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Genetically engineered viral hemorrhagic septicemia virus (VHSV) vaccines

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ABSTRACT

Viral hemorrhagic septicemia virus (VHSV) has been one of the major causes of mortality in a wide range of freshwater and marine fishes worldwide. Although various types of vaccines have been tried to prevent VHSV disease in cultured fishes, there are still no commercial vaccines. Reverse genetics have made it possible to change a certain regions on viral genome in accordance with the requirements of a research. Various types of VHSV mutants have been generated through the reverse genetic method, and most of them were recovered to investigate the virulence mechanisms of VHSV. In the reverse genetically generated VHSV mutants-based vaccines, high protective efficacies of attenuated VHSVs and single-cycle VHSV particles have been reported. Furthermore, the application of VHSV for the delivery tools of heterologous antigens including not only fish pathogens but also mammalian pathogens has been studied. As not much research has been conducted on VHSV mutants-based vaccines, more studies on the enhancement of immunogenicity, vaccine administration routes, safety to environments are needed for the practical use in aquaculture farms.

1. Introduction

Viral hemorrhagic septicemia virus (VHSV), an enveloped, non-segmented, negative-stranded RNA virus, belongs to the genus *Novirhabdovirus* of the family *Rhabdoviridae* [1,2]. The virus was first reported from rainbow trout (*Oncorhynchus mykiss*) in Europe in the 1930's, since then VHSV has been one of the major causes of mortality in a wide range of freshwater and marine fish species worldwide, bringing significant economic damages, especially, in aquaculture farms [3–5]. The isolates of VHSV are classified into four major genotypes (I, II, III, and IV) with some sublineages in genotype I (Ia, Ib, Ic, Id, and Ie) and in genotype IV (IVa, IVb, and IVc), and there is a specific correlation between genotypes and geographic distribution [1,6,7]. The linear genome of VHSV is approximately 11 kb and consists of six genes encoding nucleoprotein (N), polymerase-associated protein (P), matrix protein (M), glycoprotein (G), non-virion protein (NV), and RNA-dependent RNA polymerase (L) [8]. The N protein tightly encapsidates RNA genome, and forms ribonucleoprotein (RNP) complex with P and L proteins to replicate the whole genome and to transcribe viral genes [9]. The M protein surrounds RNP complex and bridges nucleocapsid to the viral envelope. The viral envelope G protein plays a critical role in the infectivity of VHSV through binding to cell membrane receptor and fusion to endosomal membrane at low pH to release viral nucleocapsids into cytoplasm [10]. As the G protein is the sole target for neutralizing antibodies, the development of prophylactic vaccines against VHSV has mainly focused on the G protein.

Although various types of vaccines such as whole-virus inactivated vaccines, attenuated vaccines, recombinant G protein-based vaccines, and DNA vaccines have been tried to prevent VHSV disease in cultured fishes [4,11], there are still no commercial vaccines, which may suggest that each type of the vaccines has some drawbacks to be commercialized. Among those types of vaccines, DNA vaccines have been highly successful in the protection of fish against VHSV [12]. However, as the fish immunized with plasmid vectors are regarded as gene modified organisms (GMOs) in many countries, the use of DNA vaccines in aquaculture farms has been strongly limited. Even if a vaccine company gets an approval from the nation's agency, the perspective of consumers toward GMOs is the most important factor for the successful use of DNA vaccines in aquaculture farms. Recently, the commercial use of a DNA vaccine against salmon alphavirus (Clynav) was permitted in European Union (EU), where the fish vaccinated with Clynav were not regarded as GMOs. The case of Clynav has increased the expectation for the extended use of DNA vaccines in aquaculture farms, however, a deliberate approach is still needed for the commercialization of fish DNA vaccines. Another way to develop effective VHSV vaccines would be live viral vaccines. Inactivated and recombinant subunit vaccines induce mainly humoral immune responses, while live viral vaccines can induce both humoral and cytotoxic T cell-mediated cellular immune

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responses. The MHC class I-mediated cytotoxic T cell's antiviral response against VHSV infected cells was demonstrated in rainbow trout [13,14]. Therefore, live VHSV vaccines would be superior to conventional inactivated or recombinant subunit vaccines. The highly protective ability of naturally attenuated VHSV vaccines has been reported [15,16]. However, as the virulence mechanism of VHSV is not clearly uncovered yet and the genome sequence of VHSV can be variable according to passages, the safety of naturally attenuated VHSV-based vaccines cannot be fully guaranteed.

