ABSTRACT

Title: MOLECULAR BASIS OF VIRULENCE IN INFECTIOUS HEMATOPOIETIC NECROSIS VIRUS (IHNV) USING A REVERSE GENETICS APPROACH

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Infectious hematopoietic necrosis virus (IHNV) is a pathogen of major economic importance to the aquaculture industry. The long-term goal of our work is to develop a safe and effective recombinant IHNV vaccine and possibly use IHNV as a virus vector to express foreign genes. To achieve this goal, the complete genome of IHNV 220-90 virulent strain was sequenced and characterized. Subsequently, a full-length cDNA clone of IHNV was generated by constructing the full length cDNA clone, between the cytomegalovirus (CMV) promoter and the autocatalytic hammerhead and hepatitis delta virus ribozymes. Transfection of a full-length plasmid, along with the supporting plasmids resulted in the recovery of infectious rIHNV-220-90. Characterization of the rIHNV-220-90 showed that its growth characteristics in tissue culture were comparable to those of the parental virus.

The possible role of IHNV proteins in virulence was explored to some extent. For this, the entire genome of attenuated virus (IHNV-61) was sequenced and compared with its virulent strain. The comparative sequencing analysis studies revealed that majority of differences were located in the glycoprotein gene. The M and G genes, and the trailer region between virulent and attenuated viruses were exchanged; recombinant chimeric viruses were recovered and studied for their pathogenicity in rainbow trout. The results obtained from *in vivo* studies indicate that the glycoprotein plays a major role in IHNV virulence in fish, whereas the M gene and trailer region play a negligible role in virulence of IHNV. The potential of rIHNV to serve as a viral vector was explored by expressing the VP2 protein of IPNV and hemagglutinin-estrase (HE) protein of ISAV. The recovered rIHNV-VP2 and rIHNV-HE viruses stably expressed the VP2 and HE proteins respectively for at least five serial passages and showed characteristics comparable to that of the parental virus, except that there was a one-log reduction in the virus titer. These results demonstrated that the established reverse genetics system can be utilized effectively to examine the molecular determinants of virulence, pathogenesis, and new approaches for vaccine development.

MOLECULAR BASIS OF VIRULENCE IN INFECTIOUS HEMATOPOIETIC NECROSIS VIRUS (IHNV) USING A REVERSE GENETICS APPROACH

BY

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Dedication

I dedicate this work to the infectious hematopoietic necrosis virus and to the science which made my life worth doing research.

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List of Abbreviations

aa	amino acid
bp	base pair
BSA	bovine serum albumin
°C	degrees Celsius
cDNA	complementary DNA
CPE	cytopathic effect
EGFP	Enhanced green fluorescent protein
EPC	*Epithilioma papulosum cyprini*
FBS	Fetal bovine serum
g	gram
hr	hour
GE	gene-end
GFP	green fluorescent protein
GS	gene-start
HIRRV	Hirame rhabdovirus
IG	intergenic
IHNV	Infectious hematopoietic necrosis virus
IRES	internal ribosomal entry site
kDa	kilodaltons
MEM	minimum essential medium

mg	milligram
min	minute
ml	milliliter
MOI	multiplicity of infection
mRNA	messenger RNA
MV	measles virus
nt	nucleotide
nts	nucleotides
ORF	open reading frame
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PFU	plaque forming units
p. i.	post-infection
rIHNV	recombinat IHNV
RdRp	RNA-dependent-RNA-polymerase
RT-PCR	reverse transcriptase-polymerase chain reaction
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
TNT	transcription-coupled translation
SHRV	Snakehead rhabdovirus
VHSV	Viral hemorrhagic septicemia virus
VLPs	virus-like particles
VSV	vesicular stomatitis virus
µL	microliter

Chapter 1

Introduction

Infectious Hematopoietic Necrosis Virus (IHNV), a fish rhabdovirus, is the causative agent of infectious hematopoietic necrosis. IHNV causes an acute, systemic and virulent disease in salmonid species (Wolf, 1988, Tordo *et al.,* 2005). The causative virus now exists in many wild and farmed salmonid stocks in the Pacific Northwest region of North America (McAllister, 1979). The disease was first reported in 1953 in Washington state as a cause of death in sockeye salmon (Oncorhynchus nerka) (Rucker *et al.,* 1953). In 1970's, the disease eventually spread to rainbow trout fry on Honshu Island in Japan (Sano, 1976). It has also spread to Europe and some Asian countries (Bovo *et al.,* 1987; Laurencin, 1987). It now represents a major threat to aquaculture all over Europe. Clinical infections are most common in young fish, particularly fry and juveniles. Infectious hematopoietic necrosis can have a major economic impact on farms that rear young rainbow trout or salmon; the cumulative mortality rates on these farms can reach 90-95%.

IHNV is a member of the rhabdovirus family which also includes other pathogens such as vesicular stomatitis virus (VSV) and rabies virus (RV). A unique feature distinguishing IHNV from vesicular stomatitis virus (VSV) and rabies virus (RV) is a low optimal growth temperature (12-15°C), corresponding to the natural habitat of its piscine host, a slow growth rate and lower yields of progeny virus (Leong *et al.,* 1981).

Ultrastructurally, rhabdoviruses display a bullet-shaped morphology with glycoprotein spikes projecting from the viral envelope. The rhabdovirus genome consists of an unsegmented single-stranded RNA of approximately 11-12 kb with negative polarity (Hill, 1975; Kurath and Leong, 1985). The gene organization of the IHNV is 3'-N-P(M1)-M(M2)-G-NV-L-5' (Kurath *et al.,* 1985; Kurath and Leong, 1985). IHNV was reported to produce six mRNAs rather than five mRNAs as is found for the other rhabdoviruses analysed to date (Kurath *et al.,* 1985; Kurath and Leong, 1987). These five mRNAs encode the viral structural proteins; RNA polyrnerase (L), envelope glycoprotein (G), nucleocapsid protein (N), phosphoprotein (P) and matrix protein (M). The sixth IHNV mRNA encodes a unique so-called non-structural protein (NV), which is synthesized in infected cells but appears to be excluded from mature virions (Kurath *et al.,* 1985; Kurath and Leong, 1985, 1987).

A major characteristic of rhabdovirus genomes is the presence of a conserved polyadenylation sequence present at the termini of the individual protein-coding genes. These genes are separated by intergenic regions: each of them comprises a transcription termination/polyadenylation signal and a transcription initiation signal, which allows the transcription of the genes into individual mRNAs, separated by an untranscribed intergenic dinucleotide. In IHNV, the sequence AGAYAG/C(A)7 is found at the termini of the genes, which is similar to respective sequences at the ends of the N, P, M and G genes of VHSV (Bernard *et al.,* 1990; Benmansour *et al.,* 1994; Thiry *et al.,* 1990), VSV (Rose, 1980) and RV (Tordo *et al.,* 1988). Another common feature in non-segmented negative stranded RNA virus genomes is the presence of complementary nucleotide motifs at the 3' and 5' ends of the genome.

Prior to this study, complete nucleotide sequences of IHNV were available for only two strains (Morzunov *et al.,* 1995; Schütze *et al.,* 1995). Knowledge of the entire genome sequence is a prerequisite for the genetic manipulation of the virus. The ability to introduce genetic changes directly into negative-stranded RNA viral genomes has proved to have important applications in the field of molecular biology of viruses and also in the development of attenuated vaccine strains. Because of the negative-sense nature of their RNA genomes, the ability to utilize standard molecular biological techniques to study the replicative cycles of negative-sense RNA viruses was once impossible. However, major breakthroughs in reverse-genetics technology using rabies and influenza viruses allowed investigators for the first time to genetically manipulate the genomes of these important human and animal pathogens (Luytjes *et al.,* 1989; Schnell *et al.,* 1994).

The most successful approach is the plasmid-complemented virus rescue system or the reverse genetics system. This approach is based on the co-transfection of different plasmids, one encoding the viral antigenome and others encoding the viral polymerase complex (N, P and L proteins), under the control of T7 promoter. These transfections are done in permissive cells that express the T7 RNA polymerase or cells infected with a recombinant vaccinia virus, which expresses the T7 RNA polymerase. Numerous negative-sense RNA viruses have been recovered through this plasmid-complemented rescue system (rabies virus, Schnell *et al.,* 1994; vesicular stomatitis virus, Lawson *et al.,* 1995; human respiratory syncytial virus, Collins *et al.,* 1995; measles virus, Radecke *et al.,* 1995; Sendai virus, Garcin *et al.,* 1995; rinderpest virus, Baron and Barrett, 1997; parainfluenza virus, Hoffman and Banerjee, 1997; bovine respiratory syncytial virus,

Buchholz *et al.,* 1999 and Yunus *et al.,* 2001; Newcastle disease virus, Peeters *et al.,* 1999 and Krishnamurthy *et al.,* 2000; human metapneumovirus, Biacchesi *et al.,* 2004).

Using this reverse genetics system, manipulation of the negative strand RNA virus genome not only helps us to investigate the functions of the virus genes and proteins but also to insert foreign genes into viral genome. In addition, by introducing genetic mutations into individual viral genes, the function of an individual gene and its role in pathogenesis can be studied in greater detail. Several studies on rhabdovirus pathogenesis demonstrated involvement of envelope glycoproteins in the viral pathogenesis (Clarke *et al.,* 2007; Flanagan *et al.,* 2000): (i) G protein plays a major role in synthesis of neutralizing antibodies in infected animals (Boudinot *et al.,* 1998; Lorenzen *et al.,* 1990 and 2000), (ii) targeted mutations on G allow attenuation of virulence (Bearzotti *et al.,* 1995; Gaudin *et al.,* 1999; Kim *et al.,* 1994), (iii) G is the viral protein responsible for attachment to the cell membrane receptors (Bearzotti *et al.,* 1999). The glycoprotein and the matrix protein of virulent rabies virus were exchanged with counterparts of avirulent strain, which led to attenuation of virulent virus (Pulmanausahakul *et al.,* 2008). At least two functions for M protein in an IHNV infection are: down regulation of host transcription and the induction of programmed cell death (Chiou *et al.,* 2000). The glycoprotein induces neutralizing antibodies and plays a role in the virulence of fish rhabdoviruses (Kim *et al.,* 1994; Benmansour *et al.,* 1997). It was demonstrated that NV protein of IHNV is essential for replication and also for pathogenesis (Thoulouze *et al.,* 2004).

For the purpose of developing a live attenuated viral vaccine, one requires the knowledge of the molecular determinants of virulence, which may affect the host range and tissue tropism. A detailed understanding of these factors will facilitate efforts to ensure the safety of such vaccines (Vlaycheva and Chambers, 2002). Since RNA polymerases lack proofreading ability, the replication of RNA viruses is characterized by high mutation rates, which leads to rapid adaptation to their growth environment (Domingo, 1997). This has historically been exploited to generate live attenuated vaccines. On the other hand, unrecognized adaptive mutations occurring during propagation of viruses in the laboratory can be a source of misleading results and erroneous conclusions regarding the viral life cycle in the natural host. Therefore, it is of fundamental importance to understand the molecular processes of virus adaptation to particular host cells (Mandl *et al.,* 2001). Previous studies have shown that IHNV field isolates tend to lose their virulence after serial passage in cell culture (Leong, 1988). However, the molecular determinants of viral cell adaptation and attenuation have not been studied in detail. Mammalian rhabdoviruses, like most RNA viruses, are characterized by a high mutation rate (Holland *et al.,* 1982). As a consequence, they are liable to high intra-strain variability, also described as quasispecies (Holland *et al.,* 1992; Benmansour *et al.,* 1992; Domingo *et al.,* 1993). Emmenegger *et al.,* 2003 demonstrated that the mutant spectra of natural IHNV populations are very homogeneous and the overall mutant frequency of IHNV within its host is one of the lowest reported for RNA viruses. Therefore, to study the molecular determinants of IHNV virulence, a cloned virus generated by the reverse genetics approach would be ideal. Passage of recombinant virus in cell culture and characterization of its genomic sequence would allow one to

identify markers of virulence and cell adaptation. Moreover, by comparing the sequences of an attenuated and a virulent isolate, we can make chimeric viruses by exchanging the corresponding coding region(s) of the two isolates, and identify putative amino acids involved in virulence of IHNV.

Development of a reverse genetic system for IHNV would lead to the possibility of not only studying the functions of each IHNV gene in an authentic virus system, but also other aspects of basic knowledge in IHNV molecular biology. More importantly, establishment of a rescue system will create the ability to directly create mutations into the cDNA and hence help in engineering a recombinant live-attenuated vaccine candidate. This would be particularly important for IHNV infections in the US, since there are no effective vaccines currently available to control this emerging fish pathogen. This study is, thus, proposed to establish an improved rescue system for full-length IHNV strain 220-90 and to study role of IHNV proteins in pathogenesis, keeping in perspective the long term implications and advantages of using such a system in studying the molecular biology of IHNV and generating a better vaccine for control of IHNV infections in the US and other countries.

1.1 Research objectives

The specific objectives of the present study are:

1. To determine the entire genome sequences of IHNV strains 220-90, IHNV-06 (virulent, passaged 6 times in cell culture) and IHNV-61 (attenuated, passaged 61 times in cell culture)
2. To develop an improved reverse genetic system for IHNV
3. To determine the basis for virulence of IHNV by constructing chimeras between virulent and attenuated viruses and evaluating their pathogenicity in fish
4. To develop recombinant IHNV as a vector to express foreign proteins

Chapter 2

Review of literature

2.1 History, Nomenclature and Classification

According to Wolf's (1988) historical discussion, IHNV probably dates back to the late 1940's and 1950's when severe epizootics were reported in young salmon in some hatcheries in the pacific salmon- producing region of North America (Rucker *et al.,* 1953; Watson *et al.,* 1954). It is generally accepted that these early reports of probable viral diseases were caused by the same agent that was later named IHNV by Amend *et al* (1969). IHNV is an economically important pathogen of salmon and trout (Amend, 1975). The virus is enzootic in river systems throughout western North America and has spread to Asia and Europe by movement of infected fish and eggs (Winton, 1991). The associated disease is severe, with mortality approaching 100% in some outbreaks. The disease continues to significantly impact commercial and recreational fishing activities as well as efforts to rebuild threatened or endangered fish stocks (Busch, 1983; Traxler, 1986; Winton, 1991).

Initially Genus, *piscivirus*, was proposed for IHNV and other fish rhabdoviruses, viral hemorrhagic septicemia virus (VHSV) and Hirame rhabdovirus (HIRRV). Later Morzunov *et al.,* (1995) and Bjorklund *et al*. (1996) suggested the name *Aquarhabdovirus* for a new genus incorporating these viruses, to reflect their aquatic origin. The denotation *Novirhabdovirus* was first introduced in 2000 in the seventh report of the International Committee on Taxonomy of Viruses (ICTV) (Walker *et al.,* 2000).

The term "novi-" stands for the "non-virion (NV)" gene, an additional unique gene, localized between the G and L genes, which is specific for this genus (Hoffmann *et al.,* 2005). To date, all members of the *Novirhabdovirus* genus are fish pathogens.

Although IHNV is a serologically homogenous virus, it can be separated into one variable and four distinct groups based on the relative size and molecular weights of their proteins (Leong *et al*.. 1981). Winton *et al.,* (1989) further classified IHNV isolates into four serological groups using monoclonal antibody but concluded that these groups were related to geographical origin rather than host species, virulence or date of isolation. Partial or whole nucleotide sequences of G and NV genes have been used for genotyping of worldwide IHNV isolates. Analyzing these two genes, Nichol *et al*. (1995) confirmed correlation between IHNV genotypes and their geographic origin. Subsequently, partial G gene sequence analysis of 323 IHNV North American isolates revealed 3 major IHNV genogroups designated U, M and L for the upper, middle and lower portions of IHNV geographical range in North America (Garver *et al.,* 2003; Kurath *et al.,* 2003). The genogroup U includes isolates from Alaska, British Columbia, Washington coastal watersheds and the Columbia River basin; the genogroup M includes isolates from the Columbia River basin and Idaho; and the genogroup L includes isolates from California and the southern Oregon coast. Enzmann *et al*. (2005) showed that all investigated European IHNV isolates formed one clade most closely related to the M genogroup. Recent studies (Nishizawa *et al.,* 2006; Kim *et al.,* 2007) indicated that several Japanese and Korean IHNV isolates constitute new JRt (Japanese Rainbow trout) genogroup. More recently, two additional genogroups for European and Japanese isolates were identified (Enzmann *et al.,* 2005, Nishizawa *et al.,* 2006). It is interesting that the genogroup of

European isolates shared a common source with the American genogroup M (Enzmann *et al.,* 2005), while the genogroup for Japanese isolates was closely related to the American genogroup U (Nishizawa *et al.,* 2006). Thus, at present, a total of five genogroups correlating with the geographic areas has been identified among worldwide isolates of IHNV.

2.2 Virion

Virions consist of an envelope and a nucleocapsid. Virions are bullet-shaped and measure 45-100 nm in diameter; 100-430 nm in length (Hill, 1975). Surface projections are densely dispersed, distinctive spikes that cover the whole surface except for the quasi-planar end. Capsid/nucleocapsid is elongated with helical symmetry. The nucleocapsid is cross-banded with a length of 700 nm; width of uncoiled 20 nm, or 30-70 nm. IHNV is a rhabdovirus consisting of an outer protein coat and a single-stranded RNA (molecular weight, 3.7 x 10^6) core (Hsu *et al.,* 1985; Koener *et al.,* 1987; Lorenzen *et al.,* 1999; Meyers and Winton, 1996).

2.3 Genome organization

IHNV genome RNA is enwrapped in a helical form with the nucleocapsid (N) protein and packaged within the virion together with the RNA-dependent RNA polymerase large protein (L) and the phosphoprotein (P). Analysis of the Round Butte strain (from Oregon) showed that IHNV has an approximately 11-kb genome which contains 6 genes (Kurath and Leong, 1985). R-Loop mapping experiments revealed that the 6 virus genes are located along the genome in the 3' to 5' order: The gene order of IHNV is 3'- leader-N-P-M-G-NV-L-trailer-5' (Schutze *et al.,* 1996) and the proteins are;

nucleocapsid (N), polymerase-associated phosphoprotein (P or M1), matrix protein (M or M2), surface glycoprotein (G), a unique non-virion protein (NV) and virus polymerase (L) (Kurath *et al.,* 1985). The G protein is the major antigen and is responsible for the serological properties of the virus (Hill, 1975; McAllister and Wagner, 1975). The small NV protein is absent in prototype rhabdoviruses which are known to infect mammals, and is of unknown function (Kurath and Leong, 1985).

2.4 Viral proteins

Purified infectious hematopoietic necrosis (IHN) virus contains five structural proteins which were designated L, G, N, P and M. The IHN viral polypeptides have molecular weights estimated to be 157, 72, 40, 25, and 20 kiloDaltons (kDa), respectively (Mcallister and Wagner, 1975).

2.4.1 Nucleocapsid (N) protein

The N gene is the first ORF, extending from nt 175-1350, contains 391 amino acids with a deduced molecular mass of 42 kDa (Schutze *et al.,* 1995). The N protein is the major structural protein of the virus and connected tightly with the RNA to form an RNase-resistant nucleocapsid, which serves as the template for both transcription and replication to occur. During each step of the replication reaction, both plus- and minus-strand RNAs are concomitantly enwrapped by the newly synthesized nucleocapsid (N)

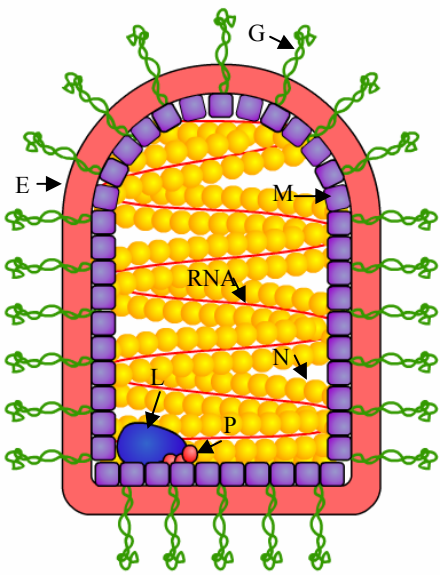

Fig. 2.1. Schematic diagram of rhabdovirus particle N: Nucleocapsid protein, P: Phosphoprotein, L: Large polymerase protein, M: Matrix protein, E: Envelope and RNA-negative-sense RNA genome. (The figure is adapted from http://www.mcb.uct.ac.za/tutorial/calgary_files/ and modified)

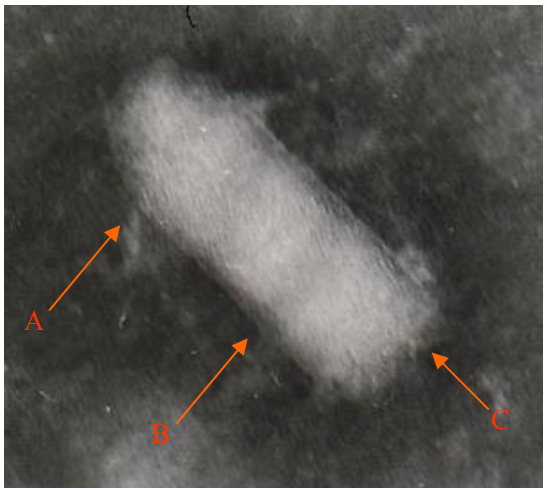

Fig. 2.2. Electron micrograph of negatively stained infectious hematopoietic necrosis virus (IHNV 220-90 strain) particles obtained from supernatant of infected EPC cells. When viewed under an electron microscope, virion is seen as bullet-shaped particles. (A) the bullet-shape of the virus. (B) tightly coiled encased genomic RNA striations (ribonucleoprotein). (C) G protein spikes in the outer membrane bilayer.

protein **(**Blumberg *et al.,* 1981; Wertz *et al.,* 1987) to form the ribonucleoprotein (RNP) complex. This encapsidated genomic RNA is also associated with the phosphoprotein P and represents the template used by the polymerase protein L to produce the six mRNAs and the full-length antigenomic RNA. Together with two major proteins, polymerase (L) and phosphoprotein (P), the genome N-RNA complex constitutes the transcribing ribonucleoprotein particle (RNP). N, P and L are the minimal set of proteins required for transcription and replication of the *Rhabdoviridae* (Stillman *et al.,* 1995). A universal phylogenetic tree of the *Rhabdoviridae* can best be constructed by using sequences of the reasonably conserved N protein gene (Hoffmann *et al.,* 2005).

2.4.2 Phosphoprotein (P) protein

The second ORF contains 690 nucleotides with a coding capacity for a 26 kDa protein consisting of 230 amino acids**.** The protein is phosphorylated to varying degrees, the most highly phosphrylated forms of which apparently have the greatest potential for supporting transcription (Emerson *et al.,* 1987; Kingsford and Emerson; 1980). It structurally stabilizes L protein to bind to the N-RNA template to form the active L-P_2 holoenzyme (Ding *et al.,* 2006). P protein forms a specific complex with N and prevents N protein from binding to cellular RNAs (Howard and Wertz 1989; Masters and Banerjee, 1988). For VSV, the P protein forms a tripartite complex with the L and N proteins to form the replicase complex to transcribe the genome and antigenome RNAs end to end with concomitant encapsidation of RNA by the N protein (Qanungo *et al.,* 2004).

2.4.3 Polymerase (L) protein

The sixth ORF is the largest gene predicted in the IHNV genome. It is capable of encoding a 1986 amino acid protein of approximately 225 kDa (Schutze *et al.,* 1995). The presence of highly conserved motifs A, B, C and D located between aa 558 and 832, which are typical for L proteins of viruses with a non-segmented single-stranded genomic RNA of negative polarity show that this ORF encodes the polymerase (L) (Tordo *et al.,* 1992). The L protein is the largest viral protein and the least abundant of all structural proteins.

2.4.4 Glycoprotein

The surface glycoprotein (G) is the antigen that determines the serological properties of a rhabdovirus (Hill, 1975). The glycosylated G proteins from VHSV and IHNV, respectively, are the targets of neutralizing antibodies (Coll, 1995). This N-glycosylated class I transmembrane protein, which forms trimeric peplomers on the virion surface (Gaudin *et al.,* 1992), exhibits several remarkable features common to all the rhabdoviruses: (i) G is the only protein responsible for the synthesis of neutralizing antibodies in infected animals (Lorenzen *et al.,* 1990; Boudinot *et al.,* 1998), (ii) targeted mutations on G allow attenuation of virulence (Bearzotti *et al.,* 1995; Gaudin *et al.,* 1999; Kim *et al.,* 1994), (iii) G is the viral protein responsible for attachment to the cell membrane receptors (Bearzotti *et al.,* 1999), and (iv) G possesses a very short cytoplasmic tail which probably interacts with other internal proteins such as N and/or M.

2.4.5 Matrix protein

The third ORF located between nts 2263 and 2850 encodes the IHNV matrix protein (M or M2). The resulting protein consists of 195 amino acids with a calculated molecular mass of 22 kDa (Schutze *et al.,* 1995). The virion contains 2000 copies of the M protein (Thomas *et al.,* 1985), which binds the nucleocapsid to the envelope and condenses the nucleocapsid into a tightly coiled helical nucleocapsid-M protein (NCM) complex that gives the virion its bullet-like shape (Barge *et al.,* 1993; Lyles *et al.,* 1996; Newcomb and Brown, 1981; Newcomb *et al.,* 1982). Within the virion, the RNP is coiled to form a tight helix and is associated with the matrix protein, which is beneath the lipid bi-layer acquired from the host. Mebatsion *et al*. (1999) have shown that the M protein of rabies virus (RV) interacts with G and is probably responsible for recruiting the G protein into the virus, necessitating the conservation of specific amino acids in the G cytoplasmic tail. For vesicular stomatitis virus (VSV), the M protein has been shown to be solely responsible for the cytopathic effect typically seen as rounding of polygonal cells in culture (Blondel *et al.,* 1990). Most recently, Ahmed and Lyles (1998) have shown that VSV M protein is capable of suppressing the transcription directed by each of the three RNA polymerases (RNAP): RNAPI, RNAPII, and RNAPIII. It was demonstrated that M acts in IHNV infection by shutting down host transcription and triggering programmed cell death (Chiou *et al.,* 2000).

