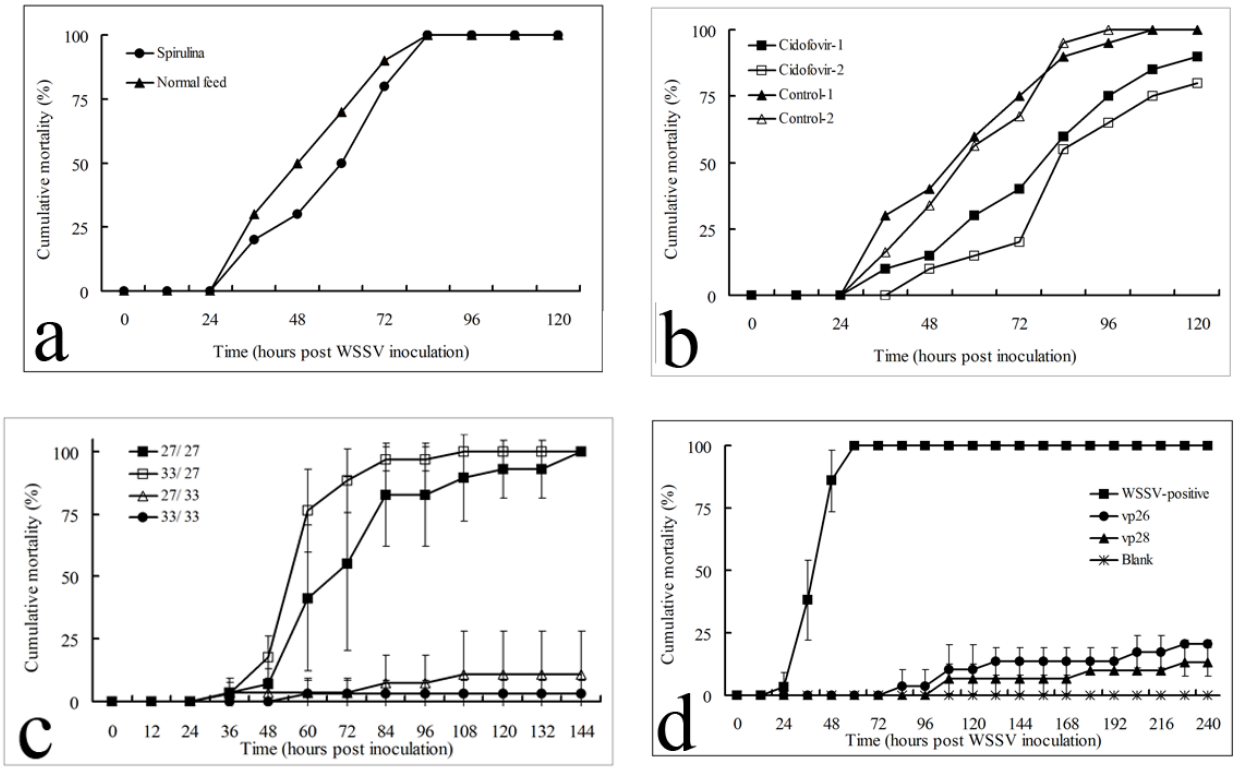

Figure 2: Comparison of efficacy of different products evaluated to control WSSV infection under standardized experimental conditions. (a) *Spirulina*; (b) Cidofovir; (c) Hyperthermia (33˚C); (d) vp28 and vp26 dsRNA. Data from (a,b) [74]; (c) [93] and (d) [108].

route was done in 2008. Molecules of dsRNA against WSSV vp28 were synthesized in RNAse III-deficient *E. coli* HT115 (DE3) transformed with a plasmid containing bidirectional T7 promoter and synthesized with T7 RNA polymerase [139]. Bacteria expressing vp28 dsRNA was harvested and inactivated with 0.5% formaldehyde for 15 min at 20˚C. Inactivated bacteria were used to coat feed at 10^8 cells per 2g pelleted feed and given to shrimp at 2% mean body weight per day for 5 days before oral WSSV challenge. Animals treated with bacteria expressing vp28 dsRNA showed 30% reduction of mortality compared to controls [139]. The efficacy of bacterially expressed dsRNA against WSSV genes vp15, vp19, vp24 and vp26 and their efficacy *in vivo* by oral delivery to shrimp were determined [140] (Table 2).