The application of reverse genetic technology to viruses have made it possible to produce artificially designed recombinant viruses that can be used not only to uncover the function of viral genes but also to develop attenuated or transmission defective viral vaccines [17–19]. Since not only the mutation of several nucleotides but also the knock-out or complete deletion of certain gene(s) are possible using reverse genetics, the safety of vaccines based on the reverse genetically produced recombinant viruses would be higher than vaccines based on naturally mutated, attenuated viruses. Furthermore, the generation of replication- or transmission-defective viruses using reverse genetics would be the way to highly enhance the safety of vaccines based on recombinant viruses. Various types of recombinant novirhabdoviruses such as infectious hematopoietic necrosis virus (IHNV), snakehead rhabdovirus (SHRV), and VHSV have been generated using reverse genetic method, and the availability of attenuated recombinant viruses as a live vaccine has also been investigated.

In this review, we focused mainly on the vaccines based on the reverse genetically produced recombinant VHSVs, but vaccines of other novirhabdoviral species are not included.

2. Reverse genetic methods for the rescue of recombinant VHSVs

Since Schnell et al. [20] had firstly developed a reverse genetic method to rescue rabies virus from cDNA clones encoding the viral antigenome and N, P, L genes, various recombinant viruses of several rhabdoviral species have been generated through reverse genetics. To transcribe the full antigenome of VHSV in a vector, T7 promoter has been used for most of studies. The use of a recombinant vaccinia virus that synthesizes T7 RNA polymerase [21] has been utilized for the production of fish rhabdoviruses such as IHNV, VHSV, and SHRV [22–24]. The supply of T7 RNA polymerase for the production of recombinant VHSVs can also be done by the insertion of the polymerase expressing cassette into cell's chromosome through a retroviral vector system [25] or a plasmid vector. Using this T7 RNA polymerase-expressing cells, recombinant VHSVs can be recovered by the co-transfection of cells with a VHSV full antigenome vector and helper vectors expressing N, P, L proteins. The end of the 3′ leader and 5′ trailer regions of VHSV genome can be precisely trimmed by the hammerhead ribozyme and the hepatitis δ virus ribozyme, respectively. Using these ribozymes, a strong RNA polymerase II promoter such as a cytomegalovirus (CMV) promoter-mediated transcription of the full antigenome is possible [26]. In this case, since no established cell line expressing T7 RNA polymerase is needed, recombinant VHSVs can be generated more conveniently compared to the method using T7 RNA polymerase.

To construct a VHSV full antigenome vector, full antigenome is divided into several fragments that possess restriction enzyme sites at both ends, and each fragment is ligated into one plasmid vector. However, in this method, site-directed mutations or extra DNA sequences are needed to make recombination sites, which lead to the production of recombinant viruses that are not perfectly same to original viruses in the nucleotide sequences. Although those differences in nucleotide sequences in a recombinant virus can be fixed by site-directed mutations, this process can be laborious. Another way to construct a VHSV full antigenome vector is the use of multiple overlap extension PCR or Gibson assembly, by which multiple fragments of VHSV genome can be united without any mutations or any extra nucleotides.

To produce recombinant VHSVs, cells are transfected by plasmids using lipid droplet-based reagents or electroporator. However, the choice of cell lines that are very susceptible to VHSV is an important factor for the successful rescue of recombinant VHSVs, since the transfection efficiency of fish cell lines is usually very low. Different genotypes of VHSV showed different preference for cell lines, e.g., bluegill sunfish BF-2 cells are more susceptible to genotype I, III, on the other hand, Epithelioma papulosum cyprini (EPC) cells are very susceptible to genotype IV. Therefore, in the rescue of a recombinant VHSV genotype IVa, EPC cells would be more advantageous than other cell lines. In addition to the low transfection efficiency, the four kinds of plasmids (full antigenome, N, P, and L) have to be co-transfected into a cell to rescue recombinant VHSVs. The probability of cells to get all kinds of plasmids in the nucleus would be very low, which would lead to the generation of a very low number of recombinant VHSVs in the cells. In our experiences, a relatively long time might be needed to get recombinant VHSVs from transfected cells or several passages of supernatants can help to enhance the chances of getting recombinant VHSVs.