2.4.6 Non-Virion (NV) Protein

The first indication of the presence of a new genus within the rhabdoviruses was the discovery of an additional gene located between the G and L encoding sequences in

IHNV (Kurath and Leong 1985; Kurath *et al*. 1985). This gene encodes a nonstructural non-virion protein, designated NV, whose product was identified in infected cells but was absent from purified virions (Schutze *et al*. 1996). In vitro translatable messenger RNA for the NV protein has been isolated from IHNV infected cells, although no corresponding protein has so far been detected in virus-infected cells (Kurath *et al.,* 1985; Kurath and Leong, 1985, 1987; Schtitze *et al.,* 1995). Genome organization is, therefore, different from those of other rhabdoviruses such as rabies virus (RV) of the genus *Lyssavirus*, where only a non-transcribed pseudogene is present between the G and L genes (Tordo *et al.,* 1986), or vesicular stomatitis virus (VSV) of the genus *Vesiculovirus*, where only genes for the five structural proteins have been detected (Rose, 1980). The function of the NV protein is not clearly known, but the conservation of an open reading frame in diverse virus species and strains may be indicative of a significant biological role. As an approach to demonstrate a biological role of NV, fish cells transiently transfected with a plasmid expressing the NV gene were found to undergo cell rounding, suggesting a possible interaction between NV protein and the cytoskeleton (Chiou *et al.,* 2000). Johnson *et al.,* (2000), applying a reverse genetics system to the snakehead rhabdovirus (SHRV), a warm-water fish *Novirhabdovirus*, have generated a recombinant virus containing a targeted nonsense mutation in the NV gene. In cell culture, this recombinant virus exhibited the same behavior as the wild-type (wt) virus, indicating that NV does not play a crucial role for in vitro replication. On the other hand, growth of NV deleted IHNV was severely impaired and the virus was non-pathogenic in fish (Thoulouze *et al.,* 2004). These results indicate that NV protein has a crucial biological role for optimal replication of IHNV in cell culture.

2.5 IHNV Replication Cycle

The rhabdoviral cycle of infection occurs by series of events in the following order: adsorption, penetration and uncoating, transcription, translation, replication, assembly and budding.

2.5.1 Attachment

IHNV is shed from infected fish and spread through water-borne contact. The most probable route of transmission is through the gills, but studies have indicated that the esophagus and cardiac stomach may also be ports of entry for the virus (Chilmonczyk and Monge, 1980; Helmick *et al.,* 1995). The first stage of IHNV replication is cellular attachment through the interaction of the viral glycoprotein G with the appropriate cell surface receptor (Coll, 1995a; Koener *et al.,* 1987; Lorenzen *et al.,* 1999). Although the IHNV cellular receptor is not known, studies conducted with other rhabdoviruses, including fish viral hemorrhagic septicemia virus (VHSV) and mammalian vesicular stomatitis virus (VSV) and rabies virus, have indicated that membrane phospholipids are involved in mediating infection (Coll, 1997; Estepa and Coll, 1996a, 1996b; Estepa *et al.,* 1999; Nunez *et al.,* 1998; Schlegal *et al.,* 1983). The phospholipid-binding region of glycoprotein G has been characterized and shown to consist of hydrophobic amino acid

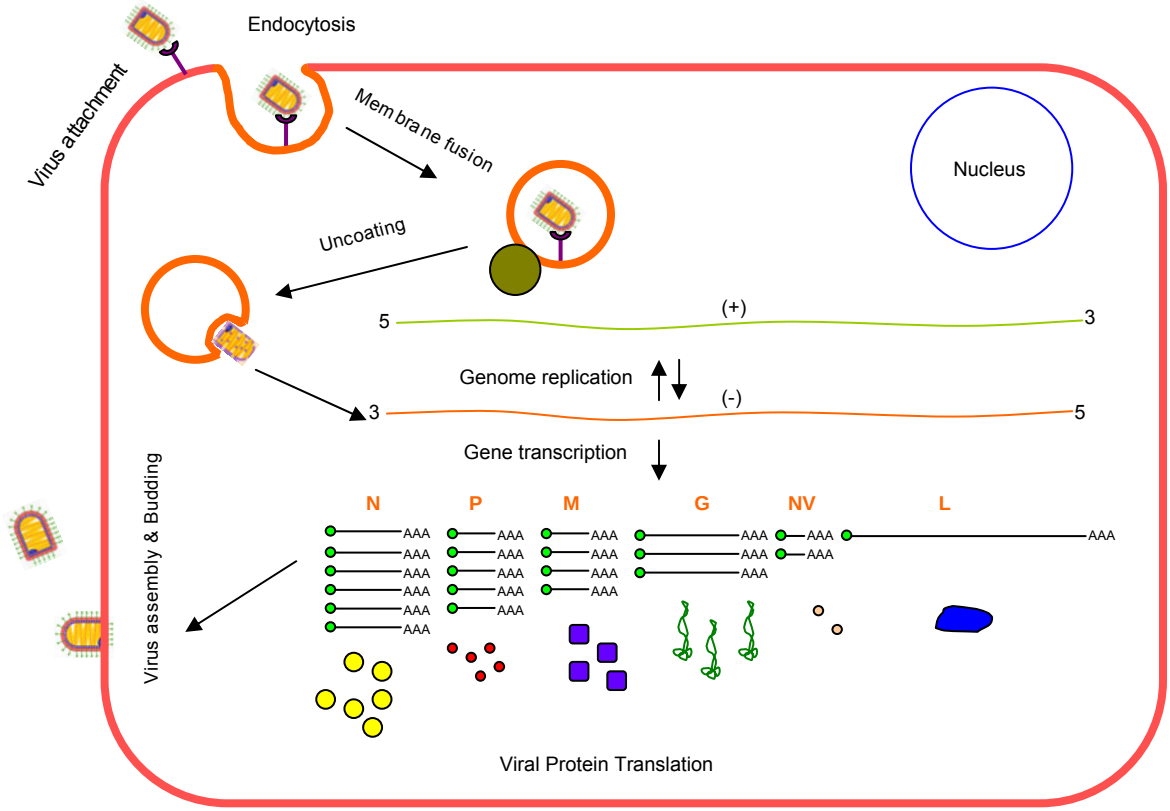

Fig. 2.3. Infectious hematopoietic necrosis virus (IHNV) life cycle. The G protein spikes bind to receptors on the surface of host cells and the viruses enter the cell by endocytosis and fusion with the membrane of the vesicle. Transcription occurs in a sequential start-stop fashion during which the polymerase transcribes the genome to produce mRNA. Six monocistronic mRNAs are produced, capped at the 5' end and polyadenylated at the 3' end and each containing the leader sequence from the 3' end of the vRNA at the 5' end of the message. Virions are assembled around the tightly coiled nucleoprotein core, and bud from the plasma membrane of the cell.

heptad repeats that bind phospholipid in a pH-dependent manner (Coll, 1995b and 1977). Bearzotti *et al*. (1999) demonstrated that antibodies recognizing fibronectin were able to block VHSV infection in fish cell cultures. They found that fibronectin specifically interacts with fish rhabdovirus and very high abundance of fibronectin in the rainbow trout muscle allowed them to hypothesize that rhabdoviruses infect fish following a two-steps: (i) passive entry of rhabdovirus into fish across the skin mucus and (ii) direct interaction to fibronectin of the superficial muscle, which is in close contact with the skin. The presence of a novel form of zebrafish fibronectin (FN2) on the cell surface increased the cell's susceptibility to infection by IHNV (Liu and Collodi, 2002).

2.5.2 Penetration

For rhabdovirus fusion with cellular membranes, the G protein trimeric spikes find and bind to their target cells and, once the viruses are endocytosed, fuse with the internal cellular membranes at low pH. However, the molecular mechanisms involved in rhabdovirus fusion are not well understood (Coll, 1999; Durrer *et al.,* 1995; Gaudin *et al.,* 1999). In many enveloped viruses, after the virus particle is internalized by a receptor-mediated endocytosis mechanism (Carneiro *et al.,* 2002), viral and cell membrane fusions are triggered by the decrease of the endosomal pH (Kielian and Jungerwirth, 1990). Conformational changes are induced by the low pH in the viral glycoproteins (Gaudin *et al.,* 1995; Weissenhorn *et al.,* 1999) to cause fusion. Uncoating occurs in the cytoplasm where replication takes place.

2.5.3 Transcription

Two modes of RNA synthesis are distinguished. Transcription starts at the 3' end of genomic RNP and involves sequential production of monocistronic mRNAs from genomic RNPs. Replication initiates at the 3' ends of both genomic and antigenomic RNPs and produces full-length RNPs. The genomic regions of IHNV and VHSV which contain the sequences for encapsidation, initiation of replication and transcription, and packaging are located at the 3’ and 5’ terminal ends of the genomes and are designated as leader and trailer, respectively. For vesicular stomatitis virus (VSV), the prototype rhabdovirus, Smallwood and Moyer (1993) and Li and Pattnaik (1999) demonstrated that optimal transcription of the genome requires the authentic 3’-terminal nucleotides which serve as a promoter region. It has long been known from studies on VSV that rhabdovirus mRNAs are sequentially transcribed starting from the 3'-terminal genome promoter (Abraham and Banerjee 1976; Ball and white 1976). Due to dissociation of the polymerase at each gene border, a progressive loss towards the 5' end is observed. This results in a gradient of transcripts following the gene order (Iverson and Rose 1981). Notably, the gene order of natural rhabdoviruses is conserved with the N and P genes needed in stoichiometric amount for RNP formation, at the first two 3' proximal positions, whereas the catalytic L protein is encoded by the most 5' terminally located gene (Conzelmann 1998; Pringle 1997). Due to transcript gradient, N mRNAs are the most abundant and L mRNAs are the least abundant viral mRNAs. This unique feature of *Mononegavirales* gene expression can modulate the level of expression of a transgene by changing the relative distance from the 3' promoter (Wertz *et al.,* 2002).

2.5.4 Replication

The viral polymerase L binds at the genomic promoter at the 3'end (leader region) of the encapsidated negative-sense genomic RNA for synthesis of subgenomic mRNAs or complete antigenomic copies of the virus genome. This is the first step in virus replication. The second step, the production of the negative-sense genome, uses the antigenomic copy of the virus genome and the antigenomic promoter at the 5'end (trailer region). Continued protein synthesis is required for the maintenance of genome replication (Pearlman and Huang, 1973). Unlike the template for primary transcription, which is the negative-strand RNP complex, the template for genome replication is the positive-strand RNP complex. The conversion of negative-strand RNP to positive-strand RNP is presumably mediated by a switch of the RNA polymerase from the transcriptive to the replicative mode. Since free positive-strand genome-length RNA is not found in infected cells, the concomitant association of the N protein with the growing positive-strand genome RNA seems to be a plausible mechanism of positive-strand nucleocapsid formation (Soria, *et al.*, 1973). The newly synthesized positive-strand RNP then serves as the template for replication, and amplification of negative-strand RNP ensues. Since the positive-strand RNP does not contain signals for transcription, this serves exclusively as a template for replication. Again, the N protein serves a vital role in the assembly of the full-length negative-strand genome RNA into RNP.

2.5.5 Assembly and Budding

The following steps outline the rhabdovirus assembly and budding (Lyles *et al.*, 1996). (i) The first step is formation of the nucleocapsid core through binding of N protein to genomic (or antigenomic) RNA while the RNA is being synthesized. N protein polymerization on the nascent RNA occurs in the cytoplasm through an exchange reaction in which P protein is released from N:P dimers as N associates with the sugarphosphate backbone of the RNA. Some cytoplasmic M protein may associate with the newly formed RNPs, but this is not sufficient for complete condensation into skeletons. (ii) While RNPs are forming in the cytoplasm, G protein in the plasma membrane localizes to, or forms, sites (i.e., microdomains) that are favorable for the initiation of budding and RNP condensation by M protein. Such sites favorable for budding must be formed soon after delivery of G to the cell surface since virus budding commences as soon as 2-3 h post-infection. (iii) When sufficient amounts of M protein have accumulated in the cytoplasm and a sub-population has localized to the inner leaflet of the plasma membrane, nucleocapsids become localized to the plasma membrane and are condensed into tightly coiled structures (skeletons) via interaction with M protein. The condensation of RNPs occurs at regions of the plasma membrane containing locally high concentrations of G protein, which results in formation of the bud site. These G-enriched microdomains may favor membrane curvature and virion extrusion. (iv) Interaction of M-RNPs with the bud site and the progressive condensation of the RNP core results in evagination of the membrane in which envelopment of the underlying condensed RNP core occurs via recruitment of both soluble and membrane-associated M

into the condensing skeleton. Thus, condensation of RNPs by M protein results in formation of bullet-shaped protrusions extending from the plasma membrane (Lyles *et al.,* 1996). (v) Cellular components, such as Nedd-4 or a related ubiquitin ligase, or other as of yet undefined proteins, associate with the PY motif of M which either directly or indirectly result in fission and release of mature virions.

2.6 IHNV infections

2.6.1 Prevalence of IHNV

The first reported epidemics of IHNV occurred in the United States at Washington and Oregon fish hatcheries during the 1950s (Rucker *et al.,* 1953). By 1973 outbreaks of IHNV in rainbow trout had occurred in Minnesota, South Dakota, Montana, Idaho, Washington, West Virginia, and Colorado (Amend *et al.,* 1973, Wolf *et al.,* 1973; Plumb, 1972). Subsequently, IHNV spread to Alaska (in 1974), throughout the Hagerman Valley to Idaho (1977 to 1980), and then was found in salmonids of the Columbia River (early 1980s), and finally was detected in the Pacific Northwest (Kurath, *et al.,* 2003). In 1971, the virus spread to Japan and subsequently to Taiwan, China, and Korea (Sano *et al.,* 1977). IHNV was detected in the common Mayfly (*Callibaetis sp*) by Shors and Winston (1989). Probably due to the expanding commercial sale of infected eggs and fish, IHNV was introduced to Europe, where it was for the first time recorded in France and Italy in 1987 (Laurencin, 1987; Bovo *et al.,* 1987), followed by detection in Belgium in 1989 and 1990, in Germany in 1992 (Enzmann *et al.,* 1992), and in Switzerland in 1993 (Knuesel *et al.,* 2003). In 1991, the first outbreaks of IHN were recorded in

hatcheries for juvenile rainbow trout and masu salmon (*O. masou*) in Kangwon Province, Korea (Park *et al.,* 1993), Japan (Kimura and Yoshimizu, 1991), and Taiwan (Chen *et al.,* 1983). The first outbreak of IHN in Austria was recognized in 1994, reported by Weber (Office International des Epizooties, [OIE]) and mentioned by Bergmann et al. (2002). IHNV was also detected in farmed rainbow trout in Iran (Fallahi *et al.* 2003) and in salmonid farm in Croatia (Vardic *et al.,* 2007).

2.6.2 Disease in Fish

The host range of IHNV includes Atlantic salmon (*Salmo salar*) (Mulcahy and Wood, 1986), chum salmon (*O. keta*), Chinook salmon (*O. tshawytscha*) (Follet *et al.,* 1987), cutthroat trout (*O. clarki*) (Follet *et al.,* 1997), Brook trout (*Salvelinus fontenalis* (LaPatra *et al.,* 1993), and brown trout (*Salmo trutta*) (LaPatra and Fryer, 1990). The disease is seen mainly in the young and juvenile stages of salmonids while the adults are more resistant and may become carriers (LaPatra, 1998). Transmission of virus was demonstrated via water, feed and contaminated eggs. It was concluded that adult carriers are the reservoir of infection and that transmission occurs primarily when carriers shed virus and expose susceptible fish or eggs (Amend, 1975). The potential for epizootics is highest at 10°C and the disease does not occur naturally above 15°C (Amend, 1970; Watson, *et al.,*1954). In cell culture, however, the virus replicates and causes cytopathic effects at temperatures up to 18°C (Amend *et al.,* 1969; Wingfield *et al.,* 1969). The principal clinical signs of disease are darkened body color, ascites, exophthalmia and petechial hemorrhages internally and externally. Severe electrolyte and fluid imbalance caused by renal failure (Amend and Smith, 1974) and degeneration and necrosis of the

hematopoietic tissues in the kidney and spleen is thought to be actual cause of mortality (OIE, 2000; Wolf, 1988). Depending on the fish species and environmental conditions, IHNV epizootics may result in mortalities of more than 90% under artificial culture conditions (Pilcher and Fryer, 1980).

2.6.3 Current control strategies

Effective vaccines have long been requested by the fish farmers, but due to limited experimental success with traditional killed or attenuated virus vaccines as well as with products based on recombinant proteins, no approved vaccines against these viruses are generally available (Lorenzen and Olesen, 1997; Winton, 1997). Amend (1976) and Nishimura *et al.,* (1985) reported having success with vaccination of rainbow trout using killed preparations of IHNV. The vaccine was most effective when delivered by injection but hyper-osmotic immersion was capable of stimulating limited immunity. Traditional strategies as well as recombinant protein vaccines have had limited success at controlling these diseases (Lorenzen *et al.,* 1999; Lorenzen and Olesen, 1997; Winton 1997). Fryer *et al.,* (1976) developed an attenuated strain of INHV by passing the virus multiple times in steelhead trout cell cultures. LD_{50} studies revealed that the attenuation reduced the virulence approximately 100-fold. This vaccine proved to be effective in eliciting protective immunity with only 5% mortality in the vaccinated group and 90% in the controls. It was also shown that one preparation was capable of giving protection when delivered by immersion; however some residual virulence was seen in some trout (Fryer *et al* 1976). These results prompted researchers to halt further experiments as the

commercial industry expressed concern about difficulties of licensing an attenuated live vaccine (Fryer *et al.,* 1976).

The DNA vaccine based on the glycoprotein gene of IHNV induces a non-specific anti-viral immune response and long-term specific immunity against IHNV (Purcell *et al.,* 2006). Genetic immunization or the direct administration of antigen-encoding DNA into animals is one of the latest technologies being exploited in the design of better viral vaccines. The administration of the DNA results in low level expression of the antigen and the subsequent induction of antigen-specific host immunity (Fynan *et al.,* 1995; Rabinovich *et al.,* 1994; Johnston and Tang, 1994; Vogel and Sarver, 1995). For viral vaccines, this approach offers many advantages: (i) The viral antigen is correctly folded and glycosylated by the host cell, (ii) The presentation of the antigen induces both humor and cellular immune responses (Ulmer *et al.,* 1993; Wang *et al.,* 1993; Xiang *et al.,* 1995), (iii) The vaccine is safe from the reversion problems inherent to live, attenuated viral vaccines. Furthermore, unlike subunit vaccines that require potentially toxigenic oil-based adjuvants to boost immunogenicity, genetic immunization requires only the delivery of properly constructed DNA plasmids to the nucleus of host cells.

2.7 Sequencing

The complete genomic sequences of IHNV were determined in 1995 in parallel by Schutze et al. (1995) and Morzunov et al. (1995). These two sequences (GenBank accession numbers X89213 and L40883, respectively) are the only complete genomic IHNV sequences available to date. Genetic analysis and phylogenetic studies of IHN viruses have previously been performed mostly on American fish samples; only a few

Asian or European IHNV isolates have been investigated thus far (Emmenegger *et al.,* 2000; Emmenegger and Kurath, 2000; Enzmann *et al.,* 2005; Garver *et al.,* 2003; Kurath *et al.,* 2003; Nichol *et al.,* 1995; Nishizawa *et al.,* 2006; Troyer *et al.,* 2000; Troyer and Kurath, 2003). These analyses exhibited surprisingly low genetic diversity within the IHNV G genes. A 303-nucleotide long part within the IHNV G gene, the so-called "mid-G" region that contains putative antigenic determinants (Huang *et al.,* 1996), has been found to be valuable for IHNV phylogenetic analyses (Emmenegger *et al.,* 2000; Emmenegger and Kurath, 2000; Garver *et al.,* 2003; Kurath *et al.,* 2003; Troyer *et al.,* 2000; Troyer and Kurath, 2003). In general, the phylogenetic relationship of IHN viruses was found to correlate with the geographic origin of virus isolates rather than with host species or with temporal factors (Troyer and Kurath, 2003). A limited correlation with host species was described by Kurath et al. (2003), and time-related divergences between Japanese isolates before and after the 1980s were observed by Nishizawa et al. (2006).

2.8 Reverse genetics

The first rhabdovirus ever recovered was rabies virus SAD B19 strain by Schnell et al. (1994) using a recombinant vaccinia virus (vTF7-3) expressing T7 RNA polymerase. After that, RC-HL strain was recovered by Ito et al. (2001) and HEP-Flurry strain by Inoue et al. (2003) using a RNA Pol II system. The other important mammalian rhabdovirus, VSV Indiana serotype, was recovered by Lawson *et al.,* 1995, Whelan *et al.,* 1995 and Harty et al., 2001. Among fish rhabdoviruses, IHNV was recovered by Biacchesi et al. (2000) and SHRV by Johnson et al. (2000) using a vaccinia virus-driven T7 RNA polymerase expression system.

As shown in Fig. 2.4, a full-length cDNA of the IHNV antigenome was assembled from subgenomic overlapping cDNA fragments and cloned in a transcription plasmid between the T7 RNA polymerase promoter and the autocatalytic hepatitis delta virus ribozyme. Recombinant IHNV (rIHNV) was recovered from fish cells at 14°C, following infection with a recombinant vaccinia virus expressing the T7 RNA polymerase (vTF7-3) and cotransfection of pIHNV together with plasmids encoding the nucleoprotein N (pT7-N), the phosphoprotein P (pT7-P), the RNA polymerase L (pT7-L), and the nonvirion protein NV (pT7-NV) (Biacchesi *et al.,* 2000).

It was demonstrated that the growth of NV-deleted recombinant IHNV (rIHNV-ΔNV) in cell culture was severely impaired but that a normal growth of rIHNV-ΔNV can be restored when NV is provided *in trans* by using fish cell clones constitutively expressing the NV protein. These results indicated that the NV protein has a crucial biological role for optimal replication of IHNV in cell culture (Thoulouze et al.., 2004). On the other hand, NV-knockout recombinant SHRV exhibited the same behavior as the wild-type (wt) virus, indicating that NV does not play a crucial role for *in vitro* replication (Johnson *et al.,* 2000). A recombinant virus expressing viral hemorrhagic septicemia virus (VHSV) G and M instead of IHNV G and M (rIHNV-Gvhsv, rIHNV-Mvhsv) was generated and was shown to replicate as well as the wild-type rIHNV in cell culture (Biacchesi *et al.,* 2001).

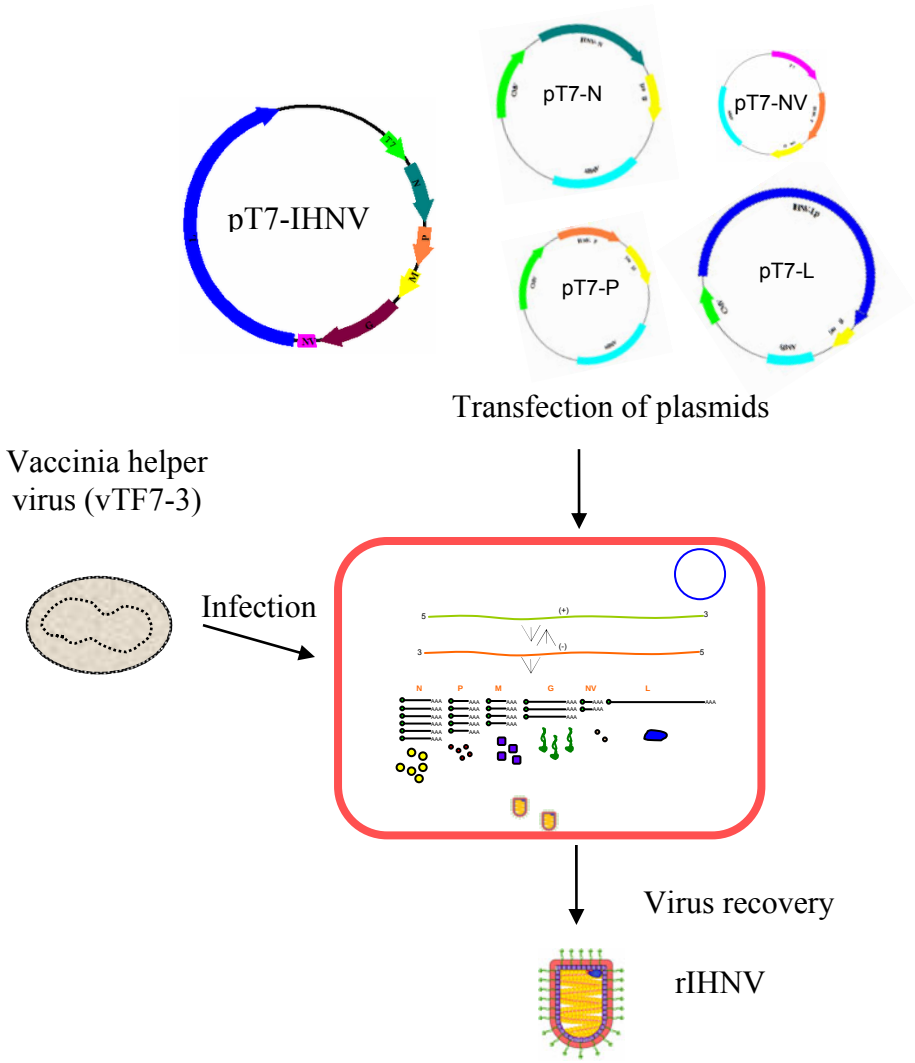

Fig. 2.4 Vaccinia virus based recovery of recombinant IHNV. EPC cells were initially infected with vaccinia virus expressing T7 RNA polymerase and followed by transfection of the antigenome plasmid along with four expression plasmids encoding N, P, NV and L proteins of IHNV.

2.9 Molecular basis of antigenic variation and virulence in IHNV

Since RNA polymerases lack proofreading ability, the replication of RNA viruses is characterized by high mutation rates. In addition, RNA viruses have large population sizes, high replication rates and short generation times. All these properties are responsible for the extremely high genetic variability of RNA virus populations (Moya *et al.,* 2000). These result in the emergence of new populations that are more adaptable to the environment, and could escape the surveillance of host immune system.

