New strategies for control, prevention and treatment of ISA virus in aquaculture

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Chile has unquestionable characteristics for exploitation of marine resources. Benefiting from this natural advantage, the Chilean salmon farming industry grew until being positioned as one of the major salmon producer worldwide. However, diseases that strongly affected the salmon production accompanied this growth. One of the most serious risks facing intensive salmon farming is the infectious salmon anemia virus (ISAV) because it produces high mortality and important economic losses. Since no pharmacological treatment has been developed for this disease, prevention strategies are essential to control ISAV. The aim of this mini-review is to discuss current and emerging alternative tools that have been developed for the treatment of ISAV and other virus of importance in aquaculture, considering their improvements, advantages and disadvantages.

Keywords antiviral, ISAV, salmon farming.

1. Infectious Salmon Anemia Virus: The Effect on the Host and its Impact on Salmon Farming.

1.1. Salmon farming and the Infectious Salmon Anemia Virus

Salmon farming is one of the main sources of food generated by the fish farming industry [1,2]. In 2012, there were 2,773,639 tons of salmonid produced worldwide, surpassed only by the cultivation of fish in the *Cyprinidae* (carp) family with 3,957,949 tons and the *Cichlidae* (tilapia) family with a total of 25,157,502 tons [2]. The most important fish that compose the salmonidae family are *Salmo salar* (Atlantic Salmon), *Oncorhynchus kisutch* (Coho Salmon) and *Oncorhynchus mykiss* (Rainbow Trout). In Chile, the salmon industry is one of the most important industrial activities; it is the second largest producer in the world, second only to Norway [3]. The industry had a big surge in the 80's and 90's largely due to advantages in environmental conditions, abundant natural resources and excellent sanitary conditions present in the country. However, the rapid expansion of salmon farming between 1992 and 2007, which established Chile as a globally relevant aquaculture producer, was not accompanied by either the technological mainstreaming or the regulatory development that the industry requires of a producer of such magnitude [4]. This had a social, cultural and ecological impact in vast areas in the south of Chile; it is believed that the stress caused to the coastal ecosystems could be related to the emergence of a sanitary crisis, between 2007 and 2008, principally due to the uncontrolled eruption of infectious salmon anemia (ISA) [5,6]. This disease provoked production losses of around 40%, in of which are primarily seen reflected in 2010 [2,70]. ISA is a systemic disease that principally affects Atlantic salmon, although it has been observed in other salmonid species [7]. It has been described to attack the circulatory system, primarily the endothelial cells and kidney tissue macrophages [8]. The clinical and pathological signs of the disease resulting in a terminal state include a severe case of anemia with less than 10% hematocrit, liver congestion, gill paleness, intestinal congestion, ascites and hemorrhaging in a variety of organs suggesting circulatory failure [8]. This disease represents a global threat; in 1984 Norway experienced an outbreak and since then there have been outbreaks detected in Canada [9], Scotland [10], the Faroe Islands and the U.S [11,12,13]. In these registered ISAV outbreaks, the accumulated mortality has varied between 10 and 95%, and in some cases there has been a complete production loss [14].

1.2. The Replicative Cycle and Virulence Factors.

Infectious salmon anemia is caused by the ISA virus (ISAV), which belongs to the genera Isavirus from the *Orthomyxoviridae* family [15]. It possesses a segmented genome of single strand RNA with negative polarity that codes eight structural proteins and two nonstructural ones. The eight segments of genomic RNA (also know as virion RNA or vRNA) are bound to multiple copies of the viral nucleoprotein (NP). In its 3' end is located a copy of the RNA-dependent RNA- polymerase (RdRp) complex formed by protein basic 1 (PB1), basic 2 (PB2) and acidic (PA); all of these in association form what are known as ribonucleoproteins. The capsid, constituted by matrix protein 1 (M1) and matrix protein 2 (M2), is surrounded by a membranous envelope where glycoproteins, hemagglutinin-esterase (HE) and fusion (F) are found inserted. Until now, not much has been known about replication and transcription cycle of ISAV, however, studies have been suggested using mechanisms based on the influenza virus, which is the model virus of the *Orthomyxoviridae* family. This is because both viruses share similar genetic, morphological and biochemical