Recently, it was reported that EPC cells passaged for a long time showed a higher susceptibility to VHSV than cells passaged for a short time [27], which suggests that the same cell lines can be different in susceptibility to VHSV according to passages or culture conditions. Therefore, finding more susceptible cell stocks can be a factor to determine the production efficiency of recombinant VHSVs. More active ways to enhance the production efficiency would be the artificial modification of cell states to give better environments for VHSV replication. Kim et al. [28] reported that the inhibition of cell apoptosis using a broad-spectrum caspase inhibitor increased the final titer of VHSV. Furthermore, CRISPR-mediated knockout of IRF9 gene in EPC cells also increased VHSV replication [29]. Consequently, the establishment of cell lines that are defect in the expression of genes involved in the inhibition of VHSV replication (e.g., genes in the pathway of type I interferon responses) can render the cells more susceptible to VHSV, which can subsequently increase VHSV titer.

3. Attenuation of VHSV by reverse genetics

Reverse genetics have made it possible to change a certain regions on viral genome in accordance with the requirements of a research. Hence, if we know the gene(s) or genomic region(s) responsible for the virulence of VHSV, we can produce safety-enhanced attenuated VHSVs through the modification of the virulence region(s) by reverse genetics. There have been many reports on the determination of VHSV virulence. Nevertheless much of those studies were based on the sequence comparison between virulent isolates and weakly virulent or avirulent isolates, through which different sequences in each N, P, NV, G, and L protein or the combination of different sequences in those proteins were suggested as the cause of differences in virulence [30–32].

Recently, production of recombinant VHSVs using reverse genetics allows analyzing the virulence mechanism of VHSV through the mutation or deletion of specific region(s) or gene(s) in a VHSV genome. The mostly studied target gene for the attenuation of VHSV is the NV gene. There have been several reports on the inhibitory activity of VHSV's NV protein against type I interferon responses [33,34], apoptosis [35], NF-κB activation [36], and the respiratory burst activity of macrophages [37]. The attenuation of VHSV using NV gene has been done through mutation(s) that can change amino acids or deletion of whole NV gene from genome. Baillon et al. [38] reported that the replacement of NV protein's R116 in virulent VHSV isolates with S116 found in weakly virulent isolates led to the production of attenuated VHSVs, which could induce protection of trout from wild virulent VHSV challenge. Ammayappan et al. [35] and Kim et al. [25] rescued attenuated rVHSV-ΔNV-EGFP genotype IVb and IVa, respectively, by replacing NV ORF with eGFP ORF, and showed the important role of NV protein in viral replication efficiency and pathogenicity. In the vaccine

experiments, olive flounder (*Paralichthys olivaceus*) immunized with the NV gene-knockout recombinant VHSV (rVHSV-ΔNV-EGFP) through either intramuscular injection or oral administration induced significantly higher protection [39,40].

The M protein of rhabdoviruses is known as a suppressor of host antiviral responses [41]. The anti-transcriptional activity of VHSV M protein was significantly reduced by the change of two amino acids (D62A E181A), and a wild-type VHSV (genotype IVb) was attenuated by the introduction of the two mutations in the viral genome using reverse genetics [42]. Kim et al. [43] reported that a wild-type marine VHSV (genotype IVa) strain that could not infect and replicate in primary cultures of rainbow trout gill cells (GECs) was able to infect and replicate in the GECs by a single amino acid mutation (I1012F) of the viral polymerase (L protein). As the positive correlation between the replication ability of VHSV strains in the GECs and in vivo virulence in rainbow trout fry (bath challenge) had been shown [44], the mutant VHSV (I1012F) might gain an in vivo virulence. In the case of N protein, Ito et al. [45] described the involvement of VHSV N protein's aa 43–46 and/or aa 168 in the different virulence of two VHSVs (genotype Ib) isolated from sea-reared rainbow trout. VHSV G protein plays an important role in the viral infection, and the involvement of G protein in the viral virulence has been suggested [30,46]. However, most of the studies on the role of G protein in VHSV virulence were conducted through the comparison of sequences among virulent and avirulent isolates [30,31,47,48], and there is no report, as far as we know, on the modification of VHSV virulence by changing G protein region using reverse genetics.

Recently, Yusuff et al. [49] recovered 8 kinds of recombinant VHSVs by the exchange of viral genes between two VHSV strains; VHSV MI03 (genotype IVb) virulent to yellow perch (*Perca flavescens*) but avirulent to rainbow trout and VHSV DK-3592B (genotype Ia) virulent to rainbow trout. In the infection experiment with rainbow trout, recombinant VHSVs based on the trout-virulent strain (DK-3592B) template induced 100% mortality, but recombinant VHSVs based on the trout-avirulent strain (MI03) template induced 0–10% mortality, indicating that virulence was not affected by the exchange of those genes.