The IHNV G protein has been shown to be the major viral protein capable of eliciting neutralizing antibodies and stimulating protective immunity in young fish (Engelking and Leong, 1989). However, in recent years there appears to be evidence for increasing serological variation among IHNV isolates, with distinct neutralizing profiles (Winton *et al.,* 1989, Groberg *et al.,* 1990, LaPatra *et al.,* 1991, Ristow and Arnzen de Avila, 1991). Whether some of these variants are indeed unique serotypes has not been thoroughly investigated and this information will be essential for development of an effective vaccine. Additionally, knowledge of antigenic variation among isolates of IHNV is important for epizootiological studies, development of accurate diagnostic tests, fish health management, and understanding the biology of the virus. It will be important to determine the nucleotide sequence of the gene coding for the G protein of the antigenic variants and to determine conserved immunogenic regions that could be targeted as candidate vaccines. It will also be important to identify potential mutations that may produce major antigenic changes and to determine the ability of the virus to develop mutations to escape protective effects of a vaccine.

Viral virulence is the relative ability of a virus to produce disease or lesions in a host. It is dependent on various host and virus factors, including changes in viral tissue tropism and alteration in the level of viral replication and transcription. Changes in viral virulence resulting from mutations in viral gene products have been observed in many viral systems. Many viral genome characteristics can influence viral pathogenesis and virulence, such as viral envelope and capsid proteins; core, matrix, and nonstructural proteins; and noncoding regions of the viral genome (Tyler and Fields, 1996). A single amino aicd change from glutamic acid to glycine at position 218 in G protein of avirulent RB-1 strain of IHNV resulted in an apparent change in the distribution of the virus in fish (Kim *et al.,* 1994). This shows an altered IHNV G may affect viral pathogenesis by changing its tissue tropism. In one class of mutants, the complete loss of pathogenicity was correlated with a single amino acid change at glycoprotein position 333 from arginine to isoleucine or glutamine (Dietzschold *et al.,* 1983, Seif *et al.,* 1985). The change in pathogenicity has been attributed to changes in the distribution of RV antigen which may have affected the movement of the virus to the brain (Kucera *et al.,* 1985; Lafay *et al.,* 1991).

Sequence analysis of IHNV isolates demonstrated a maximum nucleotide diversity of 4.40%, which confirms the suggestion that the IHNV genome has undergone only a few variations (Kolodziejek *et al.,* 2008). Previous sequence comparisons carried out by Garver et al. (2003) revealed only 30 different sequence types with a maximum nucleotide diversity of 7.3% among 120 IHNV isolates from infected fish in the Columbia River. The maximum genetic diversity found throughout the geographic range of IHNV was 8.6% (Kurath *et al.,* 2003). A later study by Enzmann et al. showed that the

European isolates varied from each other in the G gene by only 0 to 2.3% (Enzmann *et al.,* 2005), whereas the maximum nucleotide diversity among the Japanese isolates was 4.5% (Nishizawa *et al.,* 2006). A possible explanation for the highly conserved genome may lie in the acute nature of IHNV infection. Most of the viral samples analyzed were collected at the endpoint of acute-phase infections, therefore, the quasispecies of the virus population could be characterized as homogenous. It is possible that IHNV populations at chronic stages of infection may be more diverse (Emmenegger *et al.,* 2003). Potential explanations for the relatively low extent of genetic variation exhibited by IHNV compared to mammalian rhabdoviruses included a lower optimal temperature and thus a slower replication rate, a relatively short duration of actively replicating virus in an infected population, a lower level of immune selection because salmonids have a less sophisticated immune system, and finally the potential presence of vector(s) and/or reservoirs of IHNV that maintain and transmit isolates over time but place additional constraints upon variation (Oshima, 1991).

2.10 Rhabdovirus vectors

Rhabdoviruses has been utilized not only to express foreign genes but also to be used as a vaccine vector. The modular nature of their genomes makes it easy to engineer foreign genes (Conzelmann *et al.,* 1998). Homologous RNA recombination has not been demonstrated for non-segmented negative-sense RNA viruses, which contributes to the stability of these vectors (Lamb and Kolakofsky, 1996; Palese *et al.,* 1996).

Recombinant vesicular stomatitis virus (VSV) based vectors expressing foreign proteins are currently being explored as vaccines. VSV has a number of advantageous

features for use as a vaccine vector. It has a very high level of gene expression as well as rapid and extremely efficient replication in vitro. In particular, the three VSV glycoprotein exchange vectors were each engineered to express the simian immunodeficiency virus (SIV) Gag protein and HIV-1 envelope protein from additional genes. Three sequential immunizations of rhesus monkeys by the combined IM and oral routes induced a vigorous HIV-specific cytotoxic T lymphocyte (CTL) response and protection against a subsequent challenge with simian-human immunodeficiency virus (SHIV) (Rose *et al.,* 2001). VSV also has been used to express the SARS-CoV S spike glycoprotein from a foreign gene and a single intra-nasal (IN) immunization of mice provided essentially complete protection against an IN challenge with SARS-CoV (Kapadia *et al.,* 2005). VSV bearing the HA glycoprotein of human influenza A virus was highly immunogenic and protective against an otherwise lethal challenge in mice (Roberts *et al.,* 1998). VSV also has been evaluated as a vaccine vector against a number of prevalent human viruses, including papillomavirus (Reuter *et al.,* 2002), hepatitis C virus (Buonocore *et al.,* 2002), HRSV (Kahn *et al.,* 2001) and measles virus (Schlereth *et al.,* 2003). VSV is a natural pathogen of cattle, horses, and swine (Letchworth *et al.,* 1999) but human infections also do occur from contact with infected animals (Fellowes *et al.,* 1955; Hanson *et al.,* 1950). The major drawback for VSV is that, at present, there is little or no experience with its administration to humans. The central nervous system involvement observed with rodents and with a human case warrants caution.

Rabies virus (RV) causes deadly neurological disease in numerous animal species and humans. Studies with mice have demonstrated the immunogenicity of RV vectors expressing the HIV envelope or Gag protein, or the SARS-CoV S protein, from added

genes (Faber *et al.,* 2005; McGettigan *et al.,* 2001a and 2001b). The immunization of rhesus monkeys with highly attenuated rabies-based vaccine vectors expressing SIV Gag protein and HIV-1 envelope protein, followed by a boost with similar vaccine constructs in which RV G protein was replaced with that of VSV, resulted in a strong HIV-specific CTL and antibody response and protection against SHIV (McKenna *et al.,* 2006). However, whether attenuated derivatives could be used in humans is unclear, given the high neurovirulence of the parent virus.

Chapter 3

Molecular characterization of the virulent infectious hematopoietic necrosis virus (IHNV) strain 220-90

3.1 Abstract

Infectious hematopoietic necrosis virus (IHNV) is the type species of the genus *Novirhabdovirus*, within the family *Rhabdoviridae*, infecting several species of wild and hatchery reared salmonids. Similar to other rhabdoviruses, IHNV has a linear single-stranded, negative-sense RNA genome of approximately 11,000 nucleotides. The IHNV genome encodes six genes; the nucleocapsid, phosphoprotein, matrix protein, glycoprotein, non-virion protein and polymerase protein genes, respectively. This study describes the molecular characterization of a virulent strain of IHNV 220-90 and its phylogenetic relationships with available sequences of IHNV isolates worldwide. The complete genomic sequence of 220-90 strain was determined from the DNA of six overlapping clones obtained by RT-PCR amplification of genomic RNA. The complete genome sequence of 220-90 comprises 11,133 nucleotides (GenBank GQ413939) with the gene order of 3'-N-P-M-G-NV-L-5'. These genes are separated by conserved gene junctions, with di-nucleotide gene spacers. The first 15 of the 16 nucleotides at the 3'- and 5'-termini of the genome are complementary, and the first 4 nucleotides at 3'-ends of the IHNV are identical to other novirhadoviruses. Sequence homology and phylogenetic analysis of the glycoprotein genes show that 220-90 is 97% identical with most of the IHNV strains. Molecular characterization of the complete genome of IHNV virulent strain 220-90 reveals its relationship with North American and other strains. It will be helpful in

studying the pathogenesis of IHNV using a reverse genetics approach and developing efficient control strategies.

3.2 Introduction

The infectious hematopoietic necrosis virus (IHNV) is probably one of the most important fish viral pathogens causing acute, systemic and often virulent disease in both wild and cultured salmon and trout (Wolf, 1988; Winton, 1991). The first reported epidemics of IHNV occurred in sockeye salmon (*Oncorhynchus nerka*) fry at Washington and Oregon fish hatcheries during the 1950s (Rucker et al., 1953; Guenther et al., 1959; Wingfield et al., 1969). IHNV is native to salmonids of the Pacific Northwest region of North America and its current geographical range extends from Alaska to northern California along the Pacific coast and inland to Idaho (Wolf, 19881; Bootland and Leong, 1999). IHNV has spread to Asia and Europe, most likely due to the movement of infected fish and eggs (Winton, 1991).

As for all the Rhabdoviridae, the genome of IHNV consists of a single-stranded negative-sense RNA. The gene order of IHNV is 3'-leader-N-P-M-G-NV-L-trailer-5' (Kurath et al., 1985). The negative-strand RNA genome is connected tightly with the nucleoprotein N and forms the core structure of virion. This encapsidated genomic RNA is also associated with the phosphoprotein P and polymerase protein L, which is involved in viral protein synthesis and replication. Their genome codes for five structural proteins, a nucleoprotein (N), a polymerase-associated protein (P), a matrix protein (M), an RNA-dependent RNA polymerase (L) and a surface glycoprotein (G), and a nonstructural protein (NV).

To date, the complete nucleotide sequence for IHNV has been determined only for two strains (Schütze et al., 1995; Morzunov et al., 1995). In this study, we characterized the entire genome of the IHNV virulent strain 220-90, which was recovered from acutely infected juvenile rainbow trout (*Oncorhynchus mykiss)* (LaPatra et al., 1991) To understand the molecular characteristics of IHNV virulent strain 220-90, we thoroughly analyzed the entire genomic sequences and compared it with other IHNV strains and rhabdoviruses.

3.3 Materials and Methods

Cells and Viruses

The IHNV strain 220-90 was kindly provided by Scott LaPatra, Clear Springs Foods Inc., Idaho, USA. IHNV 220-90 strain was initially recovered from acutely infected juvenile rainbow trout during routine examinations of hatchery-reared fish, conducted from 1990 to 1992 in the Hagerman Valley, Idaho, USA (LaPatra et al., 1991). Specimens for virus isolation were collected when mortality increased above 200 fish day^{-1}. Viruses were isolated and identified by methods previously described (Amos, 1985). The *epithelioma papulosum cyprini* (EPC) cell line from common carp *Cyprinus carpio* (Fijan et al., 1983) was used for the isolation, propagation, and identification of IHNV isolates. Cells were propagated in minimum essential medium (MEM) supplemented with 10% fetal bovine serum and 2mM L-glutamine (ATCC, Manassas, VA). For routine cell propagation, the EPC cells were incubated at 28ºC. To propagate the virus, the cells were infected and incubated at 14ºC until cytopathic effects were complete.

The supernatant was collected 5 days post-infection, clarified and stored at -80^{o}C for further processing.

RNA extraction and amplification

Viral RNA was extracted from cell culture supernatant using Qiagen RNAeasy kit, according to manufacturer's instructions (Qiagen, Valencia, CA), and stored at -20°C. The consensus PCR primers were designed using published IHNV genome sequences (Genbank accession numbers X89213; L40883) from the National Center for Biotechnology Information (NCBI). The complete genome sequences were aligned, and highly conserved sequence segments were identified and used to design overlapping primers. The oligonucleotide primers used in this study are listed in Table 3-1. First strand synthesis was carried out in a tube containing 5µl of RNA, which was denatured at 70°C for 10 min in the presence of DMSO (3µl), 1 µl forward gene-specific primer, 1µl of 25 mM dNTPs and snap-cooled on ice for 1 min. The reaction mixture containing 2µl of 10X RT buffer, 2µl of 0.1M DTT, 4µl of 25mM $MgCl_2$, 1µl of Superscript III RT^{TM}, and 1µl of RNase OUT^{TM} was incubated at 50°C for 1 h. PCR amplifications were carried out using a *pfx*50^{TM} PCR kit (Invitrogen, Carlsbad, CA), according to manufacturer's instructions. Briefly, the following mixture was used for PCR amplification: 3µ1 of cDNA, 2µl of primer mix; 5µl of 10x PCR buffer [100 mM Tris-HCl (pH 9.0), 500 mM KC1, 1% Triton X-100], 2µ1 of 25 mM $MgCl_2$, 0.5ul of *pfx*50 polymerase, and 37µ1 of DEPC water, to make a final volume of 50 µ1. Reaction was carried in a thermal cycler (MJ Research Inc., Waltham, MA), using the following program: denaturation at 94°C for 30sec; annealing for 30sec at 60°C; and extension at

68°C for 2 min. The RT-PCR products were separated by agarose gel electrophoresis and purified using a QIAquick gel extraction kit (Qiagen, Valencia, CA).

In order to identify the 3'-terminal region of the genomic RNA, poly (A) tail was added to the 3'-end with poly (A) polymerase enzyme, according to manufactures' instruction (Applied Biosystems, USA). Tailing reaction was carried in a tube containing 30µl of RNA, 26µl of nuclease-free water, 20µl of 5X poly (A) polymerase buffer, 10µl of 25 mM $MnCl_2$, 10µl of 10 mM ATP, and 4µl of *E. coli* poly (A) polymerase. The reaction mixture was incubated at 37°C for 1 hr and then RNA was purified using a Qiagen RNAeasy kit, according to manufacturer's instructions. The cDNA synthesis and polymerase chain reaction were conducted as described above, using an oligo (dT) primer (5'-GCGGCCGCTTTTTTTTTTTTTTTTTTTT-3') for the first-strand synthesis, followed by PCR with the IHNV-specific primer NheR (5'-CGTTTCTGCTAGCTTGTTGTTGG-3'). The 5'-terminal of genomic RNA was identified by rapid amplification of the 5'-end, using a 5'RACE kit (Invitrogen, Carlsbad, USA), according to manufacturer's instructions.

Cloning and sequencing

The purified RT-PCR products were cloned into a pCR2.1 TOPO® TA vector (Invitrogen, CA). Plasmid DNA from various clones was sequenced by dideoxy chain termination method, using an automated DNA sequencer (Applied Biosystems Inc., Foster City, CA). Three independent clones were sequenced for each amplicon to exclude errors that can occur from RT and PCR reactions.

Table 3-1. Primers used in this study

IHNV primers	Sequences	Position
IHNV 1F	GTATAAGAAAAGTAACTTGAC	1-21
IHNV 1R	CTTCCCTCGTATTCATCCTC	2097-2078
IHNV 2F	GCAGGATCCCAAGAGGTGAAG	2033-2053
IHNV 2R	GGAACGAGAGGATTTCTGATCC	3819-3818
IHNV 3F	CAGTGGATACGGACAGATCTC	3767-3787
IHNV 3R	CTTGGGAGCTCTCCTGACTTG	5579-5559
IHNV 4F	GTACTTCACAGATCGAGGATCG	5523-5544
IHNV 4R	CGGGGACTCTTGTTCTGGAATG	7147-7128
IHNV 5F	CGTACCAGTGGAAATACATCGG	7098-7119
IHNV 5R	CAGGTGGTGAAGTAGGTGTAG	9018-8997
IHNV 6F	GAGGGAGTTCTTTGATATTCCC	8931-8952
IHNV 6R	ATAAAAAAAGTAACAGAAGGGTTCTC	11130-11105
IHNV NheR	CGTTTCTGCTAGCTTGTTGTTGG	525-503
IHNV 1MF	ACAGAAGCTAACCAAGGCTAT	729-749
IHNV 2MF	AGATCCCAATGCAGACCTACT	2610-2630
IHNV 3MF	GTATCAGGGATCTCCATCAG	4322-4341
IHNV 4MF	GATACATAAACGCATACCACA	6113-6133
IHNV 5MF	TCAGAGATGAAGCTCAGCAA	7546-7565
IHNV 6MF	AACACCATGCAGACCATACTC	9559-9579
IHNV 5'End	CGATATTGAAGAGAAAGGAATAAC	10692-10715
Oligo (dT)	GCGGCCGCTTTTTTTTTTTTTTTTTTT	

Table 3-2. Information about the infectious hematopoietic necrosis virus (IHNV) isolates used in this study for comparison and phylogenetic analysis

S.No	Strain	Country	Host	GenBank no.
N protein				
1.	SRCV	USA	Chinook salmon	AY442517
2.	RB-76	USA	Steelhead trout	AY442516
3.	RB-1	USA	steelhead trout	U50402
4.	IHNV-PRT	Korea	rainbow trout	AY673683
5.	LWS-87	USA	Chinook Salmon	AY4425157
6.	LR-80	USA	Chinook Salmon	AY442514
7.	LR-73	USA	Chinook Salmon	AY442513
8.	HO-7	USA	Steelhead × Rainbow Juvenile	AY442512
9.	CST-82	USA	rainbow trout	AY442511
10.	Col-85	USA	Chinook	AY442510
11.	Col-80	USA	Steelhead	AY442509
12.	Carson-89	USA	Chinook	AY442508
13.	193-110	USA	Rainbow	AY442507
14.	LB91KI	USA	Salmon	AY438975
15.	Strain K	France		X73872
P protein				
16.	IHNV-PRT	Korea	rainbow trout	AY673685
17.	Strain K	France		X73872
M protein				
18.	HV7601	Japan		AB231685
19.	IHNV-PRT	Korea	rainbow trout	AY673686
20.	Strain K	France		X73872
G protein				
21.	IHNV-PRT	Korea	rainbow trout	AY673684
22.	LR-73	USA	Chinook Salmon	L40877
23.	LR-80	USA	Chinook Salmon	L40878
24.	Carson-89	USA	Chinook	L40872
25.	RB-1	USA	steelhead trout	U50401
26.	RB-76	USA	Steelhead trout	L40880
27.	193-110	USA	Rainbow	L40871
28.	HO-7	USA	Steelhead × Rainbow Juvenile	L40876
29.	LWS-87	USA	Chinook Salmon	L40879
30.	HV7601	Japan		AB231686
31.	Auke77	USA	sockeye salmon	DQ164099
32.	CST-82	USA	rainbow trout	L40875
33.	FF030-91	USA	rainbow trout	DQ164103
34.	Cro/05	Croatia	rainbow trout	EU219616
35.	Fs62/95	Germany		AY331664
36.	Fs42/95	Germany		AY331663

37.	FsVi100/96	Germany		AY331666
38.	332	Germany		AY331657
39.	FsK/88	Germany		AY331665
40.	FR0031	USA	Chinook Salmon	DQ164102
41.	SRCV	USA	Chinook salmon	L40881
42.	Col-80	USA	Chinook	L40873
43.	Col-85	USA	Steelhead	L40874
44.	RtUi02	Korea	rainbow trout	AB288207
45.	G4	Japan	rainbow trout	AF244128
46.	Strain K	France		X73872
NV Protein				
47.	IHNV-PRT	Korea	rainbow trout	AY673687
48.	LR-73	USA	Chinook Salmon	L40877
49.	LR-80	USA	Chinook Salmon	L40878
50.	Carson-89	USA	Chinook	L40872
51.	RB-1	USA	steelhead trout	U47846
52.	RB-76	USA	Steelhead trout	L40880
53.	193-110	USA	Rainbow	L40871
54.	HO-7	USA	Steelhead × Rainbow Juvenile	L40876
55.	fs8	Germany		AY780893
56.	LWS-87	USA	Chinook Salmon	L40879
57.	HV7601	Japan		AB231659
58.	CST-82	USA	rainbow trout	L40875
59.	Cro/05	Croatia	rainbow trout	EU219617
60.	Fs42/95	Germany		AY780896
61.	Fs62/95	Germany		AY780897
62.	FsVi100/96	Germany		AY780898
63.	SRCV	USA	Chinook salmon	L40881
64.	Col-85	USA	Steelhead	L40874
65.	Strain K	France		X73872
Complete genome				
66.	WRAC	USA	Chinook salmon	L40883
67.		France	rainbow trout	X89213
Rhabdoviruses Complete Genome				
68.	**Rhabdovirus**			**GenBank no.**
69.	Bovine ephemeral fever virus (BEFV)			NC_002526
70.	European bat lyssavirus (Bat)			NC_009527
71.	Northern cereal mosaic virus (Cereal)			NC_002251
72.	Lettuce necrotic yellows virus (Lettuce)			NC_007642
73.	Maize Fine streak virus			NC_005974
74.	Maize mosaic virus (MMV)			NC_005975
75.	Mokola virus			NC_006429
76.	Orchid fleck virus (OFV)			NC_009609
77.	Rabies virus			NC_001542

78.	Siniperca chuatsi rhabdovirus	NC_008514
79.	Spring viremia of carp virus (SVC)	NC_002803
80.	Sonchus yellow net virus (SYN)	NC_001615
81.	Taro vein chlorosis virus (Taro)	NC_006942
82.	Tupaia rhabdovirus	NC_007020
83.	Vesicular stomatitis virus (VSV)	NC_001560
84.	Viral hemaorrhagic septicemia virus	GQ385941
85.	Hirame rhabdovirus (HIRRV)	NC_005093
86.	Snakehead rhabdovirus (SHRV)	NC_000903

This table shows the name of IHNV strains, countries or places from which strains were isolated and their Genbank accession numbers. These data were taken from National Center for Biotechnology Information (NCBI) website.

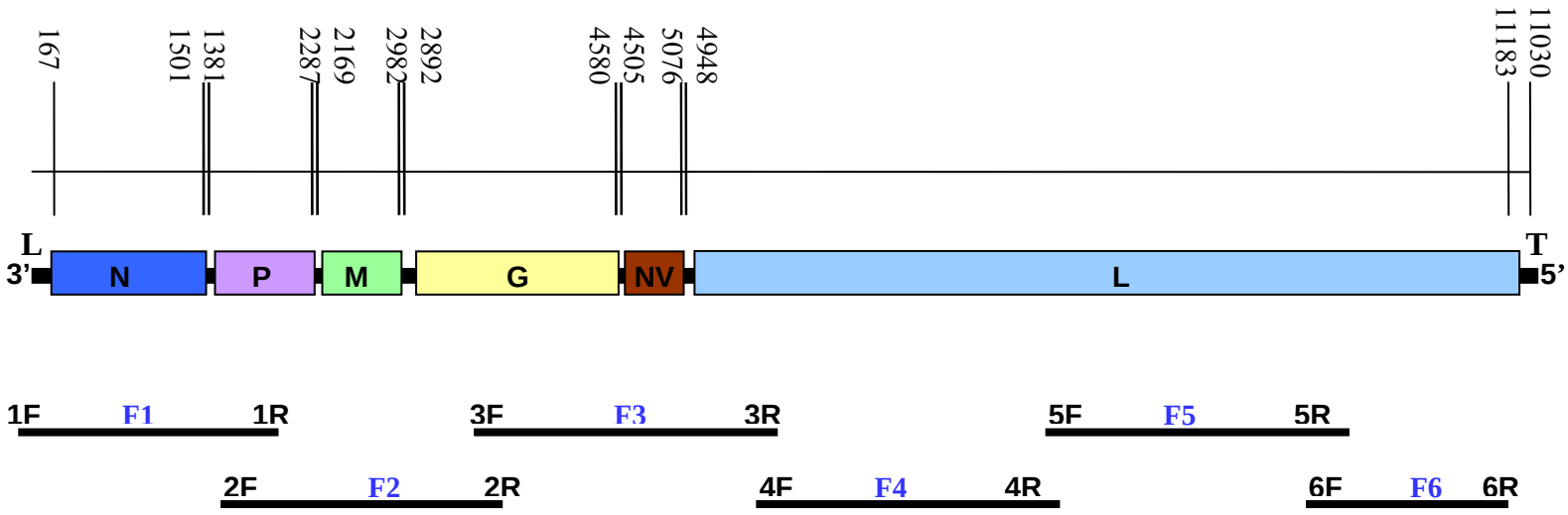

Fig. 3-1. Genetic map of IHNV genome and cDNA clones that were used for the sequence analysis. The location and relative size of the IHNV ORFs are shown; the numbers indicate the starts and ends of the respective ORFs. Six cDNA fragments (F1 to F6) were synthesized from genomic RNA by RT-PCR. The primers used for RT-PCR fragments are shown at the end of each fragment. The RNA genome is 11,133 nucleotides long and contains a leader (L) and trailer (T) sequences at its 3'-end and 5'-end respectively. The coding regions of N, P, M, G, NV and L genes are separated by intergenic sequences, which have gene-start and gene-end signals.

Sequence and phylogenetic tree analysis

The assembly of contiguous sequences and multiple sequence alignments were performed with the GeneDoc software (Nicholas et al., 1997). The pair-wise nucleotide identity and comparative sequence analyses were conducted using Vector NTI Advance 10 software (Invitrogen, CA) and BLAST search, NCBI. Phylogenetic analyses were conducted using the MEGA4 software (Tamura et al., 2007). Construction of a phylogenetic tree was performed using the ClustalW multiple alignment algorithm and Neighbor-Joining method with 1000 bootstrap replicates.

Database accession numbers

The complete genome sequence of IHNV 220-90 strain has been deposited in GenBank with the accession no. GQ413939. The accession numbers of other viral sequences used for sequence comparison and phylogenetic analysis are listed in Table3-2.

3.4 Results

The complete nucleotide sequence of 220-90

The entire genome of IHNV 220-90 strain was amplified as six overlapping cDNA fragments that were cloned, and the DNA was sequenced (Fig. 3-1). The complete genome sequence of 220-90 comprises 11,133 nucleotides (nts) and contains six genes that encode the nucleocapsid (N) protein, the phosphoprotein (P), the matrix protein (M), the glycoprotein (G), the non-virion (NV) protein, and the large (L) protein (Fig. 3-1), The gene order is 3'-N-P-M-G-NV-L-5', like other novirhabdoviruses. The genomic features and predicted proteins of 220-90 are given in Table 3-3.