characteristics [17]. In order to understand the viral cycle of ISAV (Figure 1), functions and intracellular destination of their genomic products have been inferred from the information available of influenza virus proteins. It has been suggested that the first interaction occurs with the hemagglutinin protein (HE), which recognizes a cellular receptor containing 4-0 -acetyl-sialic acid (attachment) [18]. Subsequently the particle enters the cell in vesicles that are fused to endosomes, granting the necessary acidic environment for the fusion between endosome and viral membranes (mediated by F protein), releasing the viral genome [19]. The viral ribonucleoproteins travel towards the nucleus, where viral transcription begins. The viral mRNAs produced in the nucleus are translated in the cytoplasm. The mRNA that encode HE, F and possibly M2 are translated by ribosomes associated with the endoplasmatic reticulum and pass through the Golgi secretory pathway. The mRNA that encode NP and possibly those that encode PB1, PB2, PA, non-structural protein 1 (NS1), nuclear export protein (NEP) and M1 are translated by free ribosomes. These proteins return to the nucleus, which allow for the beginning of viral replication, and subsequently, the formation of new ribonucleoproteins [19]. The assembly of mature viral particles is achieved in the cellular membrane to where glycoproteins (HE and F) and also the M2 proteins migrate; these will be the receptors of the rest of the viral particle. Finally the budding process occurs, avoiding cellular lysis of the host [19].
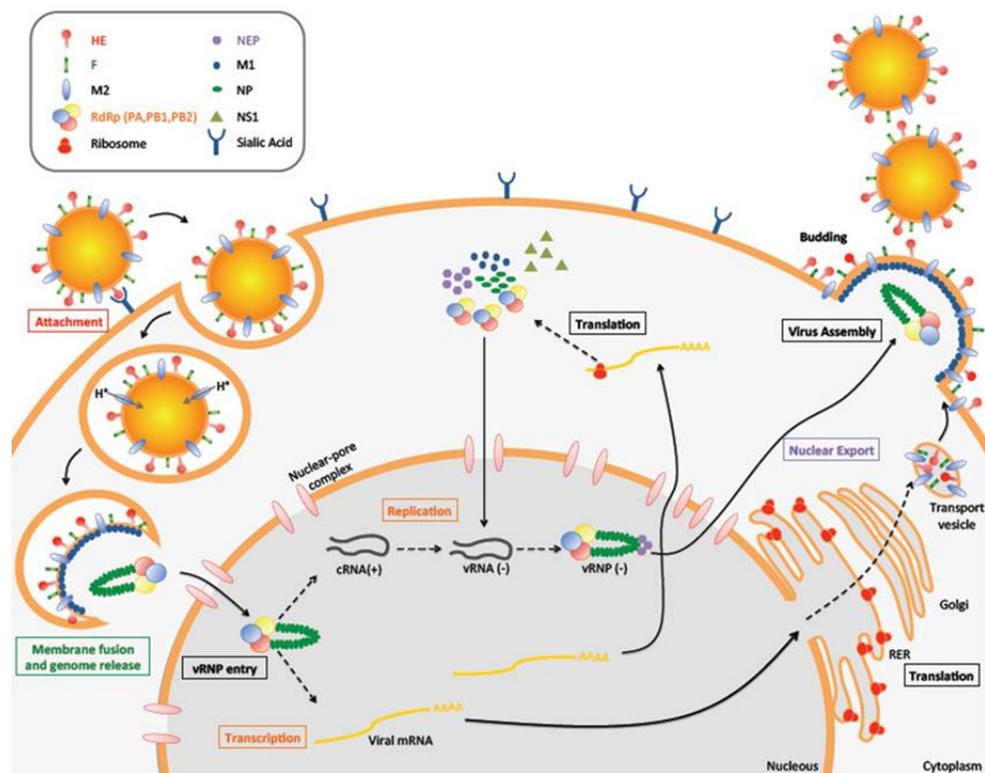

Fig. 1 Schematic representation of the viral cycle of ISAV. Here it is described in its different stages, simplified, showing only one vRNA. The solid arrows represent the transportation processes while the dashed lines indicate synthesis or interactions mediated by proteins.

In ISAV it is known that variations that occur in proteins F and HE are related to the virulence differences in several viral isolates [20]. Virulence, which is understood to be the relative ability of pathogenic agents to cause sickness, is a manifestation of the interaction between the adverse effects produced by virus components and the defense mechanisms developed by the cells to try to eliminate the infection. Nevertheless, the result of this interaction is always determined by the virus through its virulence factors, which can be attributed to any component of the viral particle [21]. It has been proposed that the most variable elements are localized in segments that encode the surface proteins (F and HE) and that these differences are directly correlated to the cytophatic effect found in cell culture and pathogenesis in fish [22, 23, 24]. The F protein has a 50-kDa precursor that suffers a proteolytic cleavage generating two subunits. This proteolytic cleavage occurs in arginine (R) residue in the 267th position allowing for the generation of functional proteins [25]. One of the reasons for proposing this protein as a virulence determinant is because it is suggested that an amino acid substitution would benefit the proteolytic cleavage, generating more functional protein, which in turn would benefit the viral cycle. This amino acid substitution would occur in the region adjacent to the site of the proteolytic cleavage, changing from glutamine (Q) into leucine (L). It is thought that the isolates that present (L) will be more virulent [20]. Another virulence determinant in the same protein would be the presence of amino acid insertions (IN), which could disturb the F0 precursor structure, instead, favoring the accessibility of trypsin and consequently, the proteolytic