There have been many reports on the effect of the nucleotide sequence change in the leader untranslated region of RNA viruses on the replication and virulence. Kim et al. [50,51] rescued a recombinant attenuated virus by the mutation of two nucleotides in the 3′-UTR (A4G and G5A) of VHSV (genotype IVa), and demonstrated the vaccine potential of the A4G-G5A strain by immersion in olive flounder.

In the attenuation of a VHSV isolate by reverse genetics, the knock-out of NV gene can generate attenuated recombinant VHSVs, because NV protein is dispensable for VHSV replication. Although several mutations in the structural genes or UTRs of virulent VHSVs can induce attenuation, the attenuated viruses can revert back to virulent forms because of no proof-reading activity of RNA polymerase, which would be problematic to be used as attenuated vaccines. Therefore, the whole deletion of NV gene in the genome would be a better way to produce attenuated VHSVs, which can lessen the possibility of reverting to virulent viruses.

The insertion of foreign genes into the anterior region of VHSV genome can retard the viral replication, which can be a way to attenuate VHSV. Kim et al. [52] reported that rVHSV containing green fluorescent protein (GFP) and red fluorescent protein (RFP) ORF between N and P gene and between P and M gene, respectively, retarded in vitro viral growth and severely weakened in vivo virulence in olive flounder. A slow replication of the recombinant viruses might confer time to hosts to express antiviral immune genes.

Insertion of species-specific antiviral cytokine gene into VHSV genome can also lead to the attenuation of VHSV. Kwak et al. [53] rescued olive flounder interferon-γ-expressing rVHSV by the insertion between N and P gene. Since interferon-γ acts in a species-specific way unlike to type I interferons, the growth of olive flounder interferon-γ-expressing rVHSV in EPC cells was similar to that of red fluorescent protein (RFP)-expressing rVHSV. However, in HINAE cells and in olive flounder, the interferon-γ-expressing rVHSV showed a retarded growth and weakened virulence compared to RFP-expressing rVHSV.

4. Single-cycle VHSV as vaccines

Although the attenuated VHSVs generated through reverse genetics can be considered as the safe type of vaccines compared to the naturally attenuated viruses because of the targeted modification of genome, they still possess replication ability in the vaccinated hosts, which can cause a disease in immunologically compromised individuals or can be pathogenic to non-targeted fish species living around farms. Considering reverse genetically generated recombinant VHSVs are classified as GMOs, the guarantee of safety is the most important thing to get permission for commercial uses. One way to enhance the safety of recombinant VHSVs is to produce them as single-cycle viral particles that are unable to produce transmissible viruses. As rhabdoviruses possess RNP complex in the viral particles, even in N, P or L gene-deleted VHSVs, the viral genome and each gene can be transcribed until the RNP complex become non-functional, which may lead to the production of viral particles that can transmit another cells one more time.

To produce single-cycle VHSV particles, a gene that is essential for viral transmission has to be knocked-out, and the protein encoding the knock-out gene has to be trans-supplied from cells. The G protein is essential for VHSV transmission, so the G gene knock-out can generate the single-cycle VHSV. A high vaccine potential of the G gene-deleted VHSV (rVHSV-ΔG) has been demonstrated in olive flounder that were immunized through an intramuscular route [54]. However, due to the inability to produce transmissible viruses, G gene-deleted VHSV particles would be less immunogenic than attenuated VHSVs. The addition of molecular adjuvants in the genome of single-cycle VHSV can be a way to enhance the immunogenicity of vaccines, and, recently, it was reported that the immunization of olive flounder with a G gene-deleted VHSV containing olive flounder CXCL12 ORF between N and P gene (rVHSV-ΔG-CXCL12) induced significantly higher protection and higher serum neutralization activity than fish immunized with rVHSV-ΔG-eGFP [55].

The G gene-deleted VHSV particles can infect host cells only once using viral envelope G proteins that were trans-supplied from G protein expressing cell lines, and the viral particles in the cells are unable to express G proteins due to the lack of G gene ORF in the genome. Recently, Kim and Kim [56] generated a single-cycle VHSV that can secrete G protein from infected cells through the deletion of just the transmembrane and C-terminal cytoplasmic region of G gene (rVHSV-GΔTM), and demonstrated the higher protective efficacy than rVHSV-ΔG.