Table 3-3. Genomic features and protein characteristics of IHNV 220-90

S.No	Gene	Start	End	5'UTR	ORF	3'UTR	Total Length[a]	Protein Size (aa)	MW [b]
1.	Leader	1	60				60		
2.	N	63	1430	112	1176	80	1638	391	42.3
3.	P	1433	2199	33	693	41	767	230	26.0
4.	M	2202	2945	53	588	103	744	195	22.0
5.	G	2948	4567	51	1527	42	1620	508	56.6
6.	NV	4570	4938	26	336	7	369	111	13.2
7.	L	4941	11031	76	5961	54	6091	1986	225.0
8.	Trailer	11032	11133				102		

[a] Total length of a gene including 5'UTR, ORF and 3'UTR

[b] Predicted molecular weight of proteins in kilo Dalton (kDa)

All the genes are separated by untranslated sequences that are called gene junctions. The untranslated regions at the 3' and 5' ends are called the 'leader' and 'trailer', respectively.

ORF 1 or Nucleocapsid (N) protein gene

The first ORF, extending from nts 175-1350, contains 391 residues and it encodes nucleoprotein (N) with a deduced molecular mass of 42 kDa. The N gene starts with the conserved sequence (CGUG) and has the putative polyadenylation signal (UCUUUUUUU). The 5'-untranslated region of 174 nts is followed by the first AUG codon of the 1176 nts open reading frame (ORF). Comparison of the published IHNV nucleoprotein sequences with IHNV 220-90 shows that it is 98% identical to the 193-110, HO-7 and LR-80 isolates (Table 3-4). The ORF 1 has 5' untranslated region of 112 nts (from putative gene start to AUG) and 3' untranslated region of 80 nts (from stop codon to the gene end).

ORF 2 or Phosphoprotein (P) gene

The P gene of 220-90 is 767 nts long and encodes a protein of 230 amino acids (aa) with a predicted MW of 26.0 kDa (Table 3-3). The predicted P protein contains 6 serine, 5 threonine and 1 tyrosine residues, identified as possible phosphorylation sites using NetPhos 2.0 server (http://www.cbs.dtu.dk/). The IHNV-P protein has an amino acid sequence identity of 6-16% with rhabdoviruses and among novirhabdoviruses, 35% with viral hemorrhagic septicemia virus (VHSV), 65% with Hirame rhabdovirus (HIRRV), and 30% with snakehead rhabdovirus (SHRV) (Table 3-5).

Table 3-4. Percent (%) amino acid identity of genomic proteins of *Rhabdoviridae* family members with IHNV strain 220-90[a]

Rhabdoviruses	3' Leader[¥]	N	P	M	G	NV	L	5' Trailer[¥]	Complete genome[¥]
BEFV	45	12	9	6	13	-	13	35	31
Cereal	34	13	8	6	7	-	12	31	30
Euro Bat	37	7	14	6	17	-	15	42	35
Maize	33	11	7	10	6	-	13	36	30
Lettuce	34	14	16	5	10	-	13	32	30
MMV	36	15	16	10	9	-	13	35	33
Mokola	40	6	12	7	17	-	15	47	34
Orchid		10	8	6	8	-	12		
Rabies	37	7	11	6	15	-	14	48	35
Siniperca	43	7	8	11	12	-	14	26	32
Spring	40	8	7	6	14	-	14	29	34
Taro	33	16	12	10	10	-	13	36	32
Tupaia	36	8	6	6	15	-	15	29	30
VSV	44	7	7	7	14	-	14	45	34
Yellow	35	10	9	9	8	-	13	35	30
HIRRV	**64**	**62**	**65**	**74**	**74**	**53**	**84**	**71**	**72**
SHRV	**44**	**42**	**30**	**35**	**39**	**10**	**58**	**36**	**55**
VHSV	**41**	**40**	**35**	**36**	**38**	**16**	**60**	**29**	**56**

[a]BEFV, Bovine ephemeral fever virus; Bat, European bat lyssavirus; MMV, Maize mosaic virus; Cereal, Northern cereal mosaic virus; Lettuce, Lettuce necrotic yellows virus; OFV, Orchid fleck virus; SYNV, Sonchus yellow net virus; SVC, Spring viremia of carp virus; Taro vein chlorosis virus (TaVCV); VSV, Vesicular stomatitis virus; VHSV, viral hemaorrhagic septicemia virus, HIRRV, Hirame rhabdovirus; SHRV, Snakehead rhabdovirus.

[¥] only nucleotide sequences were used for analysis

-Viruses belongs to *Novirhabdovirus* genus are in bold letters

Table 3-5. Percent (%) nucleotide or amino acid identity of IHNV untranslated regions and proteins with other IHNV strains[a]

IHNV Strains	3' Leader[¥]	N	P	M	G	NV	L	5' Trailer[¥]
193-110	-	**98**	-	-	**97**	96	-	-
332	-	-	-	-	**97**	-	-	-
Auke77	-	-	-	-	**97**	-	-	-
Carson-89	-	**96**	-	-	**97**	-	-	-
Col-80	-	95	-	-	**96**	-	-	-
Col-85	-	95	-	-	**96**	-	-	-
Cro/05	-	-	-	-	**97**	96	-	-
CST-82	-	**97**	-	-	**97**	96	-	-
G4	-	-	-	-	**96**	-	-	-
IHNV-PRT	-	93	95	98	**95**	95	-	-
FR0031	-	-	-	-	**96**	-	-	-
FF030-91	-	-	-	-	**96**	-	-	-
Fs42/95	-	-	-	-	**97**	97	-	-
Fs62/95	-	-	-	-	**97**	-	-	-
FsK/88	-	-	-	-	**97**	-	-	-
FsVi100/96	-	-	-	-	**97**	-	-	-
HO-7	-	**98**	-	-	**97**	97	-	-
HV7601	-	-	-	**98**	**97**	97	-	-
J04321	-	95	-	-	-	-	-	-
LB91KI	-	**96**	-	-	-	-	-	-
LR-73	-	95	-	-	**97**	96	-	-
LR-80	-	**98**	-	-	**97**	97	-	-
LWS-87	-	**96**	-	-	**97**	-	-	-
WRAC	96	**97**	**98**	**98**	**97**	96	98	96
RB-76	-	**96**	-	-	**97**	-	-	-
RB-1	-	**96**	-	-	97	97	-	-
RtUi02	-	-	-	-	94	-	-	-
SRCV	-	95	-	-	**96**	-	-	-
Strain K	-	**97**	**97**	**98**	**97**	97	98	-
X89213	96	**97**	**97**	**98**	**97**	97	98	95

[a] more than 95% identities are shown in bold letters

[¥] only nucleotide sequences were used for analysis

ORF 3 or Matrix (M) gene

The M gene of 220-90 is 744 nts long and encodes an M protein of 195 aa residues with a predicted MW of 22.0 kDa (Table 3-3). The M protein has an amino acid sequence identity of 5-10% with rhabdoviruses and among novirhabdoviruses 36% with VHSV, 74% with HIRRV, 35% with SHRV (Table 3-5). A 5'-untranslated region of 53 nts is followed by an ORF and succeeded by 103 nts 3' UTR. The deduced amino acid sequence of 220-90 M protein was compared with the other rhabdoviruses and the result is shown in Table 3-5.

ORF 4 or glycoprotein (G) gene

The gene for the G protein is located between 2948 and 4567 nts from the 3'-end of the viral genome. A 3' UTR of 51 nts is followed by an ORF (nts 1524) that encodes a polypeptide of 508 aa residues, with a calculated MW of 56.6 kDa, and succeeded by 42 nts 3' UTR. The predicted G protein contains 20 serine, 6 threonine and 6 tyrosine residues, identified as possible phosphorylation sites using NetPhos 2.0 server (http://www.cbs.dtu.dk/). Four putative N-glycosylation sites were identified at amino acids 56-59 (NASQ), 400-403 (NNTT), 401-404 (NTTI) and 438-441(NETD) and one O-glycosylation were identified at amino acid position 492. We compared the G protein of 28 IHNV strains from different parts of the world. The regions between amino acid positions 32-52, 131-204, 289-369, 380-416 are highly conserved. The regions between amino acids 247-257 and 269-276 have a greater genetic diversity than any other part of the G protein. The IHNV glycoprotein has the following domains: signal peptide at N-terminal (1-20aa), ectodomain (21-459aa), transmembrane domain (460-482 aa) and

endodomain (483-508 aa), which were predicted by SignalP server (http://www.cbs.dtu.dk/services/SignalP/).

ORF 5 or Non-virion (NV) protein gene

The NV protein gene is located between 4570 and 4938 nts from the 3'-end of the viral genome. It encodes a polypeptide of 111 aa residues, with a calculated molecular mass of 13.2 kDa. The predicted NV protein contains 1 serine, 2 threonine and 1 tyrosine residues, identified as possible phosphorylation sites using NetPhos 2.0 server (http://www.cbs.dtu.dk/). The function of NV protein is not clearly known. NV is a non-structural protein of novirhabdoviruses, which could be detected only in cell culture infected with the virus (Kurath and Leong, 1985).

ORF 6 or Polymerase (L) gene

ORF 6 encodes the largest protein, the polymerase, which starts at position 5017 and ends at position 10977. It encodes a polypeptide of 1986 aa residues, with a deduced molecular mass of 225.0 kDa. The L protein contains 67 serine, 38 threonine and 9 tyrosine residues as possible phosphorylation sites. The predicted RNA-dependent RNA polymerase (RdRp) domain is situated between residues 18 and 1159. The deduced L protein of IHNV is only 12-15% identical to the L protein of other rhabdoviruses, and it exhibits very close identity with other novirhabdoviruses [60%, 84%, and 58% identities with VHSV, HIRRV and SHRV, respectively (Table 3-5)].

Table 3-6. Comparisons of the gene junctions of the IHNV genome with that of other Novirhabdoviruses

Type Species	Gene Junctions		
	N/P	**P/M**	**M/G**
IHNV	**UCU**A**UC**U**UUUUUUU** AC **CGUG**AUAUCACG	**UCU**G**UC**U**UUUUUUU** AC **CGUG**CGUUCACA	**UCU**G**UC**U**UUUUUUU** AC **CGUG**AAAACACG
SHRV	**UCU**A**UC**U**UUUUUUU** GC **CGUG**CUCUCACG	**UCU**G**UC**U**UUUUUUU** AC **CGUG**CUCUCACG	**UCU**G**UC**U**UUUUUUU** AC **CGUG**CUCUCACG
VHSV	**UCU**A**UC**U**UUUUUUU** GC **CGUG**CUAAUAUU	**UCU**A**UC**U**UUUUUUU** GC **CGUG**CUGACAAG	**UCU**A**UC**U**UUUUUUU** AC **CGUG**UAAACACA
HIRRV	**UCU**A**UC**U**UUUUUUU** AC **CGUG**CAAACACA	**UCU**A**UC**U**UUUUUUU** AC **CGUG**CAAUCACA	**UCU**A**UC**U**UUUUUUU** AC **CGUG**UAAACACA
	G/NV	**NV/L**	
IHNV	**UCU**G**UC**U**UUUUUUU** GC **CGUG**UAAACACG	**UCU**A**UC**U**UUUUUUU** AC **CGUG**AAAACACG	
SHRV	**UCU**G**UC**U**UUUUUUU**U GC **CGUG**AUAUCACG	**UCU**A**UC**U**UUUUUUU** GC **CGUG**CAUUACACG	
VHSV	**UCU**A**UC**U**UUUUUUU** AC **CGUG**GAAAUACU	**UCU**A**UC**U**UUUUUUU** AC **CGAG**AAAACAAC	
HIRRV	**UCU**A**UC**U**UUUUUUU** GC **CGUG**UAUACAGA	**UCU**A**UC**U**UUUUUUU** AC **CGUG**AACACACG	

The gene junctions shown here are negative sense RNA sequences of respective viruses. IHNV, Infectious haematopoietic necrosis virus; SHRV,Snakehead rhabdovirus; VHSV,viral hemaorrhagic septicemia virus; HIRRV, Hirame rhabdovirus

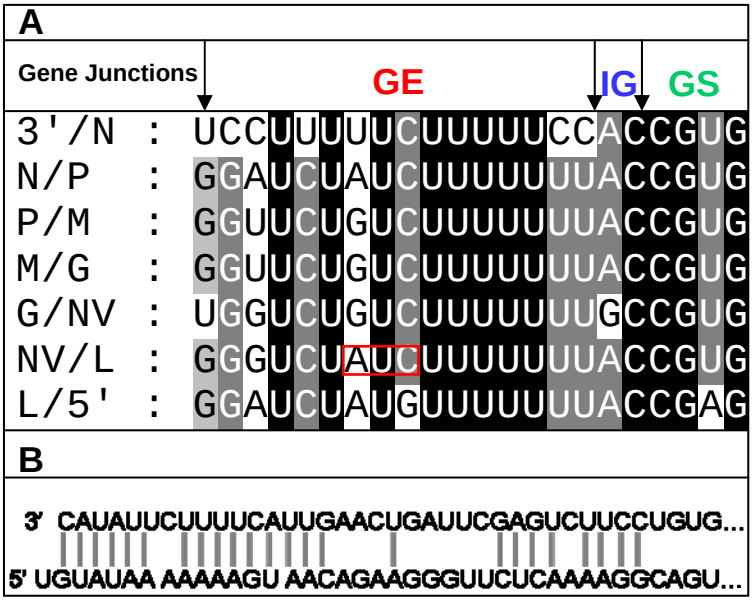

Fig. 3-2. Comparisons of the gene junctions of the genome of the IHNV 220-90

A) The seven identified gene junctions of IHNV in the negative sense of the genomic RNA are shown. 3'/N-at the junction of 3'leader and nucleocapsid gene; N/P- at the junction of nucleocapsid and phosphoprotein gene; P/M- at the junction of phosphoprotein and matrix gene; M/G-at the junction of matrix and glycoprotein gene; G/NV- at the junction of glycoprotein and non-virion gene; NV/L-at the junction of non-virion and polymerase gene; L/5'- at the junction of polymerase gene and 5' trailer. GE-Gene end; IG-Intergenic di-nucleotide; GS-Gene Start. The stop codon of NV ORF is merged with gene end sequence and is shown in red box.

B) Complementarity of the 3'- and 5'-ends of the IHNV genome. The genomic RNA sequences are shown. The first 15 out of 16 nucleotides of 3' end terminus are complementary with 5'end terminus nts. There is an extra uracil (U) residue at the 5' termini of the RNA

The Genomic termini and untranslated sequences

Rhabdoviruses have conserved untranslated regions between open reading frames for optimal translation of viral proteins (Schnell et al., 1996). These sequences consist of a putative transcription stop/polyadenylation motif ($UCURUCU^7$) which signals reiterative copying of the U sequences to generate poly (A) tail to the mRNA. This sequence is followed by an intergenic di-nucleotide AC or GC which are not transcribed, and a putative transcription start signal, CGUG (Fig 3-2A). The gene junctions of different novirhabdoviruses are shown in Table 3-6.

The untranslated region of 3' leader and 5' trailer are 60 nts and 102 nts in length, respectively. The 3'leader of 220-90 is 63% A/T rich, whereas 5' trailer is 60% A/T rich. Like other rhabdoviruses, the genomic termini of IHNV 3'-terminal nucleotides exhibit complementarities to the nucleotides of 5'-terminus of the genomic RNA (Fig 3-2B). The complementary nature of genomic termini involves the formation of a panhandle structure, which is important for replication of rhabdoviruses. We found an additional uracil (U) nucleotide at the 5'-end of the genome (Fig 3-2B).

Homology and phylogenetic analysis

Phylogenetic trees were generated from the nucleotide sequences of the ORFs and of the complete genome. The complete genome and gene proteins of IHNV were also compared with different members of *Rhabdoviridae* family. The identities of 220-90 strain with rest of the rhabdoviruses were determined by Vector NTI program and the results are shown in Tables 3-4 and 3-5. Among novirhabdoviruses, HIRRV is closely related to IHNV and has an identity of 72%. Comparison of the UTRs and protein coding

sequences of 220-90 strain with novirhabdoviruses shows that non-virion protein is highly variable than any other region of the genome (Table 3-5). The 3'- and 5'- UTRs are more conserved among *Rhabdoviridae* family members than protein coding genes (Table 3-5). The complete genome comparison of 220-90 with other two available sequences of IHNV strains reveals 96% identity with WRAC, and 95% with French strain (X89213).

The phylogenetic tree analysis of sequences of nucleocapsid (N), matrix (M), phosphoprotein (P), and non-virion protein (NV) of various IHNV strains are shown in Fig. 3-3. Phylogenetic analysis of the N gene shows clustering of 220-90 with HO-7, 193-110 and LR-80 and maintains 98% identity with those strains. Among the available sequences, WRAC strain exhibits very close identity (98%) with 220-90 for both P and M genes. All the strains display 98% identity with the 220-90 M gene, which demonstrates the highly conserved nature of M gene. When the NV genes were compared, 220-90 strain shows 95-97% identity with other IHNV strains. Previously, the North American IHNV isolates were genogrouped as U, M and L based on glycoprotein sequences (Kurath et al., 2003). Phylogenetic tree of the G genes displays that 220-90 strain belongs to the M genogroup (Fig.3-4).

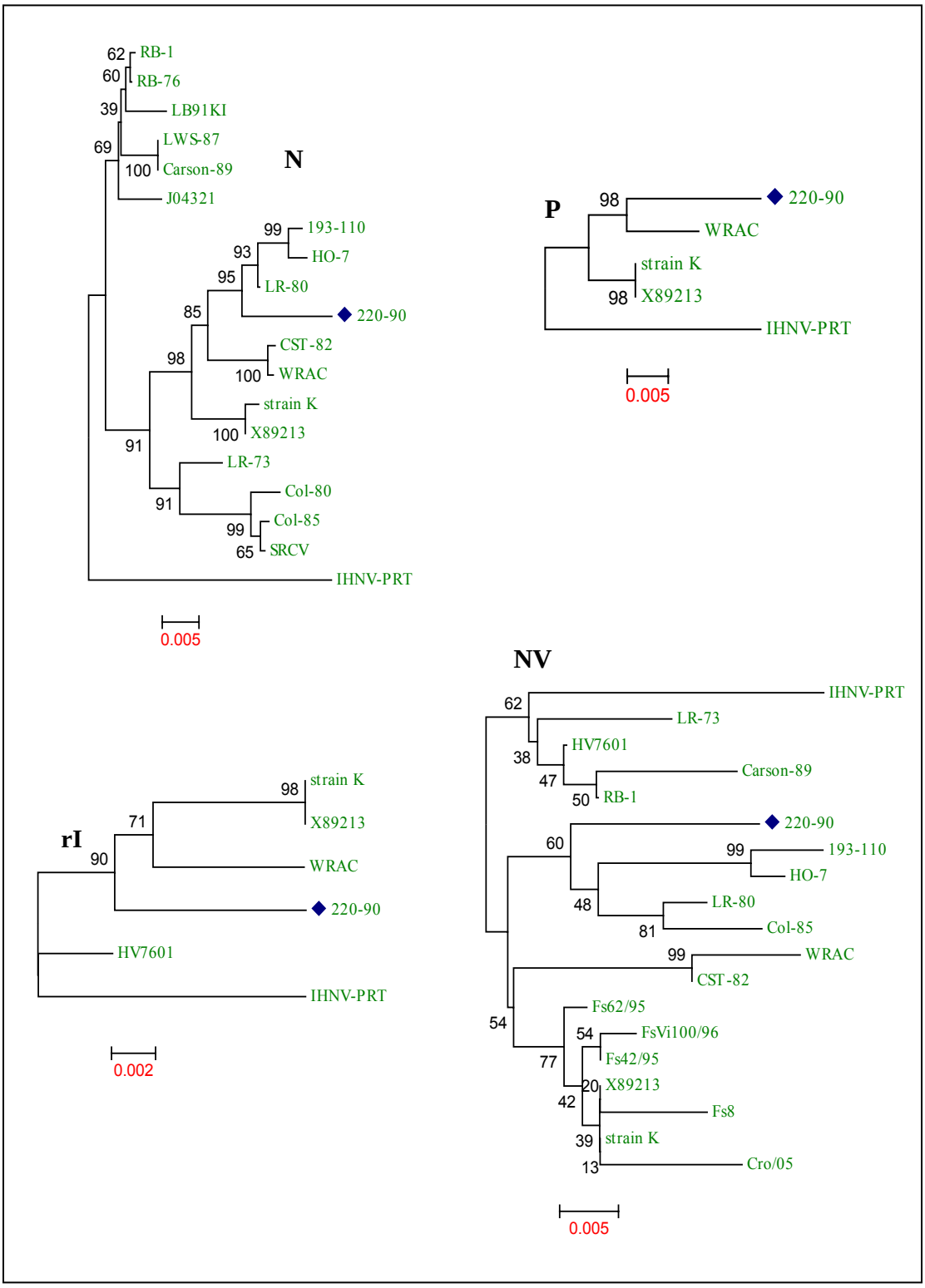
N
62
60
39
69
100
RB-1
RB-76
LB91KI
LWS-87
Carson-89
J04321
99
93
95
85
98
100
91
91
99
65
193-110
HO-7
LR-80
220-90
CST-82
WRAC
strain K
X89213
LR-73
Col-80
Col-85
SRCV
IHNV-PRT
0.005
P
98
98
220-90
WRAC
strain K
X89213
IHNV-PRT
0.005
rI
98
71
90
strain K
X89213
WRAC
220-90
HV7601
IHNV-PRT
0.002
NV
62
38
47
50
60
48
99
81
99
54
54
77
20
42
39
13
IHNV-PRT
LR-73
HV7601
Carson-89
RB-1
220-90
193-110
HO-7
LR-80
Col-85
WRAC
CST-82
Fs62/95
FsVi100/96
Fs42/95
X89213
Fs8
strain K
Cro/05
0.005

Fig. 3-3. Phylogenetic tree analysis of sequences of nucleocapsid (N), matrix (M), phosphoprotein (P), and non-virion protein (NV) of various IHNV strains. Information about the IHNV strains used in this analysis is described in Table 3-2. IHNV 220-90 strain is marked with blue diamond. Phylogenetic tree analysis was conducted by neighbor-joining method using 1000 bootstrap replications. The scale at the bottom indicates the number of substitution events and bootstrap confidence values are shown at branch nodes.

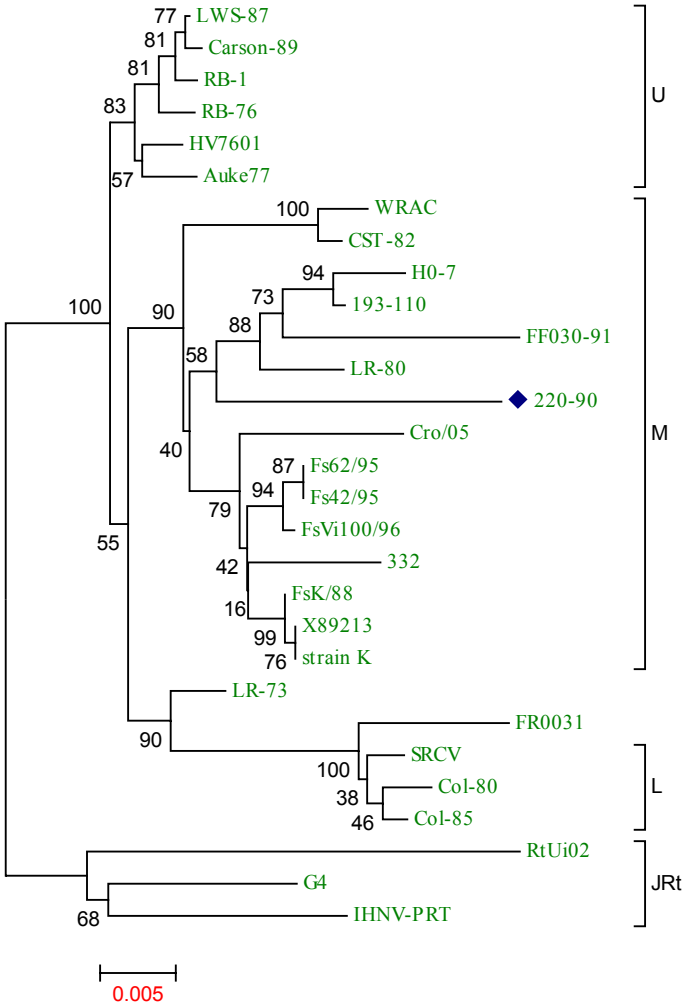

Fig. 3-4. Phylogenetic relationship of full-length glycoprotein (G) sequences of 28 IHNV strains with that of 220-90**.** Genogroups are depicted by vertical lines as described by [15]. Brackets indicate the three major genogroups, U, M and L. IHNV 220-90 (blue diamond) is grouped under M genogroup. Data of virus isolates used here are available in Table 3-2. Phylogenetic tree analysis was conducted by neighbor-joining method using 1000 bootstrap replications. The scale at the bottom indicates the number of substitution events and bootstrap confidence values are shown at branch nodes.

3.5 Discussion

A virulent IHNV strain 220-90 was isolated from the hatchery-reared juvenile rainbow trout during 90's in the Hagerman Valley, Idaho, USA (LaPatra et al., 1991). IHNV is endemic throughout the Pacific Northwest region of North America, with range extending from Alaska to northern California along the Pacific coast and inland to Idaho. It causes systemic disease in both wild and cultured salmon and trout (Wolf, 1988; Winton, 1991). The disease typically occurs in rainbow trout fry maintained in the multiple outdoor rearing units of rainbow trout farm facilities (LaPatra et al., 1991).