cleavage [20,25]. In consequence, this also would favor the generation of active protein. Until now, 4 IN regions have been characterized; IN3 and IN4 are described as highly pathogenic to isolates that are present. IN4 has only been found in ISAV reports in Chile [26] and its presence has been detected in 80% of Chilean isolates. This information shows that the ISAV isolates in Chile containing this insertion differ from the European and North American ISAV isolates. This imparts particular characteristics for the isolates that present IN4; therefore, their study is necessary in order to characterize its virulence [26,27]. HE has been characterized as another virulence determining protein. This protein possesses a high polymorphic region (HPR) in the end of C- terminal. Apart from being associated with high variability, this region is also associated with virulence. So far over 30 variations of HPR have been identified [19]. These variations can provoke a high mortality, even reaching 90%. In Chile, the isolates associated with high mortality belong to variants HPR1c and HPR7b (most abundantly) [28]. In contrast, ISAV-HPR0 has been described as one variant that does not provoke mortality or show clinical signs [29, 30]. Despite of the fact that this variant can be detected by qPCR, its isolation in cell culture has been unsuccessful. This variant is only detected in the gills; therefore, it is believed that the infection is restricted to this tissue, differing from the systemic infection caused by the isolate possessing high virulence [30]. ISAV-HPR0, which pertains to the European genotype, is considered to be the original strain that was found naturally among wild fish. In Chile there are 36 different groups of HPR, which means that the virus has experienced a high mutation rate. Nevertheless, the detection of the HPR7b strain has radically diminished in recent years, while the HPR0 variety has been steadily rising since its first detection in the winter of 2008 [31].

1.3. Immune Response

A cellular and humoral response against ISAV in Atlantic salmon has been demonstrated [32, 33, 34, 35]. In general, the innate antiviral defense mechanism in teleost fish occurs based on the production of interferon (IFN), which represents the first line of defense against pathogenic agents, in this case, ISAV. IFN molecules are secreted by nucleated cells in response to the viral infection and they trigger a signaling pathway with the expression of a number of proteins containing direct or indirect antiviral properties. There are two types of IFN: type 1(which includes IFN α and β) and is expressed by all kinds of cells and type 2 (IFNγ), which is produced by more specialized immune cells. The majority of viruses possess mechanisms allowing evasion of the interferon response. Such is the case with a non-structural protein (NS1) in influenza virus A [36]. It is known that ISAV is capable of inducing genes related to the interferon response, however, it is not inhibited by interferon, resulting in the probability that it possesses mechanisms allowing it to evade this response [37]. As a part of the humoral immune response, the presence of antibodies exclusively bound to nucleoprotein and hemagglutinin of ISAV have been identified [38]. It is believed that the increase in viral infection through antibodies could be involved in the pathogenesis since ISAV unites leukocyte cells [39]. In the leukocytes of fish the presence of immunoglobulin M receptors have been found [40]. Nevertheless, despite of the fact that an immune response against the ISAV virus in salmon has been described, this is not sufficiently efficient, therefore it is critical to search for strategies for the control, prevention and treatment of the sickness caused by this virus.

2. Control and Prevention Measures

The detailed study of the diseases and the organisms that provoke them allows for the design of diverse control, prevention and/or treatment strategies, either with an early diagnosis, combating dissemination or by attacking its origin. Nevertheless, due to the lack of knowledge about the ISA virus, the treatments in order to effectively combat it do not yet exist. In Chile, when the presence of ISAV is suspected in a salmon farm because of the clinical signs associated with the disease, a rise in mortality attributed to the virus or the detection of the virus by RT-PCR, all tanks in the center must be tested within 7 days and afterwards monitored every 15 days. In the event of a positive ISAV confirmation, the fish should be eliminated or harvested within a maximum of 15 days. Positive samples of RT-PCR lead to a sequence analysis in order to determine the virus genotype. Control measures are carried out based on the results [44]. The control regulations are primarily based on measures of biosecurity, disinfection and prevention because of the fact that the disease is acute by nature, fulminant and quickly disseminating.