One of the concerns related to the single-cycle viral particles is the possibility to revert to virulent forms by the re-gaining of a knock-out gene through the recombination with co-infected wild-type viruses, which may accelerate the disease progression. However, according to the results of Kim et al. [57], rVHSV-ΔG inhibited the replication of wild-type VHSV not only in EPC cells but also in olive flounder when simultaneously infected, which suggests that single-cycle VHSV can act as interfering viral particles. The rVHSV-GΔTM also inhibited wild-type VHSV replication like rVHSV-ΔG [56].

A bottleneck in the production of single-cycle VHSV particles is to establish a cell line that can highly express the missing gene of rVHSV, because the titer of single-cycle viral particles is largely dependent on the amount of the trans-supplied deleted protein. The transfection of cells with CMV promoter-driven expression vectors and the selection of cells with antibiotics may be the most frequently used method. However, the selected cell population usually shows a severe heterogeneity in the deleted gene expression. Furthermore, an epigenetic modulation of CMV promoter can weaken the promoter power in accordance with the number of cell passage. To overcome these drawbacks, cell cloning using a cell sorter would be a way to establish cell

populations that are highly expressing the deleted protein. The use of an inducible expression system would be better to keep the promoter power from the epigenetic modifications.

5. Attenuated and single-cycle VHSVs as vaccine vectors

Not only attenuated VHSVs but also single-cycle VHSV particles can be used as delivery vehicles for foreign antigen(s) through the insertion of the foreign gene(s) into VHSV genome. The foreign antigens can be expressed from the mutant viruses as cytoplasmic, secretory or viral envelope protein. Recombinant VHSVs displaying foreign antigen in the form of viral envelope protein can further offer the foreign antigen to immune cells through the expression of the antigen in infected cells, which may be advantageous to induce both MHC class I and II-mediated adaptive immune responses. Kim et al. [58] showed the simultaneous expression of 2 reporter genes (GFP and RFP) in the cells infected with a rVHSV that contained eGFP gene between N and P gene, RFP gene between P and M gene. Moreover, through the insertion of HIRRV G gene in front of the VHSV G gene, a recombinant VHSV expressing both viral G proteins was rescued, and was able to induce antibodies that could neutralize both viruses in olive flounder [59]. However, as the replication of recombinant VHSVs is retarded if foreign gene is inserted closer to the leader region, a long foreign gene should be inserted to the region posterior to G gene.

Recently, researches on the availability of VHSV for the platform of displaying mammalian viral antigens have been conducted, as VHSV is inactivated at temperatures higher than 20 °C. Nzonza et al. [60] rescued recombinant VHSV expressing West Nile Virus (WNV) E glycoprotein on the viral envelope using VHSV G protein's secretion signal and transmembrane C-terminal sequences. Immunization of BALB/c mice with the recombinant VHSV induced partial protection against a lethal challenge with WNV. Similarly, Rouxel et al. [61] used recombinant VHSV for a delivery vehicle of influenza virus antigens that were expressed on the surface of VHSV envelop, which conferred complete protection of immunized mice against influenza virus challenge. Although the recombinant VHSVs cannot replicate in mammals, the display of mammalian viral antigens on the VHSV envelope can be used as a tool to induce adaptive immune responses in mammals.

6. Conclusions

To fully exploit the advantages of live VHSV vaccines, immunization of fish has to be done through immersion or oral routes. Moreover, considering economic burdens of aqua-culturists related to vaccination, methods to enhance immunogenicity (that can reduce viral doses) should be developed.

Not much research has been conducted on VHSV vaccines based on reverse genetically produced mutant viruses. One of the reasons for this would be the mutant recombinant viruses are classified as GMOs. Although some artificially mutated viruses have been permitted for the use as commercial vaccines in livestock, there is no authorized GMO vaccine in aquaculture (as we mentioned, DNA vaccine itself is not GMO). Considering a high protective efficacy as prophylactic vaccines and a high applicability as combined vaccines or delivery vehicles for heterologous antigen(s), reverse genetically-produced recombinant VHSVs are fascinating items to be investigated for the development of commercial and effective vaccines. Endeavors are needed to develop methods that can guarantee the safety of attenuated-live VHSVs, single-cycle VHSVs, and VHSV vectors, and tries to change the negative perspective of consumers on GMO vaccines are also indispensable for the successful use in aquaculture farms.

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