To date, the complete genome sequences are available for only two IHNV strains (Schütze et al., 1995; Morzunov et al., 1995). Previously, only the G protein gene sequence for 220-90 strain was determined. To fully understand the molecular characteristics of a virulent IHNV, we determined the complete nucleotide sequence of 220-90 strain. The genome is 11,133 nts long and the gene organization (N, P, M, G, NV and L) is similar to all members of the *Novirhabdovirus* genus. The termini of the viral genome have conserved sequences at the 3'-end (CAUAU) and at the 5'-end (GUAUA) as other members of *Novirhabdovirus* genus. Out of first 16 nucleotides of the 3'-terminus, 15 nucleotides are complementary to 5'-terminus of the genome (Fig 3-2B), which forms the panhandle structure that may be involved in replication (Wertz et al., 1994). We found an additional uracil (U) nucleotide at the end of the 5'-trailer region, which was not reported previously in IHNV. The length of the 3'-leader of 220-90 is 60 nts, which is similar to HIRRV but slightly shorter than VHSV and SHRV (53 nts). IHNV has the second longest 5' trailer (120 nts) than other novirhabdoviruses, such as

VHSV (116 nts), SHRV (42 nts), and HIRRV (73 nts). Even though the length of 3'-leader is consistent between the members of genus *Novirhabdovirus*, the length of the 5'-trailer is highly variable (from 42nt to 116nt). It is possible that the difference in the length of trailer sequences may have some functional significance, which remains to be seen.

All the genes of VHSV start with a conserved gene start sequence (-CGUG-) like other novirhabdoviruses, followed by an ORF and conserved gene-end sequence (A/GUCUAU/ACU^7). All the genes end with 7 uracil (U) residues, which are polyadenylation signal for polymerase when it transcribes a gene. Polymerase adds poly (A) by stuttering mechanism (Banerjee et al., 1987). After this poly (A) signal, there are two conserved intergenic di-nucleotides (G/AC), which are untranscribed and act as spacers between two genes. Polymerase skips these two nucleotides to next gene start sequence and starts transcribing next gene (Banerjee et al., 1987). Transcription of rhabdovirus mRNAs is regulated by cis-acting signals located within the 3′ leader region and untranslated region between each gene ORF (Banerjee et al., 1987; Barr et al., 1997, 2001; Whelan and Wertz, 1999). In case of NV, the stop codon of NV gene is merged with gene-end sequences (Fig 3-2A). Transcription of rhabdovirus mRNAs is regulated by cis-acting signals located within the 3′ leader region and untranslated region between each gene ORF (Banerjee et al., 1987; Barr et al., 1997, 2001; Whelan and Wertz, 1999). The Kozak context for each gene was compared, as shown in Fig. 3-5. At position -3, all the genes have adenosine (A) nucleotide, except the ORF of N gene.

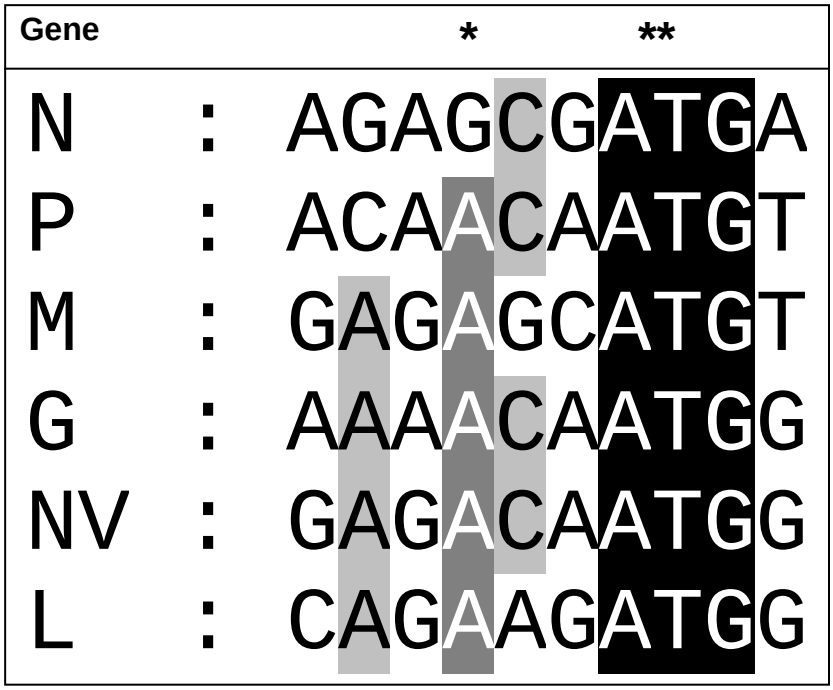

Fig. 3-5. Kozak context of each gene of IHNV 220-90

Sequences shown here are positive sense antigenome.

* Conserved adenosine (A) at position -3

** Start codon (ATG)

We observed that aa residues between 1-22, 106-150 and 206-268 are highly conserved in the N protein, whereas residues 30-31, 41-43, 177-181, 203-205 and C-terminal region from residue 312 are variable. Phylogenetic analysis of the N protein shows grouping of 220-90 with LR-80, HO-7 and 193-110 strains, with an identity of 98%. Phylogenetic tree of the P protein shows clustering of 220-90 with WRAC strain, having an identity of 98%. The matrix (M) protein is an important structural component of virion, forming a layer between the glycoprotein containing outer membrane and the nucleocapsid core. Matrix protein of IHNV is highly conserved (Table 3-4). IHNV strains used in this study exhibit very close (98%) identity with 220-90. In phylogenetic analysis of M protein, WRAC, strain K and French (X89213 strains form a cluster, which is 99-100% identical to each other, and 98% identical to 220-90. Matrix protein of rhabdovirus is involved in viral assembly, condensation of nucleocapsid, formation of bullet-shaped virion (Newcomb and Brown, 1981; Mebatsion et al., 1999) and induces apoptosis by shutdown of host cell machinery in infected cells (Finke and Conzelmann, 2005; Kassis et al., 2004). Because it is highly essential for assembly and release of virion, the matrix protein maintains highest homology among IHNV along with the polymerase protein.

The non-virion protein (NV) of 220-90 shows identity of 95-97% with other IHNV strains. The NV protein of IHNV is conserved than counterpart of VHSV, which showed high genetic diversity (Ammayappan and Vakharia, 2009a). It was demonstrated that NV-knockout IHNV replicated very slowly in cell culture and was non-pathogenic in fish (Thoulouze et al., 2004). On the contrary, NV-knockout SHRV replicated very well as wild-type virus and it was shown that NV protein of SHRV is not essential for pathogenesis (Alonso et al., 2004). These studies suggested that each species of

Novirhabdovirus genus has its own characteristics and one can not ignore the importance NV in pathogenesis. The conserved nature of NV and its importance for growth and pathogenesis suggests that NV is highly essential for IHNV. The L protein displays the highest level of sequence homology among members of various genera of *Rhabdoviridae* family (Table 3-5). All the available L sequences for IHNV strains show highest conservation (98%) as that of matrix protein. The L protein is packaged into the virus particle and is involved in both transcription and replication (Banerjee et al., 1987).

Genomic comparison of IHNV strains isolated from various marine species from different parts of the world sheds light on the correlation of genetic sequences with viral tropism and pathogenicity. The glycoprotein (G) is believed to be involved in virulence and tropism because it's involvement in viral attachment and cell entry (Bearzotti et al., 1995). Comparison of glycoproteins of various IHNV strains has shown long blocks of conserved region (data not shown). The regions between residues 8-22; 32-52; 131-214; 289-369; and 380-416 are highly conserved and the rest is showing genetic variations, which are scattered all over the protein. The major neutralizing epitopes have been mapped to two antigenic sites for IHNV, at amino acid residues 230-231 and 272-276 (Huang, 1993; Kim et al., 1994). In this analysis, we found no amino acid substitutions at positions 230-231 among 28 strains compared. On the other hand, residues 270-276 are highly variable, which supports earlier findings (Huang, 1993; Kim et al., 1994), and suggests that the involvement of this site in antigenic variation and virulence.

A wide sequence analysis of mid-G region (303 nts) within the glycoprotein gene of 323 North American IHNV isolates revealed a maximum nucleotide diversity of 8.6%, indicating low genetic diversity overall for this virus (Kurath et al., 2003). The North American IHNV isolates are genogrouped as U, M and L by phylogenetic analysis and these genogroups vary in topography and geographical range (Kurath et al., 2003). The phylogenetic analysis of the glycoprotein of 220-90 (Fig. 3-4) shows clustering with LR-80, FF030-91, 193-110 and HO-7 strains, which exhibits that 220-90 belongs to the M genogroup.

Chapter 4

Efficient recovery of infectious hematopoietic necrosis virus (IHNV) using a vaccinia-virus-free reverse genetics system

4.1 Abstract

Reverse genetics system is a powerful tool to study the function of a particular gene. The currently available reverse genetics system for *Novirhabdovirus* is based on vaccinia-driven T7 RNA polymerase expression. An efficient system for recovery of infectious hematopoietic necrosis virus (IHNV) entirely from cloned cDNAs was developed utilizing cellular RNA polymerase II machinery for transcription. A full-length cDNA clone of IHNV, flanked by hammerhead ribozyme and hepatitis delta ribozyme sequences, was assembled in an expression plasmid under the control of cytomegalovirus (CMV) promoter. Transfection of this full- length plasmid along with supporting plasmids (N, P, NV and L) into the *epithelioma papulosum cyprini* (EPC) cells resulted in the recovery of recombinant IHN virus. The authenticity of the recovered recombinant virus was confirmed by the presence of artificially-introduced restriction sites in the genome. A recombinant IHNV expressing a foreign gene - enhanced green fluorescent protein - was also recovered successfully. The recombinant IHNVs showed similar growth characteristics as the parental virus in cell cultures. The newly developed vaccinia virus-free reverse genetics system described for IHNV is highly efficient and applicable for the recovery of any *Novirhabdovirus.*

4.2 Introduction

Infectious hematopoietic necrosis virus (IHNV) is a negative-sense RNA virus belonging to the *Novirhabdovirus* genus of the *Rhabdoviridae* family (Walker et al., 2000). The genome of IHNV is composed of approximately 11-kb, single-stranded RNA which contains six genes (Kurath and Leong, 1985) and are located along the genome in the 3' to 5' order: 3'-N-M1-M2-G-NV-L-5', nucleocapsid protein (N), polymerase-associated phosphoprotein (P or M1), matrix protein (M or M2), surface glycoprotein (G), a unique non-virion protein (NV) and virus polymerase (L) (Kurath et al., 1985).

A method of recovering negative-strand RNA viruses from the full-length cDNA clones was first developed with rabies virus by Schnell et al., (1994). This method involved expressing a full-length positive-strand (anti-genomic) RNA copy of the virus genome under the control of T7 RNA polymerase (T7 RNAP) promoter. The genome was expressed in a cell line along with the viral nucleocapsid (N), phosphoprotein (P) and polymerase (L) proteins. Abundance of T7 RNA polymerase was supplied by the recombinant vaccinia virus (vTF7-3) expressing T7 RNA polymerase (Fuerst et al., 1986). After this recovery, many negative-strand viruses were recovered based on similar technique, namely vesicular stomatitis virus (VSV) (Lawson et al., 1995), measles virus (Radecke et al., 1995), Sendai virus (Garcin et al., 1995), respiratory syncytial virus (Collins et al., 1995), parainfluenza virus (Hoffmann and Banerjee, 1997), rinderpest virus (Baron and Barrett, 1997), Newcastle disease virus (Peeters et al., 1999) and many other viruses.

To avoid helper virus, researchers developed cell lines which constitutively express T7 RNAP. One of these cell lines was first used successfully for the recovery of measles virus from cDNA (Radecke et al., 1995). Later, the BSR-T7/5 (BHK-21 cell line) was generated to express T7 RNAP in cell culture (Buchholz et al*.,* 1999) and it was used successfully to recover bovine respiratory syncytial virus, and many other viruses, such as Newcastle disease virus (Romer-Oberdorfer et al., 1999), VSV (Harty et al., 2001) and rabies virus (Ito et al., 2003).

In the meantime, a different kind of reverse genetics system was developed which employed cellular RNA polymerases for transcription of viral cDNA. This system was initially developed for the influenza A virus (Neumann et al., 1999; Fodor et al., 1999; Hoffmann et al., 2000) which utilized a cellular RNA polymerase I. These systems were completely free of T7 RNAP and had significantly improved the recovery and study of the negative-strand RNA viruses. Use of autocatalytic ribozymes for efficient cleavage and production of exact termini was initially demonstrated by Le Mercier et al. (2002), for negative-strand minigenome. They demonstrated that extra nucleotides at the viral distal end affected the efficiency of viral rescue. Effectiveness of RNA polymerase II system, in conjunction with ribozymes [(hammerhead ribozyme (HHRz) and hepatitis delta virus ribozyme (HdvRz)], to rescue *Mononegavirales* was very well demonstrated by Martin et al., (2006) for Borna disease virus (BDV) and measles virus (MV). This system was utilized efficiently to recover RNA viruses; Thogoto virus (Wagner et al., 2001), rabies virus (Inoue et al., 2003), and infectious bursal disease virus (Qi et al., 2007).

For the recovery of rhabdoviruses, transfection of a full-length cDNA plasmid itself is not sufficient. To initiate viral replication cycle, minimal viral proteins namely, the nucleocapsid (N) protein, the phosphoprotein (P) and the viral RNA polymerase (L) should be provided in the form of supporting plasmids, along with the full-length cDNA plasmid (Schnell et al., 1994). A first reverse genetics for *Novirhabdovirus* was developed by Johnson et al., (2000), for snakehead rhabdovirus (SHRV) which was followed by the recovery of IHNV by Biacchesi et al., (2000). These systems utilized a vaccinia virus expressing T7 RNAP to rescue the recombinant viruses. Here, we have developed a system to rescue *Novirhabdovirus*es, which is completely free of vaccinia helper virus and T7 RNAP. In the present study, to generate recombinant IHNV (rIHNV) virus, we constructed a plasmid containing full-length cDNA copy of the virulent IHNV (strain 220-90), which is flanked by HHRz and HdvRz sequences. The viral cDNA is under the control of cytomegalovirus (CMV) immediate-early promoter and is transcribed by cellular RNA polymerase II upon transfection. This system is more efficient and convenient to recover any *Novirhabdovirus.*

4.3 Materials and methods

Cells and virus

A virulent 220-90 strain of IHNV, isolated from rainbow trout, was kindly provided by Dr. Scott LaPatra, Clear Springs Foods Inc., Idaho, USA (LaPatra et al., 1991). RNA was extracted from this virus and used for cloning of the viral genome as well as individual N, P, NV and L genes. The *epithelioma papulosum cyprini* (EPC) cells were used for virus propagation (Fijan et al., 1983). The cells were grown at 28°C in minimal

essential medium (MEM) supplemented with 10% fetal bovine serum and 2 mM L-glutamine. For preparation of virus stocks, confluent EPC cells were infected with the wild-type IHNV at a multiplicity of infection (MOI) of 0.001 in MEM with 2% fetal bovine serum. After 1 h of adsorption, the inoculum was removed, and the cells were incubated at 14°C until extensive cytopathic effect (CPE) was observed. The supernatant was collected 5 days post-infection (p.i), clarified and stored at -80^{o}C for further processing.

RNA extraction and amplification

Viral RNA was extracted from cell culture supernatant, using a Qiagen RNAeasy kit according to manufacturer's instructions, and stored at -20°C. The oligonucleotide primers used in this study are listed in Table 4-1. First strand synthesis was carried out in a tube containing 5µl of RNA, which was denatured at 70°C for 10 min in the presence of DMSO (3µl), 1 µl forward gene-specific primer, 1µl of 25 mM dNTPs, and snap-cooled on ice for 1 min. The reaction mixture containing 2µl of 10X RT buffer, 2µl of 0.1M DTT, 4µl of 25mM $MgCl_2$, 1µl of Superscript III RT^{TM}, and 1µl of RNase OUT^{TM} was incubated at 50°C for 1 h. PCR amplifications were carried out using a *pfx*50^{TM} PCR kit (Invitrogen, CA), according to manufacturer's instructions. Briefly, the following mixture was used for PCR amplification: 3µ1 of cDNA, 2µl of primer mix; 5µl of 10x PCR buffer [100 mM Tris-HCl (pH 9.0), 500 mM KC1, 1% Triton X-100], 2µ1 of 25 mM $MgCl_2$, 0.5ul of *pfx*50 polymerase, and 37µ1 of DEPC water, to make a final volume of 50 µ1. Reaction was carried out in a thermal cycler (MJ Research Inc., Waltham, MA), using the following program: denaturation at 94°C for 30sec; annealing for 30sec at

Table 4-1. Primers used in this study

Primers	Sequences[a,b,c]	Position
IHNV Primers		
IHNV 1F	GTATAAGAAAAGTAACTTGAC	1-21
IHNV PstR	ATCCTCCT*CTGCAG*CCCGAAT	2083-2063
IHNPstF	ATTCGGG*CTGCAG*AGGAGGAT	2063-2083
IHNSnaBR	CGAGATCTG*TACGTA*TCCACTG	3788-3767
IHNSnaBF	CAGTGGA*TACGTA*CAGATCT	3767-3786
IHNV 3R	CTTGGGAGCTCTCCTGACTTG	5579-5559
IHNV 4F	GTACTTCACAGATCGAGGATCG	5523-5544
IHNKpnR	GAAT*GGTACC*ATCCCGATGTAT	7132-7111
IHNKpnF	ATACATCGGGAT*GGTACC*ATTC	7111-7132
IHNV 5R	CAGGTGGTGAAGTAGGTGTAG	8997-9017
IHNV HDVR	GAGATGCCATGCCGACCCGTGTATAAAAAAAGTAAC	5'end
IHNV HDVF	GTTACTTTTTTTATACACGGGTCGGCATGGCATCTC	5'end
T7tNotR	*GCGGCCGC*ATGCTTTCAGCAAAAAACC	
IHNV HHF1	AG*ACTAGT*CTTTTCTTATACCTGATGAGTCCGTGAGGACG AAAC	
IHNV HHF2	GAGCTCGTTTAGTGAACCGCTTTTCTTATACCTGATGAG TCCGTGAGGACGAAAC	
Supporting plasmid primers		
IHNV N EcoF	*GAATTC*GCCACCATGACAAGCGCACTCAG	175-191
IHNV N NotR	*GCGGCCGC*TCAGCGGAATGAATCGGAGT	135-1331
IHNV P EcoF	T*GAATTC*GCCACCATGTCAGATGAAGAGG	1466-1481
IHNV P NotR	*GCGGCCGC*TATTGACCTTGCTTCAT	2158-2141
IHNV EcoNVF	G*GAATTC*GCCACCATGGACCACCGCGACATAAAC	4596-4616
IHNV XbaNVR	G*TCTAGA*CTATCTGGGATAAGCAAGAAAG	4931-4910
IHNV L NheF	T*GCTAGC*CACCATGGACTTCTTCGATCTTGAC	5017-5037
IHNV L NotR	*GCGGCCGC*CTATTGTTCGCCTAGT	10977-10962
T7 RNAP plasmids		
T7 RNAP NLSF	T*GCTAGC*CACCATGCCAAAAAAGAAGAGAAAGGTAGAA AACACGATTAACATCGCTAAGAAC	
T7 RNAP NotR	T*GCGGCCGC*TTAATCACTTACGCGAACGCGAAGTCCGAC TC	

[a]restriction sites are in italics, [b]Kozak context is underlined, [c]some primers has extra nucleotides ahead of restriction sites for the purpose of restriction digestion

60°C; and extension at 68°C for 2 min. The RT-PCR products were separated by agarose gel electrophoresis and purified using a QIAquick gel extraction kit (Qiagen, CA).

Cloning and sequencing

The purified RT-PCR products were cloned into a pCR2.1 TOPO® TA vector (Invitrogen, CA). Plasmid DNA was sequenced by dideoxy chain termination method, using an automated DNA sequencer (Applied Biosystems, CA). All the DNA sequence analyses were performed by the Center of Marine Biotechnology (COMB), University of Maryland Biotechnology (UMBI) core facility, which houses an ABI 3130 XL Genetic Analyzer having 16-capillary electrophoresis channels (Applied Biosystems, CA).

Construction of a full-length clone of IHNV

A full-length cDNA copy of the IHNV RNA genome was constructed by assembling six overlapping cDNA fragments generated through RT-PCR by standard cloning techniques, as described (Biacchesi et al., 2000). The clones were ligated serially by natural or artificially created unique restriction sites (Fig. 4-1). The hammerhead ribozyme (HHRz) cDNA sequence (5'CTGATGAGTCCGTGAG GACGAAACTATAGGAAAGGAATTCCTATAGTC3') and hepatitis delta virus ribozyme (HdvRz) sequence (5' GGGTCGGCATGGCATCTCCACCT CCTCGCGGTCCGACCTGGGCATCCGAAGGAGGACAGACGTCCACTCGGATGG CTAAGGGAGAGCCA 3') were fused with fragment 1 (F1) and fragment 6 (F6) respectively by overlapping PCRs.

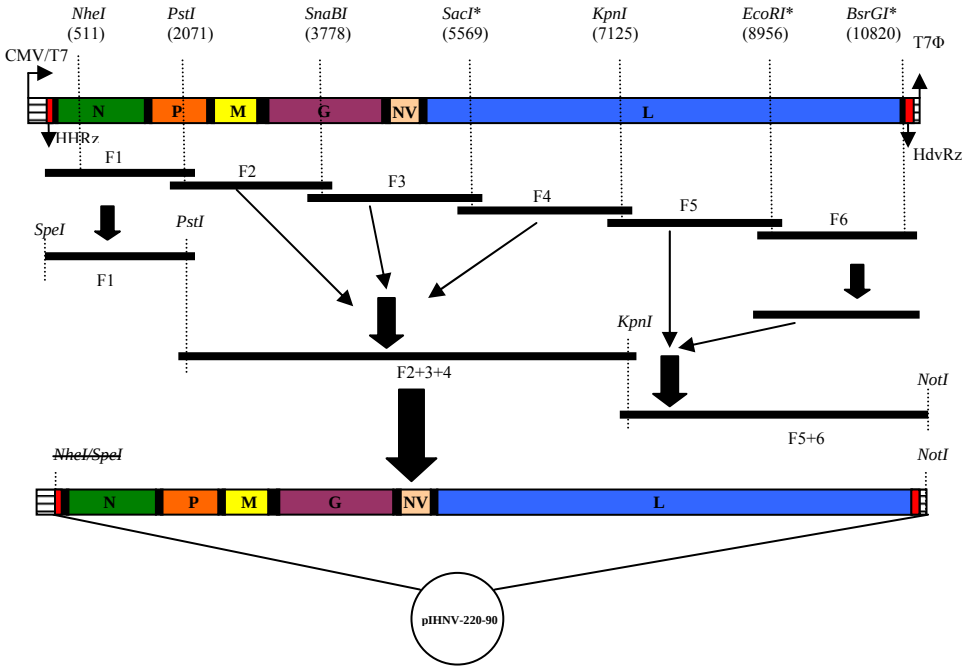

Fig 4-1. Vector construction. Locations of the six cDNA fragments used to construct the full-length IHNV genomic clone and relevant restriction sites. Six overlapping cDNA fragments covering the entire IHNV genome were assembled by ligation into the modified multiple cloning site of the pSmart plasmid using the *SpeI, NheI, PstI, SnaBI, KpnI, EcoRI, BsrGI* and *NotI* restriction enzyme sites. Assembly was done by 3 steps ((i) Topo cloning of six fragments (F) separately and addition of hammerhead ribozyme (HHRz) at the 5' end of F1 and hepatitis delta ribozyme (HdvRz) at the 3'end of F6; (ii) Construction of F2+3+4 and F5+6; (iii) Assembly of full length clone by ligating there major constructs (F1 thru' F6)). Restriction site lines (dotted vertical lines) indicate restriction sites that were designed into the primer or present naturally (*) at the indicated genome positions. -CMV/T7, cytomegalovirus immediate-early enhancer & promoter / T7 RNA polymerase promoter; T7Φ/SV40 poly(A), T7 transcription termination and SV40 late poly adenylation signal.

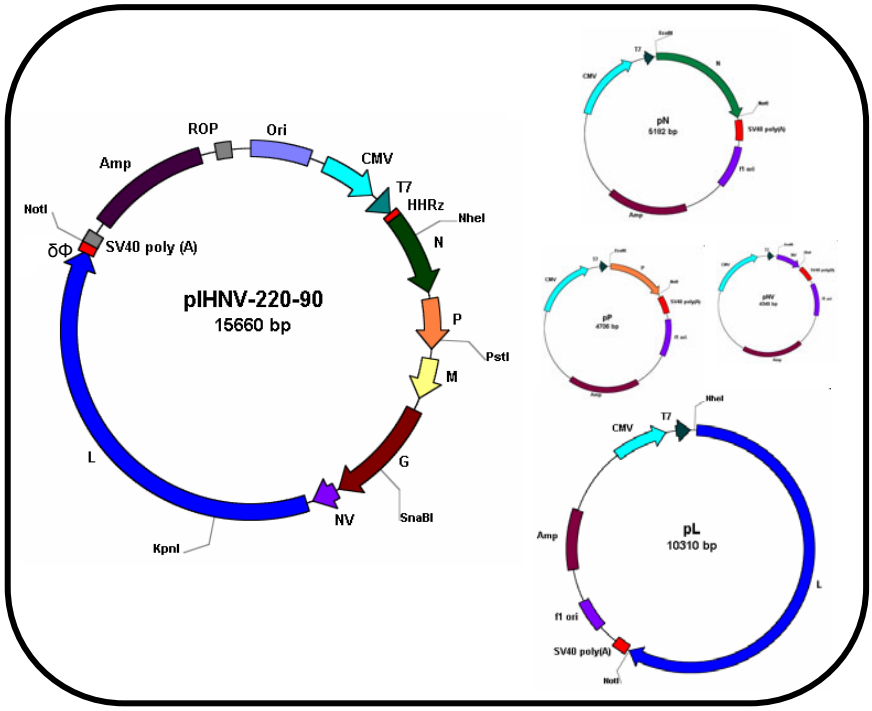

Fig. 4-2. Schematic of the five plasmids used to produce recombinant IHNV in EPC cells. CMV, Cytomegalovirus immediate-early enhancer/promoter; T7, T7 RNA Polymerase Promoter; HHRZ-hammerhead ribozyme; δΦ, hepatitis delta virus ribozyme and T7 termination; SV40 poly(A), SV40 late polyadenylation signal; Amp, ampicillin resistance gene; ROP, Repressor of primer (lowers plasmid copy number); Ori, origin of replication; f1 ori, Phage f1 region.pIHNV-220-90 is the full-length plasmid of IHNV 220-90 strain and the pN, pP, pNV, and pL are helper plasmids for the expression of nucleoprotein, phosphoprotein, non-virion protein, RNA-dependent RNA polymerase respectively. Restriction sites shown in the full length plasmid serves as genetic tags except *NotI* and in the supporting plasmids were used for insertion of respective ORFs into pCI vector. Approximate size of plasmids with its name is shown in the middle of each plasmid.