2.1. Control Strategies

Massive salmon farming, concentrated in high density, facilitates the spreading of diseases in farms and because of this, producers should maintain an environment that ensures sanitation. Managing effective sanitary conditions consists of adopting practices and procedures that emphasize the prevention of outbreaks of diseases, whether they be infectious or noninfectious, of bacterial origin, fungal, parasitic or viral as is the case with ISA [41]. Control and biosecurity procedures that are used in Chile were adopted from the measures taken in Norway in order to control viral outbreaks in the 90's. Even though a biosecurity program cannot completely avoid or prevent the entry of pathogens, biosecurity measures can keep them under control. This consists of practices and procedures that reduce the introduction of pathogens into an installation reduce the risk of pathogens disseminating and reduce the conditions that could augment susceptibility of infection and sickness. Currently, biosecurity is emerging as one of the most indispensable topics in the

national and international community. It can be defined as a set of management measures or practices aimed at preventing the entrance and exit of diseases and controlling the dissemination of microorganisms capable of producing disease. Biosecurity, including the process control, involves adoption of pertinent measures in order to solve the problem [41]. The need to apply measures of biosecurity has intensified with globalization, due to high-risk factors in carrying out production such as: rapid technology development, accessibility of transportation and international business. Along with this, there are risk factors linked to the spread of diseases. In case of ISAV, the risk factors involved in the dissemination of the virus, are found in the transportation of the fish from high-risk zones to zones free of the pathogen, the entrance of staff and visitors to the farms with inadequately disinfected implements and equipment. For each of these identified risk factors, regulations have been implemented, in of which the cleanliness and disinfection are considered fundamental in controlling ISAV [42]. The disinfection of equipment and surfaces necessary to conduct work in fish farms should be preceded by a thorough cleaning, whose role is fundamental. If not accomplished, the effectiveness of the disinfection will be compromised since disinfectants become reduced in germicidal strength in the presence of residual organic material [43]. The National Fishing Service Sanitary Program (Servicio Nacional de Pesca, SERNAPESCA) [44] stipulates that each center must have a hygiene and disinfection manual, which should be developed with the ultimate aim of preventing the transmission and dissemination of pathogenic agents, being either for the center itself, to or from other centers or for the environment. The use of disinfectants as viral neutralizing mechanisms has been utilized against a series of pathogens in the aquaculture industry. They should have three important qualities: be 100% biodegradable (short term and leaving no traces), have the ability to dissolve in fresh and salt water and be of a high microbial spectrum for viruses, bacteria and fungus. In studies, diverse agents against ISA virus have been reported. Iodophors, chloramine T and mixtures of peracetic acid, hydrogen peroxide and acetic acid have resulted in varying levels of effectiveness [45]. Other disinfectants, such as quaternary ammoniums, glutaraldehyde and sodium hypochlorite have also been evaluated, showing effectiveness in laboratory conditions. In terms of disinfectant agents, it is generally accepted that the resistance of an enveloped virus, such as ISAV, is low. In Chile, studies conducted by Centrovet laboratory tested the ISAV neutralizing capabilities of six products: Carsept 50% (50% Quaternary ammonium bromide), Bixler (4.2% Quaternary ammonium + 12% Glut + 18% detergent), Climber 20% (20% glutaraldehyde), IPN Killer (established inorganic oxidants), Cress 50% (50% bronopol) and ViroKiller (80% chloramine T) with the goal of evaluating the capacity of different disinfectant products that neutralize or eliminate ISAV. To do this, samples of the virus with the different products were incubated for the minimum incubation period in order to imitate natural conditions and the disinfectant was removed no longer than 30 seconds after contact with the virus. The post-treatment clearance of the virus was determined by immunofluorescence and RT-PCR, suggesting total inactivation. In the evaluated conditions, the disinfectants were capable of eliminating at least five viral logarithms, therefore effective in the elimination of 99.999% of the virus in less than 30 seconds [46]. Further studies carried out by ADL laboratory show that in the presence of a low amount of organic material (2%), the use of 100ppm of ClO_2 for 5 minutes presented an antiviral effect when medial titers of ISAV are tested and, therefore, ClO_2 eliminated 99.99% of ISAV titers on all evaluated surfaces, which coincides with the existing international protocol for disinfection. However, in the presence of larger amounts of organic material (5 and 20%), the same procedure loses effectiveness in deactivating ISAV. This means that before any disinfection, it is fundamental to complete a detailed cleaning of all surfaces in order to ensure an effective disinfection. Not all the ClO_2 presents the same effectiveness in combating ISAV, because there is a high variability between the different products [47].