Other molecules such as long dsRNA (up to 500 bp) against YHV have also been expressed in bacteria using a hairpin-RNA expression vector [141]. This unexpensive approach produced up to 5 mg dsRNA per 130 ml bacterial culture. The dsRNA against YHV reduced mortality up to 65% compared to controls. A WSSV vp28 siRNA was produced in bacterial cells but inactivated bacteria was injected to shrimp. Results indicate that siRNA contained in inactivated bacteria was twice as effective (60 vs. 30% inhibition of mortality) compared to isolated vp28 siRNA intramuscularly injected to shrimp [142].

Another approach was to isolate the dsRNA produced in bacteria and using different macromolecules as delivery vehicle. Chitosan nanoparticles (0.2% w/v) containing 35 µg vp28 dsRNA, were adsorbed (100 µl) in 2g of pelleted feed. Animals were treated with feed coated with chitosan at 2% mean body weight for 5 days before oral WSSV challenge. Results showed that chitosan nanoparticles were more effective than inactivated bacteria to inhibit shrimp mortality [139]. Liposomes have also been evaluated to deliver RNAi molecules in vertebrates [143]. The main drawback of these macromolecules is that they are rapidly cleared by the liver and lack target tissue specificity. Other molecules used to deliver siRNA include cholesterol-conjugated siRNA and antibody-protamine bound siRNA [143]. Immersion of nematodes (*Caenorhabditis elegans*) into a solution containing dsRNA molecules was also evaluated as a delivery approach. Animals immersed in dsRNA solution showed a successful inhibition of an endogenous target gene giving the animals a lethal phenotype [144].

The need to produce high amounts of RNAi molecules and its massive delivery to an animal population is still in development. The use of RNAse III-deficient bacterial strains to produce RNAi molecules seems an efficient and cheap method to obtain massive amounts of these molecules. Different approaches to deliver RNAi to animals have been evaluated and many of them have proven effective. Feeding animals with RNAi-coated feed seems a promising method for the massive delivery to a large number of animals and to elicit an antiviral effect. More studies are required to optimize and enhance the efficacy of a massive RNAi delivery method.

Perspectives and Conclusions

Shrimp aquaculture is an important industry for many developing countries providing high quality food and generating jobs and revenues. Due to the appearance of infectious diseases the further development of shrimp aquaculture has been compromised. Effective control methods and strategies to curb diseases have been sought but so far no effective method has been successfully applied in the field.

Under experimental conditions several strategies and methods have been evaluated and have given different results. Some have been more effective than others to reduce infection and shrimp mortality. Nonetheless, it is difficult to compare the efficacy of different control methods since most of them have been done using undefined virus titers and with different experimental conditions. Recently, a standardized inoculation procedure has been developed in order to determine the exact amount of infectious doses of WSSV used in experimental challenges [145]. Using standardized virus inoculation procedures makes it possible to evaluate and to compare the efficacy of different products and strategies against viral pathogens (Figure 2).

The application of RNAi to control shrimp viral diseases in the field is a promising strategy. Large-scale production of RNAi through bacterial cells is a cheap, attractive method. This strategy coupled to massive delivery of RNAi molecules through feed either using inactivated bacterial cells expressing RNAi, or through macromolecules encapsulating or binding RNAi represents an easy and effective method to distribute RNAi in shrimp farming facilities such as hatcheries or grow-out ponds. More research is required to address issues related to environmental safety and food safety of RNAi-treated shrimp.

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