The subcloning of fragments was initially carried out in pSmart® vector (Lucigen, WI), which was modified (TopoSmart) such that it would contain multiple cloning sites of TOPO® TA vector. From these subclones, a full-length clone of IHNV was assembled in the pCI vector (Promega, WI) as follows. A *PstI* site present in the intron region of pCI vector (down stream of CMV promoter) was deleted by mutational PCR for the ease of cloning. A T7 transcription termination signal, taken from pQE-TriSystem vector (Qiagen), was added to the F6 fragment containing the HdvRz sequence by PCR. To assemble a full-length clone, the F1 fragment was double digested with *SpeI* and *KpnI* restriction enzymes, and cloned between *NheI* and *KpnI* sites of the pCI vector. Second, the F2, F3 and F4 fragments were ligated serially in a separate Toposmart vector, and the ligated, single F2+3+4 fragment was excised by digesting it with *PstI* and *KpnI* enzymes, and subcloned between the unique *PstI* and *KpnI* sites of pCI vector harboring F1 fragment. Like wise, fragments 5 and 6 were ligated in a different TopoSmart vector and then subcloned into the pCI vector by digesting with *KpnI* and *NotI* restriction enzymes. Finally, a full-length plasmid of pIHNV-220-90 was obtained (Fig. 4-2), and its DNA completely sequenced for its integrity using an automated DNA sequencer (Applied Biosystems, CA).

Construction of supporting plasmids

Using a full-length clone of pIHNV-220-90 as a template, the open reading frames (ORFs) of N (1,176 bp), P (693bp), NV (336bp), and L (5,958bp) genes were amplified by PCR using respective primer pairs (Table 4-1). A Kozak consensus sequence was incorporated in front of the start codon of each ORF, as shown in Table 4-2

Table 4-2. Plasmids used to transfect EPC cells and generate IHNV

S.No	Plasmidsa	Promotersb	Kozak Sequence
1.	pIHNV-220-90*	CMV/T7	NA
2.	pIHNV-EGFP*	CMV/T7	NA
3.	pN	CMV/T7	GCCACCATGA
4.	pP	CMV/T7	GCCACCATGT
5.	pNV	CMV/T7	GCCACCATGG
6.	pL	CMV/T7	GCCACCATGG
7.	pT7NLS	CMV/T7	GCCACCATGG

[a]Plasmids used in this study to recover IHNV 220-90

[b]Promoter(s) present in each plasmid.

*Plasmid has hammerhead ribozyme at the 5'-end and hepatitis delta virus ribozyme at the 3'-end of cDNA.

-pIHNV-220/90 is the full-length plasmid of IHNV 220-90 strain and the pN, pP, pNV, and pL are helper plasmids for the expression of nucleoprotein, phosphoprotein, non-virion protein, RNA-dependent RNA polymerase.

-pIHNV-EGFP is the full-length plasmid of IHNV 220-90 strain, which contains EGFP ORF along with extra transcription unit between P and M genes

-pT7NLS- phage T7 RNA polymerase plasmid containing a nuclear localization signal

-N/A - Not Available

(Kozak, 1987). The N and P ORF was cloned between *EcoRI* and *NotI* restriction sites, whereas the NV ORF was between *EcoRI* and *XbaI* sites, and the L ORF was cloned between *NheI* and *NotI* restriction sites of the pCI vector (Promega) by digesting with appropriate restriction enzymes (Fig. 4-2). The DNA from the resulting pN, pP, pNV, and pL support plasmids was sequenced using an automated DNA sequencer (Applied Biosystems, CA).

Construction of T7 RNA-polymerase expression plasmid

The T7 RNA-polymerase expressing plasmid, pT7NLS, which contains eight amino acids nuclear location signal (NLS) derived from the SV40 large T antigen, was constructed according to Wu and Rupprecht (2008). The T7 RNAP gene was amplified from the pTF7-3 plasmid (ATCC, VA**)** with respective primers T7 RNAP NLSF and T7 RNAP NotR (Table 4-1). Amplified T7 RNAP gene was cloned between *NheI* and *NotI* restriction sites of a pcDNA3.1 (+) (Promega, WI) vector. The transcription of pT7NLS is under control of both the cytomegalovirus (CMV) immediate-early promoter and the T7 promoter.

DNA transfection and virus recovery

The plasmids pIHNV-220-90 (1 µg), pN (0.5µg), pP (0.2 µg), pL (0.2 µg), and pNV (0.15 µg) were diluted in 500ul µl Opti-MEM® medium (Invitrogen, CA). Next, Lipofectamine™ LTX reagent (Invitrogen, CA) was added slowly, according to manufacturer's instructions, and incubated for 30 min at room temperature. The plasmid – Lipofectamine reaction mixture was added to the EPC monolayer in a six-well plate

without replacing the growth medium. The transfection mixture was removed after 8 h of incubation at 28^{o} C, and the transfected cells were washed and maintained in Eagle's MEM (ATCC, VA) containing 10% fetal bovine serum at 14°C for 5 days. Cell monolayer was observed for the development of virus-induced cytopathic effect (CPE) and also expression of EGFP (cells transfected with the EGFP plasmid). After 5 days of incubation, the cells were submitted to three cycles of freeze-thawing. Supernatant was clarified by centrifugation at 8,000 x *g* in a microcentrifuge, and used to inoculate fresh cell monolayers in T-25 flasks at 14°C. The supernatant was harvested and clarified for further processing of the recombinant viruses.

RT-PCR and confirmation of the genetic tags

RT-PCR was performed on the RNA extracted from the recovered viruses to confirm the presence of artificially introduced genetic markers. Briefly, the viral RNA was extracted from partially purified virus obtained after ultracentrifugation (collected from 26% sucrose cushion), using RNeasy® Mini Kit (Qiagen). RT-PCR was performed to verify the presence of *NheI*, *PstI, SnaBI* and *KpnI* restriction sites that were artificially introduced during the cloning process. Restriction analysis of the PCR products was carried out on a 1% agarose gel. The obtained RT-PCR products were also subjected to DNA sequencing to confirm the presence of artificially introduced genetic tags.

Construction of pIHNV-EGFP plasmid

The EGFP ORF was amplified from pIRES2-EGFP (Promega, WI) plasmid using EGFP-specific primers (Table 4-1). An additional transcription unit, comprising of

untranslated regions between the P and M ORFs, was fused at the C-terminus of EGFP ORF. Without introducing any restriction sites, EGFP ORF was inserted (with additional transcription unit) between *Pst*I and *Xho*I restriction sites between P and M genes. The *Pst*I-*Sap*I fragment was excised from the full-length pIHNV construct by restriction enzyme digestion and replaced it with the modified *Pst*I-*Sap*I fragment derived from the F2+F3+F4 plasmid construct.

Virus titration

To analyze the growth characteristics of rIHNVs, confluent EPC cells were infected with the recombinant virus stocks at an MOI of 0.01. Infected cell cultures were removed at different time intervals and stored at -70°C; the supernatants were centrifuged and titrated on EPC cells by plaque assay, as described previously (Burke and Mulcahy, 1980) with modification. Briefly, the confluent monolayers of EPC cells, grown in six-well plates, were infected with serially diluted supernatants from virus stock. After a 1-h incubation at 14°C, the cells were washed once by PBS and overlaid with 0.75% methylcellulose (Difco) in Eagle MEM containing 10% FBS. After 5 days of incubation at 14°C, the overlays were removed and the cells were fixed and stained with a solution containing 25% formalin, 10% ethanol, 5% acetic acid, and 1% crystal violet for 5 min at room temperature. After rinsing of the cells with distilled water, the plaques were counted.

Experimental fish infection

Virulence comparisons of wild type and recombinant viruses were performed in fingerlings of rainbow trout using the method of LaPatra et al. (1994). Briefly, duplicate

25 fish groups of rainbow trout (*O. mykiss*) juveniles (mean mass 12·0 g) were infected by intra-peritoneal injection in 10^5 PFU/ml of representative IHNV isolates. Mock-infected control groups were injected with cell culture media only. Fish mortalities were recorded over a 2 weeks period of time.

4.4 Results

Construction of full-length cDNA clone of IHNV Genome

We constructed an infectious clone of IHNV-220-90 by inserting the 11,134 nucleotides of IHNV-220-90 genome and the sequences of HHRz and HDVRz into the pCI vector (Promega, WI). The IHNV 220-90 strain was isolated from rainbow trout and maintained in Scott E LaPatra lab (La Patra *et al.,* 1991). The complete genome of IHNV 220-90 strain sequence has been taken from GenBank (accession number GQ413939). The complete IHNV RNA genome was amplified by RT-PCR as six fragments using overlapping primers. All the six fragments were assembled together in a mammalian expression vector, pCI as depicted in Fig 4-1. Some mutations resulting in altered amino acids were corrected by mutational PCR. With silent mutation, unique restriction sites (*NheI, PstI, SnaBI* and *KpnI*) were introduced into the full length clone. The nucleotide differences between cDNA clone and wild type virus is listed (Table 4-3). The plasmid is under the control of both CMV and a T7 RNAP promoter. To favor the virus rescue and efficiency, full length cDNA was fused with self-cleaving ribozyme sequences at both the ends. This allows precise cleavage at the termini of RNA and leaves authentic 3' and 5' viral RNA ends.

Table 4-3. Nucleotide differences between the wild-type IHNV and cloned cDNA

S.No	Nucletide position	Wild type[a]	Full length clone[b]	Amino acid change	Gene involved	Restriction sites created[c]
1.	516	T	A	silent	N	*NheI*
2.	2071	G	T	Silent	M	*PstI*
3.	2074	G	A	Silent	M	
4.	2989	T	C	Silent	M/G*	NA
5.	3778	G	T	Silent	G	*SnaBI*
6.	6570	C	T	Silent	L	NA
7.	7125	C	T	Silent	L	*KpnI*
8.	10230	A	G	Silent	L	NA

[a] Nucleotides naturally present at indicated positions in the wild type IHN virus

[b] Differences in the cDNA sequence are due to creation of restriction sites or simply mutations in the genome sequence.

[c] Restriction sites listed were artificially introduced by silent mutation for cloning purpose as well as genetic tags.

*Untranslated region between the ORFs M and G.

NA-not applicable

Expression of T7 polymerase in EPC cells

To evaluate the boosting effect of T7 RNA polymerase on viral rescue from cDNA plasmids, T7 RNAP expressing plasmid (pT7NLS) was constructed. The T7 RNAP gene was amplified from pTF7-3 plasmid and subcloned into the vector pcDNA3.1. A nuclear localization signal derived from the SV40 large T antigen (Bruce *et al.,* 1987; Kalderon *et al.,* 1984) was added to the T7 RNA polymerase at its N-terminus. The efficiency of such construct was demonstrated for the recovery of rabies virus (Wu and Rupprecht, 2008). The T7NLS plasmid is an autogene, transcription of which is controlled by its own product (Brisson *et al.,* 1999; Bruce *et al.,* 1987). After T7 RNAP is produced in the cytoplasm, a majority of the T7 RNAP is transported to the cell nucleus, and the transfected full-length and helper plasmids in the nucleus are transcribed by both T7 RNAP and cellular RNA polymerase II.

To test the T7 RNAP activity, a reporter vector was constructed by cloning the EGFP gene downstream of the T7 promoter. Cotransfection of the T7NLS RNAP and EGFP plasmids into the EPC cells yielded green fluorescence, which was absent in the control cells transfected with EGFP plasmid alone (data not shown).

Supporting plasmids

The ORF of all the supporting plasmids (pN, pP, pNV and pL) were amplified by PCR from the full-length clone and subcloned into the expression vector pCI (Fig. 4-2). The vector, pCI, was selected to express all the proteins because it contains both cytomegalovirus promoter, which has been shown to be highly active in fish cells (Lopez

et al., 2001; Anderson *et al.,* 1996), and a T7 promoter to utilize T7 RNAP. A Kozak consensus sequence was included in front of the start codon of each ORF to provide an optimal sequence context for protein translation (Kozak, 1987). Expression of the corresponding IHNV proteins from supporting plasmids was checked by analysis of in-vitro translated products (TNT-T7-coupled system) by sodium dodecyl sulfate-polyacrylamide (SDS-PAGE) gel electrophoresis (data not shown).

3.4 Rescue of recombinant IHNV from cloned cDNA

Transfection experiments were carried out on the EPC cell monolayers in six-well plates at 28°C. Cells were transfected with a mixture of full-length and supporting plasmids and then shifted to 14°C after 8hr. Initially we observed some rounded cells due to transfection reagents, but because this is vaccinia free system, the CPE produced by recombinant viruses was evident as early as 72 hrs post-transfection. There was absolutely no need for additional passages to confirm the recovery of rIHNVs. High titer viruses were produced at transfection level itself (Table 4-4). To verify that the rescued viruses that were derived from the cDNA plasmids, genomic RNA extracted from the pelleted virus was subjected to RT-PCR and restriction enzyme digestion.

Table 4-4. Titers of viruses recovered after transfection of EPC cells with different combinations of plasmids

pIHNV-220-90	pIHNV-EGFP	pN	pP	pNV	pL	pT7NLS	Virus titer (PFU/ml)
+	-	+	+	+	+	-	8.5×10^5
+	-	+	+	+	+	+	8.7×10^6
-	+	+	+	+	+	-	2.3×10^5
-	+	+	+	+	+	+	3.1×10^6
+	-	+	+	-	+	-	5.2×10^5
+	-	-	+	+	+	-	1.1×10^3
+	-	+	+	+	-	+	NA
-	+	+	+	+	-	+	NA

EPC cells ($6x10^5$ cells/well in six-well plates) were transfected with the indicated plasmids. Supernatants of the transfected cell culture were collected after 5 days and the virus was titered by plaque assay.

-Plasmid pIHNV-220-90 is a full-length clone of IHNV 220-90 strain and pN, pP, pNV, and pL are the helper plasmids for expression of nucleoprotein, phosphoprotein, non-virion protein, RNA-dependent RNA polymerase.

-pT7NLS- phage T7 RNA polymerase plasmid containing a nuclear localization signal

-pIHNV-EGFP is a full-length plasmid of IHNV 220-90 strain containing EGFP ORF (along with extra transcription unit) between the P and M genes.

(+) plasmids included in the transfection procedure.

(-) plasmids excluded in the transfection procedure.

NA- Not available as virus was not recovered.

Negative-control PCRs were performed with recombinant virus RNA which had not been reverse transcribed. All four restriction sites that were introduced during cloning were present and cut by the respective restriction enzymes (Fig. 4-3). In addition, sequence analysis of the amplified fragment confirmed the existence of genetic markers (Fig. 4-4). These results show that the recovered viruses were indeed derived from the cDNA plasmids. The efficiency of recovery from different experiments was calculated by titrating the recovered viruses in EPC cells (Table 4-4). A high tittered rIHNV was rescued from almost all wells which didn't require further amplification. The expression of recombinant IHNV proteins was confirmed by Western blot analysis (Fig.4-5).

The rescue efficiency was improved when T7NLS plasmid was co-transfected with IHNV plasmids (Table 4-4). The recombinant virus, IHNV-EGFP, was rescued from the cDNA as described in materials and methods, and the expression of the EGFP gene was confirmed by fluorescent microscopy (Fig. 4-6). In order to optimize the transfection conditions for recovering the virus, we performed several transfections with different amount and ratio of supporting plasmids and full-length plasmid. Even though we found that increasing the concentration of pIHNV-220-90 and pN plasmids improves the recovery of virus and increase the virus titer, it was concluded that the optimal transfection conditions were 1.0 μg of full-length plasmid, 0.5 μg of pN, 0.2 μg of pP, 0.2 μg of pL, and 0.15 mg of pNV plasmids. Our results demonstrate that the pNV plasmid is not essemtial for virus recovery (Table 4-4).

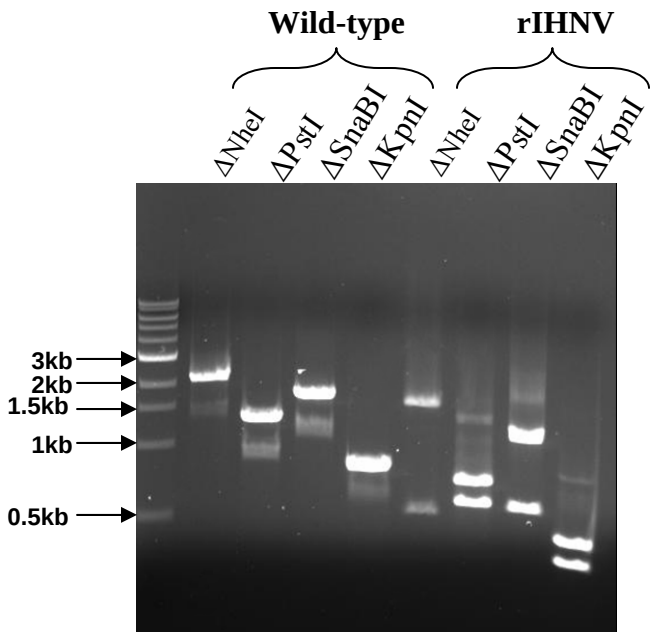

Fig 4-3. Gel analysis of the RT-PCR products used to identify recombinant IHNV

Reverse transcriptase PCR (RT-PCR) was done with the RNA of wild-type and recombinant IHNV (rIHNV) extracted from the pelleted virus. Primers covering restriction sites were used for RT-PCR. The four RT-PCR products of wild-type IHNV and rIHNV (approximate sizes 2100, 1696, 1387 and 1387) were electrophoresed on 1% agarose gel. These RT-PCR products were digested with restriction enzymes *NheI* (lanes 1 and 5), *Pst I* (lanes 2 and 6), *SnaBI* (lanes 3 and 7), and *KpnI* (lanes 4 and 8). Lane M; molecular size marker; lanes 1-4, wild-type IHNV; lanes 5-8, rIHNV. The RT-PCR products of rIHNV were cut by respective restriction enzymes, whereas the wild-type were not cut. The sizes of the products (approximate): *NheI* (518bp and 1579bp); *PstI* (574bp and 726bp); *SnaBI* (572bp and 1128bp); *KpnI* (325bp and 385bp). The sizes of the molecular marker are indicated on the left.

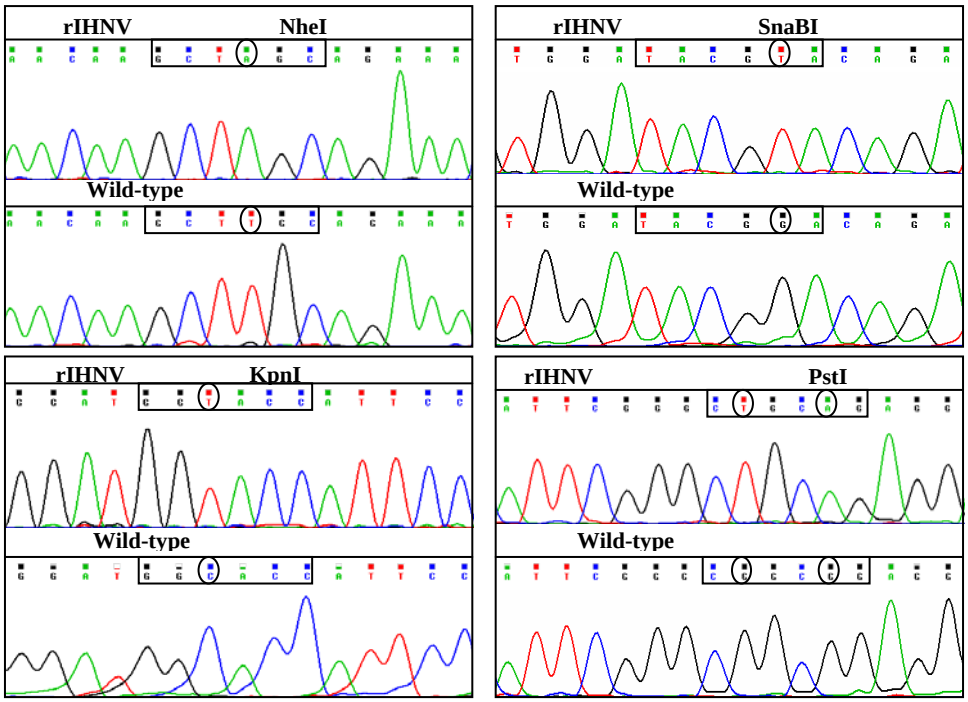

Fig 4-4. Identification of genetic markers in the genome of rIHNV

Chromatograms showing the nucleotide sequence of RT-PCR products generated from the wild-type (wtIHNV) and recombinant IHNV (rIHNV) 220-90 genomes which were sequenced across each of the four tags (rectangular mini boxes). The nucleotides (circled) were modified during the cloning procedures to accommodate *NheI, PstI, SnaBI* and *KpnI* restriction sites that served as genetic markers. Two nucleotides were mutated to create *PstI* site. All the mutations to create restriction sites were silent.

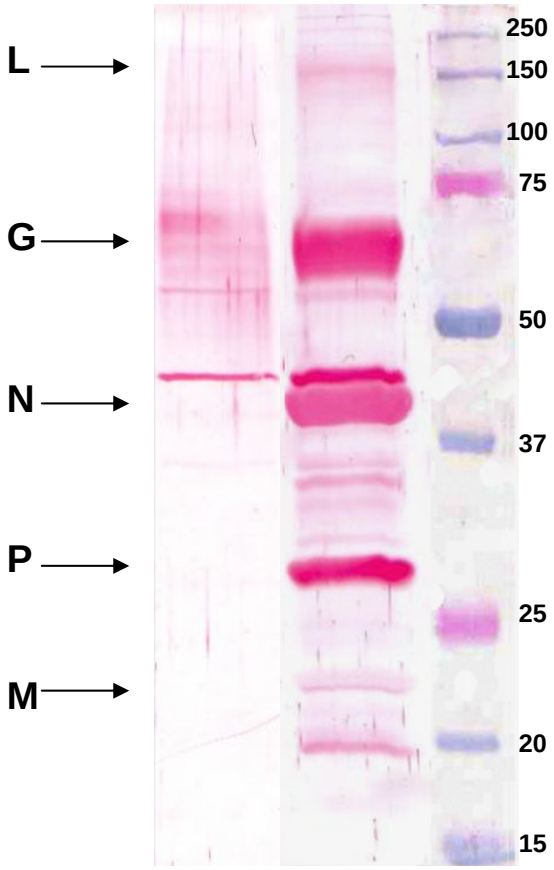

Fig 4-5. Western blot analysis of recombinant IHNV proteins

EPC cells were infected with the rescued rIHNV 220-90 at an MOI of 0.1 or mock infected. Five days later or after completion of CPE, supernatants were collected, clarified and ultra centrifuged using 26% sucrose cushion. The pellet was loaded onto 10% SDS-polyacrylamide gel. IHN viral proteins were detected by Western blotting using polyclonal antibodies against wild-type IHNV. Lane1: Mock infected Lane2: rIHNV Lane M: Molecular weight marker (molecular weight in kDa is shown on the right)

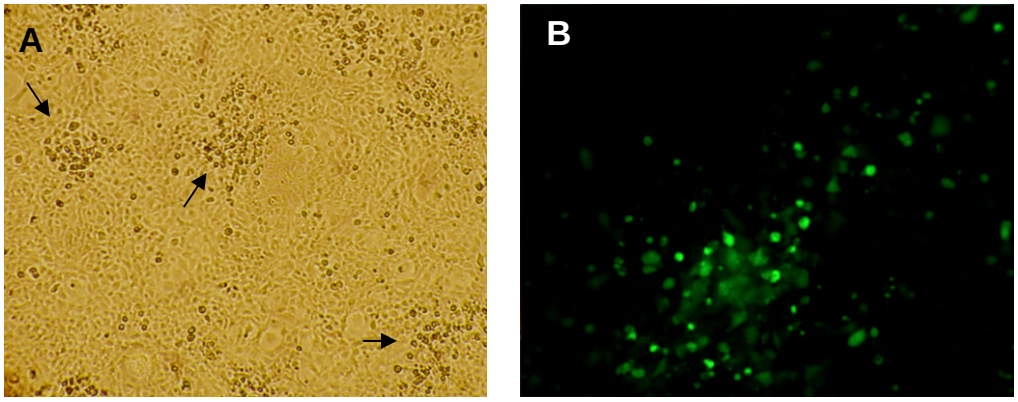

Fig 4-6. Cytopathic effect of recombinant IHNV in the EPC cells

EPC cells were infected with (A) recombinant IHNV (rIHNV) and (B) recombinant IHNV expressing EGFP (rIHNV-EGFP) (**A**) The IHNV induced cytopathic effect (CPE); rounding of cells and foci of dead cells (shown by black arrows) in infected EPC monolayer. (**B**) Expression of the EGFP gene in cells infected with rIHNV-EGFP. EPC cells were examined under UV-light microscope at 405 nm; cells infected with rIHNV-EGFP yielded green fluorescence.

3.5 Biological characteristics of the rescued IHNVs

Growth kinetics of wild-type and recombinant viruses were done in EPC cells by plaque assay, as described (Burke and Mulcahy, 1980). The cells were stained with neutral red, and the plaques were counted. The replication efficiency of the recombinant viruses appeared to be no different from that of the wild-type virus (Fig. 7). The morphology and size of plaques produced by rIHNVs and wild-type viruses were compared. The plaques from all these viruses had approximately the same size and appearance, indicating that the growth characteristics of recombinant viruses in cell culture were not significantly different (data not shown).

Virulence comparisons of the wild type and rIHNVs were performed in fingerlings of rainbow trout (Fig. 8). The rIHNV caused approximately 10% less mortality than the wild-type IHNV. This slight virulence difference between wild-type IHNV and rIHNV is probably because of the quasispecies nature of the wild-type virus. The recombinant IHNV carrying EGFP gene (rIHNV-EGFP) causes only 5% less mortality than that of rIHNV. This result shows that insertion of a foreign gene does not affect the virulence of virus drastically.