2.2. Preventative Measures

2.2.1. Traditional Vaccines

By definition, a vaccine is a preparation of antigens that is administered to produce antibodies and, in consequence, a defense response against pathogenic microorganisms. In some cases, this response generates immune memory, eventually producing immunity against the corresponding pathogen. In order for vaccines to achieve an optimal effect, it is essential to maintain good hygiene and low levels of stress in the fish. Currently, vaccination is an integral part in the vast majority of the salmon farming industry; Northern Europe, Chile, Canada and the United States are the principal consumers since in these places the value of a healthy population of salmon and/or trout justifies the cost of the vaccine. In salmon farming, vaccines have been vital in the control of bacterial diseases, however this has not been the case with viral ones [48]. Today, the majority of viral vaccines used in fish farming are based on an inactive virus or recombinant protein subunits. In the case of ISAV, there is innate immune response activation during the infection, but there is evidence that it does not provide the necessary protection. Therefore, it is the activation of the adaptive immune system that will assume a central role in the survival of the host and the total elimination of the virus [35]. While there are known vaccines to provide protection against virulent ISAV isolates [49], and in Chile some of them have been used and registered by the Agriculture and Cattle Service (Servicio Agrícola Ganadero, SAG) [50], the protecting effects presented by these vaccines have not (yet) been described in detail. While the existence of mucosal immunity in teleost fish is known, it is a field that has not yet been well explored and very little is known about the mechanisms capable of inducing protection and immunization. Inactivated virus or killed virus vaccines generally are not effective unless administered via injection. For this reason, in Chile, the majority of current vaccines used against ISAV, fall under the

injectable emulsion category. The protection that these kinds of vaccines provide can last approximately six months to a year. However, the disadvantages in this method are that in order to induce protection the inactive virus must be accompanied by oil-water emulsion adjuvants, which can slow the fish growth. Furthermore, the intraperitoneal injections apart from meaning hard labor (if it is not automated), require that the fish be anesthetized, which is not recommended for reproducers. This is added to the combination of vaccines with adjuvants that are used to better the administration and modulation of the immune system; however, the oil-based adjuvants that have had a positive effect on vaccines against ISAV have had negative effects when used on salmon. In addition, this method is laborious, requires time and specialized personnel. On the other hand, the production of large quantities of the virus required to prepare an inactive vaccine needs extra security, furthermore the technology is time consuming and complex, since it is difficult to develop. Additionally, there are inactive vaccines that are inconsistent in their effectiveness [52]. The viral vaccines that use attenuated viruses, have many advantages since they induce heightened protective immunity and furthermore disseminate from vaccinated fish, which implies a simple delivery and a low required dosis. However, they run a great risk of reverting back to virulence and disseminating, therefore they cannot be used in the environment. Oral live vaccines have been tested on fish with good results; they are optimal from the point of view of protection, administration and cost [53]. Nevertheless, its principle problem, similar to the case of vaccines utilizing attenuated viruses, is safety in terms of the ecosystem, which is enormously difficult when used as a commercial vaccine. In general, vaccines administered orally are integrated into the diet whether it be through mixture, aspersion or bioencapsulation. This kind of administration is the most simple, but the most common problem is maintaining the integrity of the vaccine when it passes through the intestine. To improve this situation, efforts have been focused on protecting vaccines from the digestive enzymes [54].

2.2.2. DNA Vaccines

Also known as genetic immunization, the most recent technology is used on fish and is based on the use of bacterial plasmids that encode a viral protein antigen whose expression is found under the control of eukaryotic elements (promoter hCMV and terminator SV40) and a gene that encodes for antibiotic resistance in plasmid construction [55]. The characterization of the etiological agent is essential to identify the key virulence factors that could help in narrowing the search of potential antigens for the construction of these third generation vaccines. DNA vaccines that are used on fish must pass through several tests *in vitro and in vivo* in order to discard possible undesired effects. With the use of genetic engineering techniques, the vaccines could be optimized to mitigate unwanted effects or on the contrary increase wanted ones. The chosen vaccine then should go through production development process, where the method of large-scale production and the delivery must be optimized. The economic cost associated with the production should be in accordance with the final cost of the potential product. The ideal vaccine should be safe for the fish, the handler and the consumer. It should have a long-term wide protection spectrum, be easy to administer, easy to manufacture, be low cost and easy to register. To date, various DNA vaccines have been tested directed towards viruses that possess DNA and RNA genomes, especially those directed against virus in the Haemorrhagic Septicemia (VHSV), the Rirame Rhabdovirus (HIRRV) and the Red Seabream Iridovirus (RSIV). These vaccines are primarily used as intramuscular injections dispersed in water. And although the development of this kind of vaccine is low cost, effective and secure, it had been a difficult task. For example, the use of DNA vaccines based on glycoprotein G from the rhabdovirus Infectious Hematopoietic Necrosis (IHN) is licensed in Canada, but cannot be used within the European Union due to problems related with biosecurity [57]. In ISAV, DNA vaccines that contain the HE gene can give protective immunity [58], which indicates that the surface glycoprotein is antigenic. However, not much is known about the antigenic variability of HE, expect that this protein presents in its C-terminal end, a highly polymorphic region (HPR). It is not known if these vaccines are capable of issuing cross-protection against different variants of ISAV, including HPR0 [59]. On the other hand, this kind of vaccine has demonstrated very low protection or has been non reproducible to be utilized commercially [58].