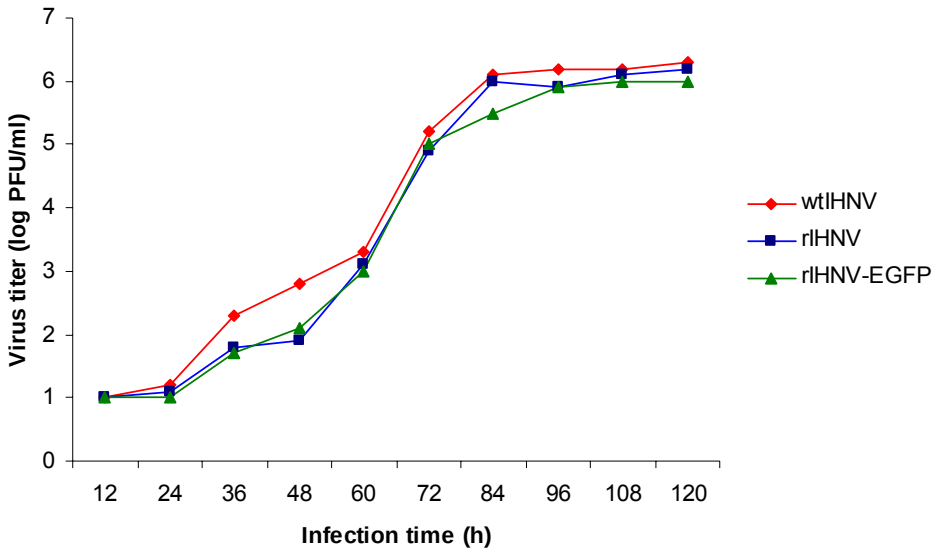

Fig 4-7. Growth kinetics of the recombinant IHN viruses in EPC cells.

EPC cells were infected (at 0.01 MOI) with the wild-type IHNV (♦), recombinant IHNV (rIHNV) (■), or recombinant IHNV expressing EGFP (rIHNV-EGFP) (▲). The cell culture supernatant was collected at 12hr intervals post-infection. Supernatants were serially diluted and each dilution (100 µl) was used for infection of cells in 12-well plates. After 1 h of virus adsorption, supernatants were removed from the wells and then overlaid with 0.75% methylcellulose. The infected cells were incubated at 14°C for 7 days or until the development of countable plaques. Then, the cells were stained with neutral red and the plaques were counted.

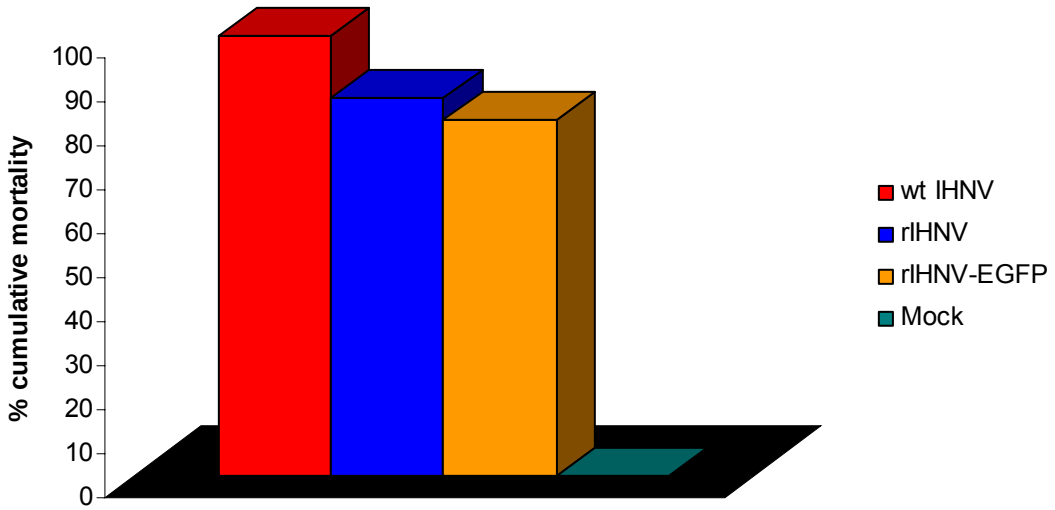

Fig 4-8. Virulence comparison of the recombinant viruses in trout

Juvenile trout (n = 50; mean mass, 3.0 g) were infected by intra-peritoneal injection with 10^5 PFU/ml of the wild-type IHNV (wt IHNV), the recombinant IHNV (rIHNV), the recombinant IHNV expressing EGFP gene (rIHNV-EGFP) or mock infected. Mortalities were recorded every day for 2 weeks and are expressed as a percentage of cumulative mortality. Mock-infected trout were treated under the same conditions.

4.5 Discussion

IHNV, belonging to genus *Novirhabdovirus*, was first recovered successfully by Biacchesi *et al.,* (2000) using recombinant vaccinia virus expressing the T7 RNA polymerase (vTF7-3). SHRV, another *Novirhabdovirus* was recovered by Johnson *et al.,* (2000) also using the vTF7-3 vaccinia system, and by Alonso *et al.,* (2004) using EPC cell line stably expressing T7 RNA polymerase**.** In this present study, we exploited cellular RNA polymerase II to drive the expression of CMV promoter-driven plasmids. This is the first report of successful recovery of non-mammalian rhabdovirus using vaccinia- and T7 RNA polymerase-free system.

In this study, we describe the recovery of IHNV entirely from cloned cDNA, using vaccinia/T7RNAP-free system. Recombinant IHNV, derived from the virulent strain 220-90, was generated after transfection of EPC cells with CMV-driven plasmids allowing simultaneous expression of antigenomic copy of IHNV RNA, and N, P, NV and L proteins. The recombinant IHNV, which showed growth characteristics indistinguishable from the wild-type virus, possesses four genetic tags that were introduced into the cDNA copy. A similar approach has been used to generate infectious virus from cloned full-length cDNA of other nonsegmented negative-strand RNA viruses (Wagner *et al.,* 2001; Inoue *et al.,* 2003; Martin *et al.,* 2006).

The CMV promoter is a strong eukaryotic promoter and can be recognized efficiently by cellular RNA polymerase II. It was demonstrated that protein expression was very high when CMV promoter was used to express gene of interest in the EPC cell line when compared to T7 promoter (Lopez *et al.,* 2001). Similarly, the efficiency of

RNA polymerase II was higher than that of T7 RNA polymerase (Inoue *et al.*, 2003). The viral full-length antigenomic RNA is made with authentic 5'- and 3'- terminal ends by flanking ribozyme mediated autocatalytic cleavage. As the *cis*-acting elements, hammerhead ribozyme (HHRz) and hepatitis virus ribozyme (HdvRz) can raise the efficiency of virus rescue. The cellular RNA polymerase II itself can help the recovery of virus if all the plasmids were cloned under the control of the CMV promoter. To test, whether inclusion of T7 RNAP expressing plasmid will increase the efficiency of viral recovery, T7NLS RNAP plasmid was co-transfected with viral plasmids. We found that viral titer was one log higher in wells in which pT7NLS plasmid was included. Even though T7 plasmid increase the efficiency of recovery, it is absolutely not essential for the IHNV rescue because transfection of IHNV plasmids itself produced very high titered virus (Table 4).

The optimal transfection conditions and the ratio of full-length plasmid to the helper plasmids determined in the present study could be optimized for other fish cell lines also. IHNV usually grows at lower temperature (14°C), which is not an optimal temperature for vaccinia virus infection and T7 RNA polymerase transcription. For efficient production of T7 RNAP and transcription, the EPC cells have to be kept at higher temperatures (Biacchesi *et al.,* 2000). Both high temperature and vaccinia virus toxicity hinders the efficient recovery of IHNV. The major advantage of this CMV-based system is that the transfection could be done at optimal temperature for EPC cells and after reasonable time, the cells could be transferred to 14°C. Even after the cells are transferred to 14°C, the CMV-driven transcription will continue but at slower rate. This not only increases the chances of virus recovery but also the titer of recovered viruses.

The identity of the recombinant viruses was verified by RT-PCR using viral genomic RNA extracted from pelleted virus and sequencing. Analysis of PCR products by restriction digestion and sequencing verified both that the RNA was indeed IHNV and that the mutations were maintained. It was suggested that when vaccinia helper virus is used for viral recovery, it makes homologues recombination between the full-length plasmid and transfected supporting plasmids. This leads to correction of nucleotides which are deliberately mutated in the viral genome and loss of restriction site markers (Biacchesi *et al.,* 2000; Garcin *et al.,* 1995). This hinders further mutational studies on viral genome. These drawbacks have been overcome by this vaccinia virus-free recovery system. In this study, the recovered viruses maintained all the restriction sites which were introduced as genetic markers.

Earlier studies demonstrated that expression of one or two foreign genes did not significantly affect the biological properties of rhabdoviruses. (Mebatsion *et al.,* 1996; Schnell *et al.,* 1996; Haglund *et al.,* 2000; Biacchesi *et al.,* 2000; Alonso *et al.,* 2004). Our findings also showed that the insertion of EGFP as a foreign gene between the P and M ORF does not affect the replication of the recombinant virus drastically. The recombinant viruses, rIHNV and rIHNV-EGFP, developed in this study showed similar growth characteristics in tissue culture as those of its wild-type parental virus (Fig. 7). The CPE induced by both wild-type IHNV and rIHNV in cultured EPC cells were very similar and the plaques formed by rIHNV in EPC cells were also indistinguishable in size and shape from those of the parental virus (data not shown).

Although it is feasible to rescue viruses using vaccinia expressing T7 RNAP as a helper-virus, it may be problematic, particularly when developing a vaccine for commercial use. It warrants stringent purification processes to remove all traces of vaccinia virus to ensure vaccine purity. To circumvent dependence on helper-virus, researchers developed continuous cell lines that stably express T7 RNAP. Several recombinant viruses were recovered using cell line constitutively expressing the T7 RNAP (Buchholz *et al.,* 1999; Enterlein *et al.,* 2006; Finke and Conzelmann, 1999; Harty *et al.,* 2001; Volchkov *et al.,* 2001; Alonso *et al.,* 2004). Although stable cell lines expressing T7 RNAP helps in recovery of recombinant viruses without vaccinia virus, there are limitations to this approach. The major limitation is cell lines that express T7 RNAP to support rescue can be difficult to develop and maintain.

The low fidelity of T7 RNA polymerase and failure to remove extra nucleotides at the termini of antigenomic RNA may interfere with the virus rescue. These drawbacks could be circumvented by RNA polymerase II-based reverse genetics system and by self cleaving ribozymes (HHRz and HdvRz), respectively. This strategy was successfully used to rescue rabies virus (Inoue *et al.,* 2003), Borna disease virus (Martin *et al.,* 2006; Yanai *et al.,* 2006) and measles virus (Martin *et al.,* 2006). These studies demonstrated that CMV promoter system allows much more efficient production of rIHNVs compared with the conventional T7 polymerase driven system **(**Biacchesi *et al.,* 2000). Coupled with the markedly enhanced recovery rates, this new system makes production of recombinant *Novirhabdovirus* much easier than conventional T7 vaccinia system in fish cell line. This recovered virus can be engineered to carry foreign genes or immunogenic

epitopes of other fish pathogens and hence can be effectively used as a multivalent virus-vectored vaccine in aquaculture.

IHNV is an important model system for *Novirhabdoviruses* and this plasmid-based rescue system could facilitate further investigations into the molecular aspects of IHNV replication. This newly developed recovery system for IHNV entirely from cDNA is a powerful tool to decipher not only IHNV genome, but also rest of the species of *Novirhabdovirus*. In this study, we achieved an important milestone in the reverse genetics of *Novirhabdovirus* by implementing a vaccinia/T7RNAP-free system. This system abolishes the drawbacks of the T7 RNAP based system; mainly, (i) efficiency of virus recovery is higher than the T7 based system (ii) avoids vaccinia virus contamination with stock virus (iii) effective vaccine production for commercial use (iv) no special handling of the cell line and helper virus is needed (v) mutational studies could be carried out without worrying about vaccinia virus recombination (vi) eliminates the burden of making and maintaining T7 RNAP expressing cell lines.

Chapter 5

Molecular determinants of virulence in Infectious Hematopoietic Necrosis Virus

5.1 Abstract

Infectious hematopoietic necrosis virus (IHNV) loses its virulence when passaged in cell culture. However, the molecular basis of virulence in IHNV and its adaptation to cell culture is not known. In this study, virulence of IHNV field strain 220-90 (IHNV-06) and its cell culture adapted counterpart, IHNV-61, was compared in rainbow trout and it was found that IHNV-06 causes 71% mortality, whereas the cell culture adapted causes 16% mortality. To identify the genes involved in virus attenuation, complete genome sequences of both virulent and cell culture adapted strains were analyzed. Comparison of the complete nucleotide and deduced amino acid sequences of these viruses reveals major amino acids substitutions in the glycoprotein gene and minor changes in the matrix protein gene and 5'-trailer sequence. To determine the gene(s) or nucleotides responsible for virulence or virus attenuation, chimeric rIHNV-06/61G, rIHNV-06/61M, and rIHNV-06/61T were generated, in which glycoprotein and matrix protein genes, and trailer sequence of the virulent virus was replaced with the attenuated one, respectively. *In vitro* analysis showed that these chimeric viruses have similar growth and replication kinetics in the cell culture as the recovered rIHNV-06. *In vivo* studies demonstrated that fish inoculated with rIHNV-06/61M and rIHNV-06/61T viruses induced similar cumulative mortality (26%) in rainbow trout as the parent rIHNV-06. However, fish mortality caused by rIHNV-06/61G was reduced to 4% when the glycoprotein of virulent virus was

swapped with the attenuated one. These results demonstrate that the molecular determinants of virulence in IHNV reside in the glycoprotein gene.

5.2 Introduction

Infectious hematopoietic necrosis virus (IHNV) is an important rhabdoviral pathogen of salmonid fish that causes a large economic impact on commercial fish farms as well as hatcheries (Winton, 1991). IHNV is a member of the genus *Novirhabdovirus*, within the family *Rhabdoviridae* (Tordo *et al.,* 2005). It has a linear single-stranded, negative-sense RNA genome of approximately 11 kb, with six genes encoding five structural proteins and one non-structural protein (Kurath *et al.,* 1985; Schütze *et al.,* 1995; Morzunov *et al.,* 1995). The virus replicates in the cytoplasm and produces six monocistronic mRNAs, which are capped and polyadenylated. The virions comprise of nucleoprotein, N; a polymerase-associated protein, P; an RNA-dependent RNA polymerase, L; a matrix protein, M; and a glycoprotein, G. The gene order of IHNV is 3'-N-P-M-G-NV-L-5'. The negative-strand RNA genome is connected tightly with the nucleoprotein N and forms the core structure of virion. This encapsidated genomic RNA is also associated with the phosphoprotein P and polymerase protein L, which are involved in the viral protein synthesis and replication.

Since IHNV is a RNA virus, the replication of RNA viruses is characterized by high mutation rates. In addition, RNA viruses have large population sizes, high replication rates and short generation times. All these properties are responsible for the extremely high genetic variability of RNA virus populations (Moya *et al.,* 2000). Viral virulence is the relative ability of a virus to produce disease or lesions in a host. It is

dependent on various host and virus factors, including changes in viral tissue tropism and alteration in the level of viral replication and transcription. Many viral genome characteristics can influence viral pathogenesis and virulence, such as viral envelope and capsid proteins; core, matrix, and nonstructural proteins; and noncoding regions of the viral genome (Tyler and Fields, 1996). Previous studies have shown that few amino acid changes in the G protein of IHNV or VHSV would result in an apparent change in the virulence and distribution of the virus in fish (Kim *et al.,* 1994; Bearzotti *et al.,* 1995). This demonstrates that an altered G may affect viral pathogenesis by changing its tissue tropism.

Earlier studies have demonstrated that virulent strains of IHNV lose their virulence potential after serial passage in cell culture (Fukuda *et al.,* 1989; Michael, 1974; Leong et al, 1988). However, the molecular basis for cell adaptation and attenuation is not known because none of these viruses were cloned and characterized by nucleotide sequence analysis. In this study, virulence of IHNV-06 (passaged 6 times in cell culture) and its cell culture adapted counterpart IHNV-61 virus (IHNV 220-90 passed 61 times in cell culture), was compared in rainbow trout and it was found that the IHNV-06 causes high mortality, whereas the cell culture adapted caused low mortality. While it is possible to study the evolution of viruses and its impact on viral pathogenicity by comparing genomic sequences of heterologous strains, the analysis of homologous strains provides a unique opportunity to understand specific genes likely to be involved in virulence and pathogenicity. To identify genes or putative residues that might be associated with the viral virulence or cell adaptation, we have carried out a comparative analysis of newly determined complete genome sequences of the virulent and attenuated viruses of IHNV

220-90 strain. We utilized an IHNV reverse genetic system developed in our laboratory to make chimeric viruses by substituting the virulent virus candidate genes with that of attenuated virus. The growth kinetics and pathogenicity of the recombinant viruses were studied.

5.3 Material and methods

Virus and cells

The virulent (IHNV-05) and attenuated (IHNV-60) viruses of IHNV 220-90 strain were kindly provided by Dr. Scott LaPatra, Clear Springs Foods Inc., Idaho, USA. These viruses were amplified one more time in cell culture to make stock viruses. The *Epithelioma papulosum cyprini* (EPC) cells were used for virus propagation (Fijan *et al.,* 1983). The cells were grown at 28°C in minimal essential medium (MEM) supplemented with 10% fetal bovine serum and 2 mM L-glutamine. For preparation of virus stocks, confluent EPC cells were infected with the IHNV at a multiplicity of infection (MOI) of 0.01 in MEM with 2% fetal bovine serum. After 1 h of adsorption, the inoculum was removed, and the cells were incubated at 14°C until extensive cytopathic effect (CPE) was observed. The supernatant was collected 5 days post-infection (p.i), clarified, and stored at -80^{o}C for further processing.

RNA extraction and amplification

Viral RNA was extracted from cell culture supernatant and stored at -20°C using Qiagen RNAeasy kit, according to manufacturer's instructions. The oligonucleotide primers used in this study are listed in Table 5-1. First strand synthesis was carried out in

a tube containing 5µl of RNA, which was denatured at 70°C for 10 min in the presence of DMSO (3µl), 1 µl forward gene-specific primer, 1µl of 25 mM dNTPs and snap-cooled on ice for 1 min. The reaction mixture containing 2µl of 10X RT buffer, 2µl of 0.1M DTT, 4µl of 25mM $MgCl_2$, 1µl of Superscript III RT™, and 1µl of RNase OUT™ was incubated at 50°C for 1 h. PCR amplifications were carried out using a *pfx*50™ PCR kit (Invitrogen, CA), according to manufacturer's instructions. Briefly, the following mixture was used for PCR amplification: 3µ1 of cDNA, 2µl of primer mix; 5µl of 10x PCR buffer [100 mM Tris-HCl (pH 9.0), 500 mM KC1, 1% Triton X-100], 2µ1 of 25 mM $MgCl_2$, 0.5ul of *pfx*50 polymerase, and 37µ1 of DEPC water, to make a final volume of 50 µ1. Reaction was carried in a thermal cycler (MJ Research Inc., Waltham, MA), using the following program: denaturation at 94°C for 30sec; annealing for 30sec at 60°C; and extension at 68°C for 2 min. The RT-PCR products were separated by agarose gel electrophoresis and purified using a QIAquick gel extraction kit (Qiagen, CA).

Cloning and sequencing

The purified RT-PCR products were cloned into a pCR2.1 TOPO® TA vector (Invitrogen, CA). Plasmid DNA was sequenced by dideoxy chain termination method, using an automated DNA sequencer (Applied Biosystems, CA). All the DNA sequence analyses were performed by the Center of Marine Biotechnology (COMB), University of Maryland Biotechnology (UMBI) core facility, which houses an ABI 3130 XL Genetic Analyzer having 16-capillary electrophoresis channels (Applied Biosystems, CA).

Table 5-1. Oligonucleotides used to create marked restriction sites in the full-length IHNV clone[a].

Primers	Sequence	Position
P/M PciF	AAATCCAAGAG*ACATGT*CAAACGAGAGC	2227-2254
P/M PciR	GCTCTCGTTTG*ACATGT*CTCTTGGATTT	2254-2227
M/G PacF	G*TTAATTAA*CATGCCATCCCTCACTCAC	2842-2869
M/G PacR	GGCATG*TTAATTAA*CTATTTTTCCTTCC	2856-2829
M/G SmaF	*CCCGGG*CAAAACAATGGACACCATGA	298-3011
M/G SmaR	TTGTTTTG*CCCGGG*CTCTGCGAGTTGCGTT	2999-2970
G/NV SacIIF	TAA*CCGCGG*TCAATCTTCACCTCTT	4523-4547
G/NV SacIIR	ATTGA*CCGCGG*TTAGGACCGGTTTGCCA	4536-4509

[a]Restriction enzyme sites are in italics.

Construction of chimeric plasmids

A full-length cDNA clone of virulent IHNV 220-90 strain made earlier was utilized for this study to recover chimeric viruses. In this study, we also constructed full-length cDNA clone of attenuated virus, IHNV-61 by assembling six overlapping cDNA fragments generated through RT-PCR by standard cloning techniques, as described earlier (Ammayappan and Vakharia, 2009b). Two restriction sites, *PciI* and *PacI*, were introduced at the beginning and end of the M protein ORF, respectively. Likewise, two more restriction sites, *SmaI* and *SacII*, were introduced at the beginning and end of the G protein ORF respectively by overlapping PCR using primers listed in Table 5-1. The IHNV-61 M and G ORF was amplified with PciF/PacR and SmaF/SacIIR primers respectively, and digested with the respective restriction enzymes. The digested fragments were used to replace the G and M ORFs from the IHNV-06 cDNA clone and this creates pIHNV-06/61G and pIHNV-06/61M constructs, respectively. The trailer region of the virulent strain was replaced with the attenuated virus by digesting with *BsrGI* and *NotI* restriction enzymes, and this gives rise to pIHNV-06/61T construct. All the DNAs from full-length clones were sequenced completely for its integrity using an automated DNA sequencer (Applied Biosystems, CA).

DNA transfection and virus recovery

The full-length plasmid (1 µg), pN (0.5µg), pP (0.2 µg), pL (0.2 µg), and pNV (0.15 µg) were diluted in 500ul µl Opti-MEM® medium (Invitrogen, CA). Subsequently, Lipofectamine™ LTX reagent (Invitrogen, CA) was added slowly, according to manufacturer's instructions, and incubated for 30 min at room temperature. The plasmid

and Lipofectamine mixture was added to the EPC monolayers in six-well plates without replacing growth medium. The transfection mixture was removed after 8 h of incubation at 28^{o} C, and the transfected cells were washed and maintained in Eagle's MEM (ATCC, VA) containing 10% fetal bovine serum at 14°C for 5 days. Cell monolayer was observed for the development of virus-induced cytopathic effect (CPE). After 5 days of incubation, cells were submitted to three cycles of freeze-thawing. Supernatant was clarified by centrifugation at 8,000 x *g* in a microcentrifuge and used to inoculate fresh cell monolayers in T-25 flasks at 14°C. The supernatant was harvested and clarified for further processing of the recombinant viruses.

RT-PCR

RT-PCR was performed on the RNA extracted from the recovered viruses to demonstrate the presence of the chimeric genes. Briefly, the viral RNA was extracted from partially purified virus obtained after ultracentrifugation (collected from 26% sucrose cushion), using RNeasy® Mini Kit (Qiagen). Part of the RNA genome was amplified by RT-PCR using primer pairs covering M, G and trailer regions. The obtained RT-PCR products were then subjected to DNA sequencing to confirm the presence of the chimeric genes. Control reactions without RT were included to show that the PCR products were derived from RNA and not from transfected plasmid DNA.

Virus titration

Recombinant viruses were titrated on EPC cells by plaque assay, as described previously (Burke and Mulcahy, 1980) with modification. Briefly, the confluent

monolayers of EPC cells, grown in six-well plates, were infected with serially diluted supernatants from virus stock. After a 1-h incubation at 14°C, the cells were washed once by PBS and overlaid with 0.75% methylcellulose (Difco) in Eagle MEM containing 10% FBS. After 5 days of incubation at 14°C, the overlays were removed and the cells were fixed and stained with a solution containing 25% formalin, 10% ethanol, 5% acetic acid, and 1% crystal violet for 5 min at room temperature. After rinsing the cells with distilled water, the plaques were counted.

Growth curves in cell culture

To analyze the growth characteristics of rIHNVs, confluent EPC cells were infected with the recombinant virus stocks at an MOI of 0.01. Supernatants were collected at indicated time points post-infection and replaced by an equivalent volume of fresh medium. The collected samples were stored at -80°C, and titrated later in parallel by plaque assay. Each growth curve is based on the average of the virus titers from two infected monolayers.

Experimental fish infection

Virulence determinations were performed using the method of LaPatra *et al.* (1994). Briefly, groups (25 fish/group) of rainbow trout (*O. mykiss*) in duplicate (mean mass 12·0 g) were challenged by intraperitonial injection of 10^3 to 10^5 PFUs of representative IHNV isolates. Mock-infected control groups were exposed to cell culture media only. Experimental groups were held separately in 19 l aquaria at 15 °C which received ultra-violet light treated spring water, and fish were monitored minimum for 21 days.

5.4 Results

Fish studies of virulent and cell culture adapted IHN viruses

Fish studies were performed using the method of LaPatra et al. (1994) as described in the material and methods section. Rainbow trout juveniles were infected by intraperioneal injection of 10^5 PFUs of the virulent and cell culture adapted IHN viruses. The cumulative percent mortality in fish is shown in Fig. 5-1, which indicates that the virulence of cell culture adapted virus, IHNV-61, was reduced from 71% to 16%, compared to its parent virulent virus, IHNV-06.