3. The search for effective treatments in controlling ISAV

In the case of RNA genome virus, such as orthomyxoviruses, they have been shown to possess a high mutation and recombination rate giving origin to new strains, which have impeded the success of the prevention by use of vaccines, since the new viral strains continually escape the immune response produced by the vaccine [60]. Despite the measures of control and biosecurity that have been implemented in order to control ISAV, it is a fact that the ISAV-HPR0 variant is on the rise and considering that this virus is an RNA virus with a high mutation rate, there is the danger that it could generate new variants and as a result, cause new outbreaks. Since an effective treatment does not exist, if a new ISA outbreak were to occur, the only possible alternatives would be an early harvest or the elimination of the fish, both of having huge economic repercussions within the industry. For this reason, new solutions that are capable of controlling or eradicating this virus are being looked for. Taking into consideration the commercial and ecological importance of this problem, the search for highly effective treatments is a matter of great relevance.

3.1. Candidates in the development of ISAV antiviral treatments

3.1.1. Nucleotide Analogues

To date, approximately 40 antiviral compounds have been approved for use on humans, primarily for the treatment of infections produced by the Human Immunodeficiency Virus (HIV), Hepatitis B (HBV) and herpes. However, the number of approved antivirals that can be used to treat infections caused by an RNA virus (with the exception of HIV) are very limited. Among these one can find amantadine and rimantadine (inhibitors of the M2 channel in influenza virus) [61] and oseltamivir and zanamivir (neuraminidase inhibitors) [61] and ribavirin for the treatment of the Sincitial respiratory virus (RSV), hepatitis C (HCV) and also it is being used in the treatment of Lassa fever. Ribavirin is a synthetic nucleoside, scientifically named, D-ribofuranosil-l/-/-l,2,4-triazol-3-carboxamida. Several proposed action mechanisms for ribavirine have been suggested [62], in of which dehydrogenase IMP enzyme inhibition (IMPDH) which reduce the GMP, GDP and GTP intracellular pools further reducing the mRNA capping [63]. Ribavirin is a wide spectrum antiviral agent, although it has proven to be a good inhibitor of viruses in the *Orthomyxoviridae* family. It has demonstrated positive effects in inhibiting influenza. In addition, effect has taken place in inhibiting the infective cycle of some viruses such as Viral Haemorrhagic Septicemia (VHSV), Infectious Pancreatic Necrosis (IPNV) and Infectious Hematopoietic Necrosis (IHN) [64]. In ISAV, ribavirin has shown that it can dramatically inhibit infections in the SKH-1 cells and furthermore, studies carried out on *Salmo salar* have shown that this antiviral is capable of inhibiting this virus *in vivo* as well [65]. In this case, it has been suggested that the inhibition mechanism is dual. On one hand, (it suggests) the direct inhibition of RNA-polymerase, because the removal of the antiviral does not produce a reversion of inhibitory effect, possibly indicating that ribavirin bonded to the polymerase with high affinity. On the other hand, it also suggests that the inhibition of the association of viral mRNA with ribosomes [65]. With the studies that have been done up until now, ribavirin has been proposed as a palliative treatment that alleviates the symptoms of ISA, however, it is not capable of clearing the virus.

3.1.2. Antivirals of Natural Origin (Phytotherapy)

Continuing with research that looks to develop an effective antiviral therapy in aquaculture, attempts have been made to obtain natural based compounds, that are safe, biodegradable, do not alter the environment and that possess antiviral activity. It is widely known and accepted that there are plants that possess antiviral activity, some of them, have been investigated for use in aquaculture. For example, some plants have been found to possess antiviral properties against the virus in tissue cultures [66] and others impact a virus that infects shrimp [67]. Other investigation groups [68] succeeded in controlling VHSV which affects salmon, utilizing leaf extract from olive trees (*Olea europaea*) and its active compound, oleuropein. Additionally diverse plants have been tested such as: *A. marmelos, C. dactylon, L. camara, M. charantia* y *P. amarus*. They have shown antiviral activity against White Spot Syndrome (WSS) [69]. It is probable that the active compounds can inhibit or block viral transcription in order to reduce replication in the host cells, or increase immunity of the host acting as immunostimulants. Despite having obtained positive results, the continued research of natural antivirals for salmon is barely existent. In this regard, the only patent describing a therapeutic agent of natural origin is the U.S Pat. Nº6.440.466 which provides a prophylactic and/or therapeutic composition that contains effective quantities from extracts obtained from plants *Lantena camera*, *Aegle marmelos*, *Ocimum sanctum*, *Mimosa púdica*, *Cynodon dactylon*, *Curcuma longa* y *Allium sativum,* with the option of combining with pollutants or pharmacologically acceptable excipients. Currently in Chile, studies are being conducted on the activity of antivirals derived from terpenic isolates from species of the *Heliotropium* genus. From this research, it has been determined that senecionilic ester of filifolinol accomplished a significant inhibition of IPNV replication with non-cytotoxic dose (EC50 of 160 μg/mL). The study of protein synthesis showed that this compound acts as an inhibitor of viral RNA messenger synthesis [70]. In ISAV, two studies have been conducted to evaluate the antiviral activity *in vitro* and *in vivo* of three natural flavonoid isotopes from the resin exudate of the plant specie *Heliotropium sinuatum* (*Heliotropiaceae*) against ISAV. Results showed that one of them (7-O metileriodictiol) was able to suppress the infectivity of ISAV *in vitro.* The study also showed that *in vivo* this compound protects the infected fish, maintaining a high percentage of viability. These results suggest that 7-O metileriodictiol is a good candidate for use as an antiviral therapy for ISAV in the salmon farming industry [71]. Furthermore, recent studies have been carried out using algae as natural antiviral sources against the ISAV [72].

3.2. Proposal of new strategies that could be used in treating ISAV.

3.2.1. Interference RNA

The use of RNA interference (RNAi) sequences is an innovative technology, consolidating an wide range of studies done up until now, where great advances *in vitro* and *in vivo* in controlling different viruses are shown. Studies around the intervention of Respiratory Syncytial Virus (RSV) are the most developed. DeVicenzo [73] did an *in vivo* study using artificially infected patients with RSV and as a therapy RNAi was used in a double blind random model

controlled with a placebo. The individuals were pretreated with RNAi via nasal (spray) and then exposed to RSV in the same way. This accomplished a more than 90% reduction in the acquisition of the infection and it shows that the RNAi technologies can protect and block entrance routes from the virus. This further opens up the use of this technology in countering viral infections *in vivo* for humans. In addition, there are examples of treatments on important species in aquaculture. The inhibition of the replication of RSIV, a pathogenic marine virus, has been reported in fish cell lines using RNAi against surface protein [74]. Also, control of the viruses WSS and/or the YVH (Yellow Head Virus) which infect shrimp, has been achieved [75,76,77]. Additionally, an approach *in vivo* has been described in of which RNAi against surface protein of the WSS is synthesized by bacteria, which were inactivated and introduced in the food, achieving a 68% of protection from this pathogen which in normal conditions causes a 100% mortality rate [78]. The most relevant is that in shrimp subjected to the RNAi strategy, the viral amount was reduced to undetectable levels in real time RT-PCR. This strategy would be therefore a very promising approach in treatment of ISAV. While studies exist that suggest genetic arrangements and recombination as factors which will lead to genetic evolution of ISAV generating new variants [20], it is also true that within each genome segment there are conserved zones. The genomic analysis of ISAV [20, 28], allows for the clear identification of an important number of regions that are conserved, and therefore, could be utilized as target sequences for RNAi. Nevertheless, it must be taken into account that the future of the application of RNAi technique for controlling the virus in the industry is still distant. Studies that have been carried out present many inconsistencies, suggesting that the antiviral activity by RNAi could be affected by various factors, including target genes, sequence, the delivery system and the dose. However, the primary inconvenience, and at the same time primary challenge that this technique presents, is the search for an effective delivery method of RNAi on a large scale into open aquaculture systems.

3.2.2. Small viral RNA (svRNAs)

Within the same field of research of regulatory RNAs, svRNAs are found. Previous research has shown that the genomic non-coding regions of influenza virus form fork-type structures, which are recognized by the RpRd with the involved proteins to copy and replicate the virus and give the "green light" for viral replication [80]. The svRNAs are sequences of RNA measuring from 22 to 27 nucleotides in length and correspond to 5' end of each segment of viral genome. The expression of these svRNAs correlates with the accumulation of vRNA and a change in which the activity of the RpRd changes from transcription to genome replication. Pérez y cols. [80] demonstrated that the depletion of svRNA, while not having an impact on mRNA and complementary RNA, does impact dramatically the loss of viral RNA. It is believed that the svRNA trigger the viral transcription change into replication through interactions with the viral polymerase machine. Therefore, it is proposed that these svRNAs could be a potential therapeutic target against influenza [80]. Similarly to what occurs with influenza, nucleotides that form a fork-type structure have been identified in ISAV, using a nuclear magnetic resonance data. It remains unanswered however, which proteins are relevant, if there is regulation by svRNAs present in ISAV and which are the molecular characteristics that enable the protein to recognize this RNA. Having this knowledge will facilitate the development of new approaches to interfere with the replication of ISAV in order to control the effects in salmon farming [81].

3.2.3. Antimicrobial peptides

The antimicrobial peptides (AMPs) are one of the non-specific components found in the immune system that operate as the first line of protection in many animal species, including fish. They exercise a wide spectrum of antimicrobial activity, apart from other potentials, in innate immunity and therefore, represent a promising class of antiviral agents. In order to understand the antiviral action mechanism, the latest studies carried out have indicated that this mechanism is dual, focusing both on the virion (antiviral) and also on the host cell (immunostimulant). Despite of the serious problems with viral diseases and the restrictions of the use of chemical products in aquaculture, very few reports have been attempted in evaluating the success of the antiviral activities of AMPs in fish. As a consequence, it has become unavoidable to conduct the necessary studies, in order to understand if AMPs can be utilized as model molecules for the design of antiviral pharmaceuticals, and solving the problems with viruses in the worldwide fish farming industry [82]. Although cecropin, pleurocidin, defensins and piscidines have been studied, little is known about the effect of AMPs. In general, it is known that these peptides are the most effective against enveloped viruses, as is the case with ISAV. There are studies that have shown that the direct effect of AMPs in the majority of pathogens occurs by the interruption of lipidic cellular membranes, through diverse mechanisms. In all of these models, the peptides are capable of forming transient pores or ionic channels, which can produce membrane permeation, loss of cellular contents and osmotic instability and/or the spreading of peptides to intracellular targets. Consequently, the death of the microorganism occurs. However, this only is attributed with cationic peptides. In order to explain the direct antiviral action of the AMPs, two mechanisms have been proposed: one is the inactivation of the viral particles by disturbing the lipidic components of its membranes and the second is the prevention of viral dissemination in the host cell to inhibit the fusion of the cellular and viral membrane [83]. Other studies have shown that the AMP itself can act against different viruses by way of diverse mechanisms [82]. The ability to understand how AMPs function in fish could be the starting

point in designing new DNA constructs that incorporate molecular adjuvants in the form of AMP sequences and, in turn be used as antivirals.

4. Conclusion

In the last years, ISAV has been maintained at low levels essentially due to measures of control and biosecurity that have been implemented. However, facing the latent danger that new outbreaks of the disease could occur, and considering that there have not been efficient treatment developments, it is imperative to study both the molecular biology of the virus and it is reproductive cycle in order to develop new efficient antiviral strategies that allow for the control or eradication of the infectious salmon anemia virus (Figure 2).
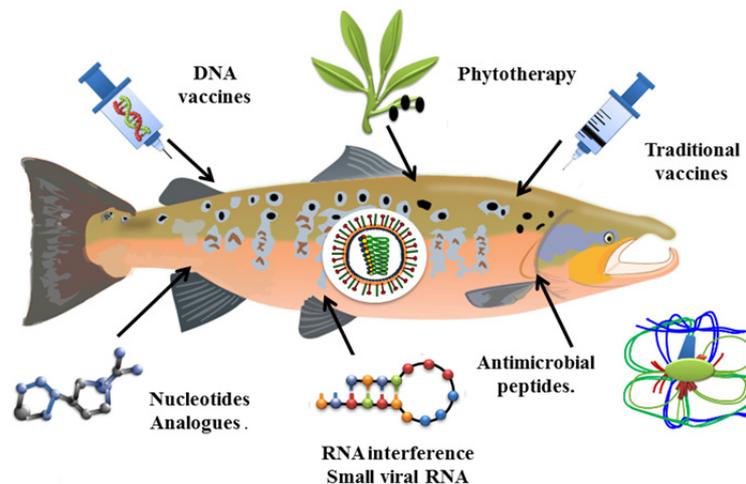
Fig. 2 Current and proposed treatments that could be used in controlling the infectious salmon anemia virus.

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