Sequences of cell-adapted IHNV variants

To determine the molecular basis of virus adaptation in cell culture, and possible mechanism for virus attenuation, the serially passaged IHNV-61's genome was sequenced in its entirety. At the indicated passage, the viral RNAs were extracted, amplified by RT-PCR and their products were directly sequenced. The nucleotide and deduced amino acid sequences of both virulent and attenuated strains were compared. Table 5-2 shows the differences in the nucleotide sequences between the two viruses and resulting amino acid substitutions. The matrix protein gene shows 4 nucleotide differences that resulted in only one amino acid substitution, whereas glycoprotein gene shows 53 nucleotide differences, which are scattered all over the gene, and resulted in 17 amino acid substitutions. There are minor nucleotide differences in the UTRs between P and M (1 nt) and between M and G (5 nts) genes. There are 4 nucleotides differences in the trailer region between the virulent and cell culture adapted virus (IHNV-61).

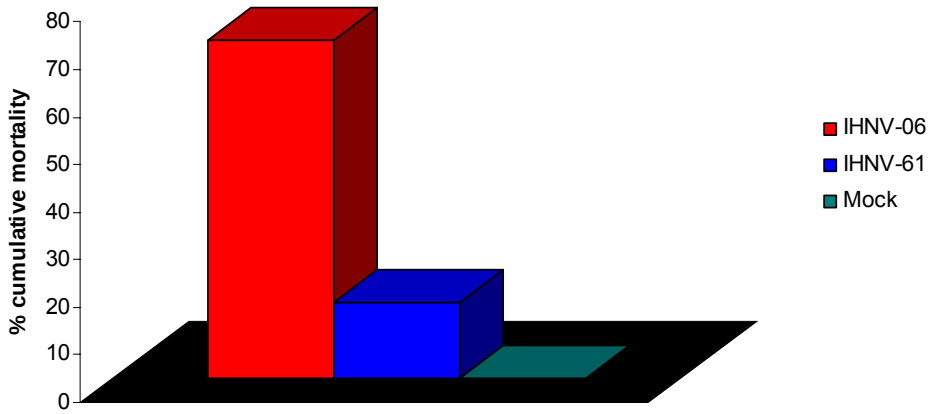

Fig. 5-1. Virulence comparison of virulent and cell culture adapted IHNV 220-90 in trout. Juvenile trout were infected by intra-peritoneal injection with 10^5 PFU/ml of the wild-type IHNV (wtIHNV-06), cell culture adapted IHNV (wtIHNV-61), or mock infected. Mortalities were recorded every day for 4 weeks and are expressed as a percentage of cumulative mortality. Mock-infected trout were treated under the same conditions as for other virus infected trout.

Table 5-2. Nucleotide and deduced amino acid differences between the virulent and attenuated viruses of IHNV 220-90 strain.

ORF	Nucleotide differences	Amino Acid differences
N	0	0
UTR	0	NA
P	0	0
UTR	1	NA
M	4	1
UTR	5	NA
G	53	17
UTR	0	NA
NV	0	0
UTR	0	NA
L	0	0
Trailer	4	NA

UTR-untranslated region, NA- not applicable

Recovery of chimeric viruses

In order to identify the genes involved in virus attenuation, we constructed chimeric viruses, rIHNV-06/61M, rIHNV-06/61G and rIHNV-06/61T in which the ORF of matrix and glycoprotein genes and trailer region of the virulent virus were replaced with the counterparts of attenuated strain, respectively. Unique restriction sites were created at the beginning and end of the M and G ORFs by overlapping PCR for the ease of swapping the fragments. By using the established reverse genetics method in our laboratory, full-length plasmids were cotransfected with pN, pP, pNV, and pL supporting plasmids into the EPC cells and incubated for 5 days at 14°C. The recombinant viruses were amplified by few cell passages and used for further studies. The genomic RNAs of the recovered viruses were analyzed after RT-PCR amplification and sequence analysis of the RT-PCR products confirmed the presence of chimeric sequences (data not shown).

Characterization of chimeric IHNVs *in vitro*

To investigate whether the M and G proteins or trailer region are involved in the viral adaptation *in vitro*, EPC cells were infected with each virus at MOI of 0.01, and the virus titer was analyzed by plaque assay. Figure 5-2 depicts the growth curve of each virus in EPC cells at different time points post-infection. All the viruses exhibiting typical CPE of IHNV at 36 hrs post-infection in EPC cell culture reached maximal virus production, approximately at 84 hrs p.i (Fig. 5-2). The recombinant rIHNV-06, rIHNV-61 and chimeric viruses did not exhibit significant differences in their replication kinetics in EPC cells, albeit the growth of rIHNV-06 is slightly lower than rIHNV-61 (Fig. 5-2).

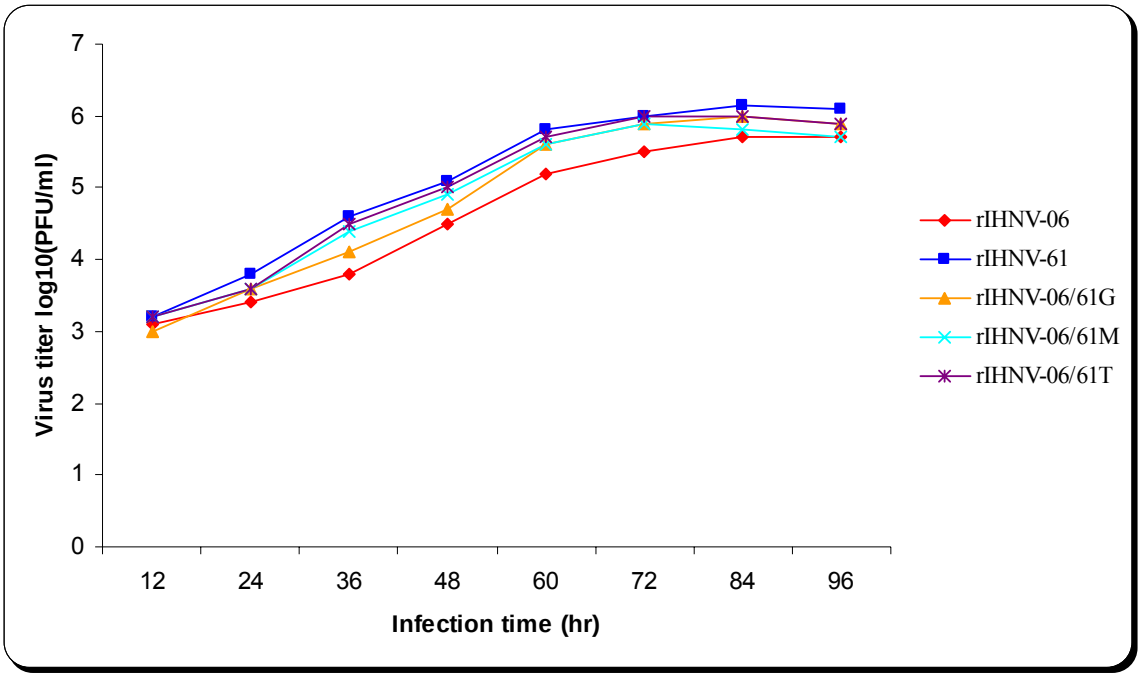

Fig. 5-2. Replication kinetics of the recombinant IHNVs in cell culture. Monolayers of EPC cells were infected with the indicated viruses at MOI of 0.01, harvested at the indicated time points, and infectious titers were determined by plaque assay.

Plaque phenotype of IHNV chimeras

To determine the role of viral proteins in plaque phenotype, the chimeric and recombinant parental viruses were subjected to plaque assay on EPC cells, and analyzed 9 days post-infection. The cells were then stained with crystal violet, and the plaque sizes and morphology were compared (data not shown). The plaques of all the three chimeric and two parental viruses had approximately the same size and appearance, indicating that the biological activity of chimeric viruses in tissue culture cells was not substantially altered.

Virulence studies in trout

The abilities of all of the recombinant viruses to replicate *in vivo* and to induce disease symptoms in trout were compared. Duplicate groups of 25 juvenile trout (mean weight, 12 g) were infected by intra-peritoneal injection of 10^3 PFUs of the rIHNV-06, rIHNV-61, rIHNV-06/61M, rIHNV-06/61G, and rIHNV-06/61T or mock infected. Mortalities were recorded every day for 4 weeks after virus exposure. As shown in Fig. 5-3, rIHNV-06 is more pathogenic for trout since the cumulative percent mortality (CPM) reached a plateau of 26% by 1 month p.i. Trout infected with these viruses developed typical symptoms of IHNV infection, and fish mortality started at day 7. In contrast, trout infected with the rIHNV-61 had a delayed mortality (started at day 10), and the CPM reached a plateau of 4% by 1 month p.i. The chimeric virus, rIHNV-06/61G, in which the virulent glycoprotein gene was swapped with the attenuated one, induced CPM of only 4% by 1 month p.i, which is similar to the attenuated recombinant rIHNV-61. The pathogenicity of other two chimeric viruses, rIHNV-06/61M and rIHNV-06/61T, was similar to that of virulent recombinant rIHNV-06.

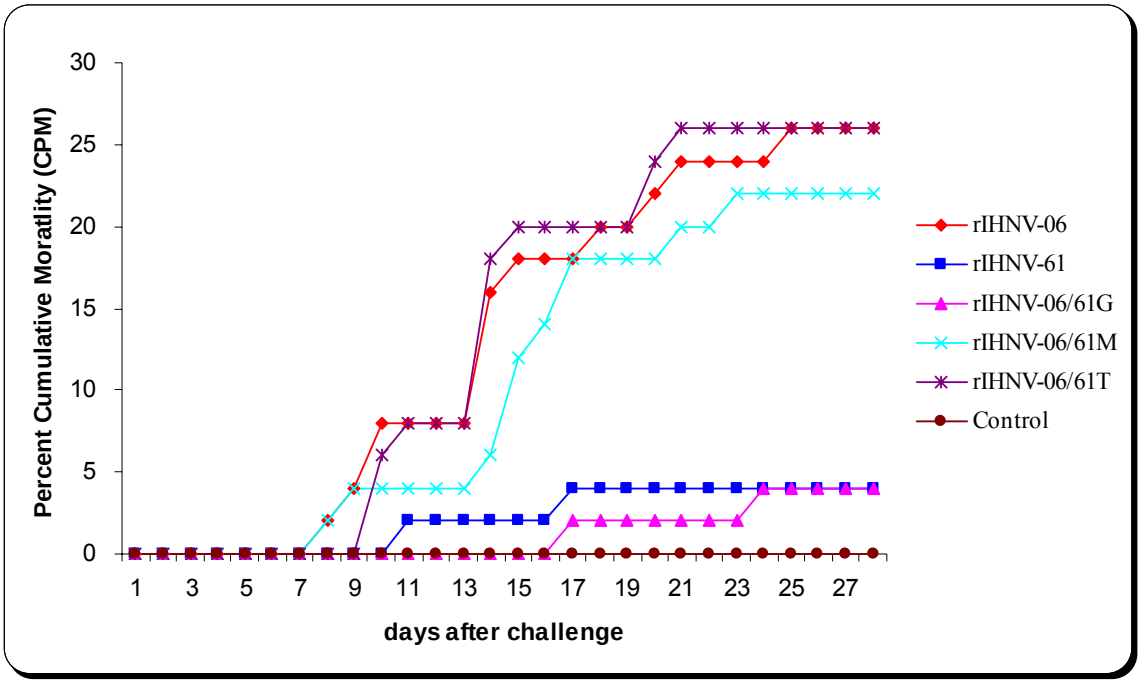

Fig. 5-3. Percent cumulative mortality caused by the recombinant viruses in trout. Juvenile rainbow trout were inoculated by intraperitoneal injection with 10^3 PFUs of rIHNV-06, rIHNV-61, rIHNV-06/61M, rIHNV-06/61G or rIHNV-06/61T or mock-infected with PBS. Mortalities were recorded every day for 4 weeks and are expressed as a percentage of cumulative mortality.

5.5 Discussion

Since RNA polymerases lack proofreading ability, the replication of RNA viruses is characterized by high mutation rates, which leads to rapid adaptation to their growth environment. This has historically been exploited to generate live attenuated vaccines. On the other hand, full understanding of the molecular processes of virus adaptation to particular host cells would ensure that no unrecognized adaptive mutations occur during propagation of virus in cell culture, which could be a source of misleading results and erroneous conclusions regarding the viral life cycle in the natural host (Mandl *et al.,* 2001). Cell-adapted viruses often confer reduced virulence in natural host animal. It would be of great interest to assess virulence of these cell-adapted and chimeric viruses in fish to identify specific residues important for virulence of IHNV.

In this study, we compared the virulence of IHNV strain 220-90 (IHNV-06) and its cell culture adapted counterpart, IHNV-61 (passaged 61 times in cell culture) in rainbow trout and found that the IHNV-06 induced CPM of 71%, whereas the cell culture adapted caused only 16% CPM. This fish experiment clearly indicates the attenuation of virus after serial passages in cell culture. Previous studies have shown that IHNV field isolates tend to lose their virulence after serial passage in cell culture (Fukuda *et al.,* 1989; Michael, 1974; Leong *et al.,* 1988). However, there was no complete sequence data to reveal the molecular basis of this process. Therefore, we determined the complete nucleotide and deduced amino acid sequences of both IHNV genomes to identify the molecular determinants of virulence. In earlier studies, (Ammayappan and Vakharia, 2009c), we determined the complete genome sequence of virulent IHNV 220-90 and in this study we also sequenced the entire genome of attenuated virus IHNV-61 for

comparison. Determination of the entire genome sequences of both viruses is necessary for the development of infectious clones of IHNV and to make chimeric viruses.

Comparison of the nucleotide and deduced amino acid sequences of the virulent and attenuated IHNV 220-90 strains revealed minor substitutions in the matrix protein gene (1 aa), UTRs (P/M-1 nt; M/G-5nts) and trailer sequence (4 nts), and major substitutions (53 nts) in the G protein gene (Table 5-2). The majority of nucleotide changes in the G gene is synonymous and resulted in only 17 amino acid substitutions. The presence of an excess of non-synonymous mutations (those which result in a predicted amino-acid change) compared to the number of synonymous mutations (those which are silent) in a gene or region of a gene has been interpreted as evidence for positive selection acting on that region (Nei and Gojobori 1986; Hughes and Hughes 1995; Seibert *et al.,* 1995). Earlier studies have shown that when 72 IHNV isolates from Haggerman Valley, Idaho were sequenced for its 303 nt region (mid-G) of the glycoprotein (G) gene (from nt 686 to 988), amino acids at positions 252, 256 and 270 each had an excess of non-synonymous mutations (Troyer and Kurath, 2003). In this study, glycoprotein gene exhibited maximum substitutions that are scattered all over the glycoprotein. Therefore, the precise residues involved in virulence and cell culture adaptation could not be identified. Hence, we utilized the reverse genetics approach to identify the molecular determinants of virulence.

Using infectious clones of IHNV, we were able to produce chimeric viruses of IHNV by substituting the M and G protein genes and trailer sequence of the virulent 220-90 strain with that of the attenuated one. To recover all the recombinant and chimeric

viruses, we used supporting plasmids pN, pP, pNV and pL of the virulent strain. RT-PCR and sequencing of the M, G and trailer region of the chimeric viruses were performed. Sequence analysis of the swapped regions of recombinant viruses showed the presence of chimeric genes. Surprisingly, the recovered viruses, rIHNV-06 and rIHNV-61, and chimeric viruses, rIHNV-06/61M, rIHNV-06/61G and rIHNV-06/61T, showed no difference in growth kinetics in cell culture.

To evaluate the pathogenicity of recombinant chimeric viruses in trout, groups of fish (25 fish/group, in duplicate) were injected intraperitoneally with 10^3 PFUs of the recombinant viruses. Fish injected with recombinant rIHNV-06 showed CPM of 26% at 28 days p.i., whereas fish injected with the rIHNV-61 exhibited only 4% CPM (Fig. 5-3). These results are as expected since rIHNV-06 was derived from the virulent virus (IHNV-06) that causes higher mortality than the cell culture adapted virus, IHNV-61. The chimeric rIHNV-06/61G in which the virulent G gene is exchanged with the attenuated one induced CPM of 4%. These results demonstrate that the virulence determinants of rIHNV-06 were lost (due to swapping) and the rIHNV-06/61G displayed pathogenesis which is comparable to that of the rIHNV-61. It is important to note that the virulence of the other two chimeric viruses, rIHNV-06/61M (virulent matrix gene is replaced with attenuated one) and rIHNV-06/61T (virulent 5' trailer is replaced with attenuated one), was between 22-26% CPM, suggesting that the matrix gene and 5'-trailer are not involved in the virulence of IHNV.

In earlier studies, virulent and mildly virulent strains were compared only for their glycoprotein gene sequences, especially the 'mid-G' region. Kim et al. (1994) proposed

that substitutions in the IHNV escape mutants at residues 78 and 218 were responsible for altered tissue tropism and loss of virulence. Bearzotti et al. (1995) demonstrated that as few as two concomitant amino acid substitutions in antigenic regions at residues 140 and 430 of the glycoprotein are sufficient to reduce the virulence of the VHSV in fish. To demonstrate that the glycoprotein is the sole determinant of virulence in IHNV, we exchanged the G protein of virulent virus with attenuated one, which results in the loss of virulence in rIHNV-06. However, we could not pinpoint the residues involved in virulence of IHNV. Therefore, mutational studies should be carried out on non-synonymous residues which have potential to be involved in pathogenesis. Sequence analysis of the virulent and attenuated viruses revealed no amino acid substitutions in the polymerase gene, suggesting that polymerase protein is not involved in the virulence and adaptation of IHNV to cell culture. In conclusion, we have demonstrated that the G protein carries the determinants for IHNV virulence and cell culture adaptation.

Chapter 6

Recombinant Infectious Hematopoietic Necrosis Virus as a Vector

6.1 Abstract

The two major pathogens of fish, infectious pancreatic necrosis virus (IPNV) and infectious salmon anemia virus (ISAV) cause disease in juvenile trout and salmon. Currently there are no efficient vaccines available for these diseases. Using the reverse genetics approach, we constructed a recombinant infectious hematopoietic necrosis virus (IHNV) vector from an attenuated vaccine strain of 220-90 to express the host-protective immunogen VP2 of IPNV and hemagglutinin-esterase (HE) of ISAV. The gene encoding the VP2 protein of the IPNV was inserted into the most 3'-proximal locus of a full-length IHNV cDNA, whereas the HE ORF was inserted between the N and P genes for high-level expression. We successfully recovered the recombinant IHNVs expressing the VP2 (rIHNV-VP2) and HE protein (rIHNV-HE) using a vaccinia virus-free reverse genetics system. The recombinant viruses, rIHNV-VP2 and rIHNV-HE, are genetically stable after at least five serial passages in cell culture and expressed high levels of VP2 and HE proteins. Our results indicate that recombinant IHNV could be a suitable vector to express immunodominant proteins of fish pathogens. The *in vivo* challenge studies are yet to be carried for rIHNV-VP2 and rIHNV-HE to investigate the efficiency of these viruses in protecting the fish against IPNV and ISAV, respectively.

6.2 Introduction

Infectious pancreatic necrosis virus (IPNV) is the causal agent of a highly contagious and destructive disease of juvenile rainbow and brook trout as well as Atlantic salmon (Wolf, 1988). Highly virulent strains of IPNV can cause greater than 90% mortality in hatchery stocks less than 4 months old. Survivors of infection can remain lifelong asymptomatic carriers of the virus, and these carriers serve as reservoirs of infection, shedding virus in their urine, feces, and reproductive products (McAllister *et al.,* 1987).

IPNV is the prototype virus of the *Birnaviridae* family and belongs to the *Aquabirnavirus* genus (Dobos, 1995a). The IPNV genome consists of two segments of double-stranded RNA that are surrounded by a single-shelled icosahedral capsid (Dobos, 1976). The larger of the two genomic segments, segment A encodes a 106-kDa precursor protein in a single large open reading frame (ORF) which is cotranslationally cleaved by the viral nonstructural (NS) protease to generate mature VP2 and VP3 structural proteins (Dobos, 1977, Duncan *et al.,* 1987). The genomic segment encodes VP1, a 94-kDa minor internal protein, which is the virion-associated RNA-dependent RNA polymerase (Dobos, 1995b; Duncan *et al* 1991). VP2 is the major outer capsid protein of virion, and type-specific neutralizing antibody is produced against this protein (Dobos, 1995b, Nicholson, 1993). It is also involved in the cell attachment (Granzow *et al* 1997; Kuznar *et al.,* 1995).

Many attempts have been made to express the structural proteins of IPNV as subunit vaccines for the control of this disease. It has been demonstrated that the recombinant VP2 protein expressed in different expression systems provided significant

protection against the disease (Manning and Leong, 1990; Shivappa *et al.,* 2005; Allnutt *et al.,* 2007). However, those efforts have not been translated for practical use, due to limitations of the delivery systems and these vaccines are not completely effective.

Infectious salmon anemia virus (ISAV) is a pathogen of economic importance affecting Atlantic salmon (*Salmo salar*) in Norway, Scotland, the Faeroe Islands, Canada, and the United States (Lovely *et al.,* 1999; Mullins *et al.,* 1998; Rodger and Richards, 1998; Schyth *et al.,* 2003; Thorud and Djupvik, 1988) and Coho salmon (*Oncorhynchus kisutch*) in Chile (Kibenge *et al.,* 2001). ISAV is the type species of the genus *Isavirus* belonging to the *Orthomyxoviridae*. The ISA virus (ISAV) has a negative-stranded RNA genome consisting of eight single-stranded segments that range from 1.0 to 2·3 kb (Mjaaland *et al.,* 1997) and the virions have two surface glycoproteins; haemagglutinin-esterase (HE) protein encoded by segment 6 and fusion (F) protein encoded by segment 5. ISAV HE has been shown to possess both haemagglutinating and receptor-destroying activity; the latter has been suggested to be an acetylesterase (Falk *et al.,* 1997). Eliassen *et al*. (2000) demonstrated that ISAV replicates in a manner similar to the influenza viruses. Immunoblot analyses indicated that ISAV HA, in contrast to influenza virus HA, is not post-translationally cleaved (Krossøy *et al.,* 2001). The presently available whole virus inactivated vaccine that is used in Maine, USA, and Canada does not fully protect against the virus (Kibenge *et al.,* 2003). It was suggested that the HE protein is the major determinant of virulence in ISAV (Cunningham *et al.,* 2002; Nylund *et al.,* 2003; Plarre *et al.,* 2005). The immunization studies against ISA using plasmids expressing the ISA virus HE demonstrated only moderate protection after challenge with ISA virus, with relative percent survival of 39.5 and 60.5 % in two parallel groups (Mikalsen *et al.,* 2005). For over

25 years, ISAV has caused major disease outbreaks in the Northern hemisphere, and remains an emerging fish pathogen because of the asymptomatic infections in marine wild fish and the potential for emergence of new epidemic strains (Kibenge *et al.,* 2009).

Infectious hematopoietic necrosis virus (IHNV) is a pathogen of major economic importance to the aquaculture industry. It causes infectious hematopoietic necrosis (IHN) disease in cultured trout and salmon worldwide, and is the most important viral disease of salmonid in the Northern hemisphere. Epizootics of IHNV are particularly devastating in rainbow trout hatcheries, and it infects primarily the juvenile stages of fish and also adults (Busch 1983.) IHNV is a nonsegmented negative-strand RNA virus from the *Rhabdoviridae* family, genus *Novirhabdovirus*. The genome of IHNV contains six open reading frames (ORF) in the order 3'-N-P-M-G-NV-L-5' (Kurath *et al.,* 1985).

We recently recovered IHNV from a full-length cDNA clone using a vaccinia virus-free reverse genetics system (Ammayappan and Vakharia, 2009b). This DNA-based recovery system for IHNV makes it possible to genetically engineer infectious virus. The highly efficient expression of viral and foreign proteins via negative-strand RNA virus vectors may have additional biotechnological applications. It is also possible that purification of expressed proteins could be made easier if they were incorporated into the extracellular virus particles. IHNV is an especially useful system because the virus can be grown to very high titers in fish cells and it is easily purified in large quantities. The major advantage of rhabdoviruses is the modular nature of their genomes which makes it easy to incorporate additional genes with extra transcription unit. The foreign genes can be engineered in any positions along the genome depending upon the need for

the level of expression and can be controlled by selecting an upstream or downstream location to insert the additional gene (Conzelmann *et al.,* 1998). Earlier studies have shown that recombinant IHNV could be used as a vector to express foreign genes (Biacchesi *et al.,* 2000; Ammayappan and Vakharia, 2009b). In this report, we describe the recovery of recombinant attenuated strain of IHNV 220-90 strain expressing the VP2 of IPNV and the HE of ISAV, respectively.

6.3 Materials and methods

Virus and cells

An attenuated strain of IHNV 220-90 (IHNV-61) was used for cloning of the full-length cDNA copy of the IHNV genome. IHNV-61 was kindly provided by Dr. Scott LaPatra, Clear Springs Foods Inc., Idaho, USA. The infectious pancreatic necrosis virus (IPNV) Buhl strain and infectious salmon anemia virus (ISAV) Maine-2003 strain were maintained in our laboratory. The e*pithelioma papulosum cyprini* (EPC) cells were used for IHNV propagation (Fijan *et al.,* 1983). The cells were grown at 28°C in minimal essential medium (MEM) supplemented with 10% fetal bovine serum and 2 mM L-glutamine. For preparation of IHN virus stocks, confluent monolayers of EPC cells were infected with the virus at a multiplicity of infection (MOI) of 0.01 in MEM with 2% fetal bovine serum. After 1 h of adsorption, the inoculum was removed, and the cells were incubated at 14°C until extensive cytopathic effect (CPE) was observed. The supernatant was collected 5 days post-infection (p.i), clarified and stored at -80^{o}C for further processing.

RNA extraction and amplification

Viral RNA was extracted from cell culture supernatant, using a Qiagen RNAeasy kit according to manufacturer's instructions, and stored at -20°C. The oligonucleotide primers used in this study are listed in Table 6-1. First strand synthesis was carried out in a tube containing 5µl of RNA, which was denatured at 70°C for 10 min in the presence of DMSO (3µl), 1 µl forward gene-specific primer, 1µl of 25 mM dNTPs, and snap-cooled on ice for 1 min. The reaction mixture containing 2µl of 10X RT buffer, 2µl of 0.1M DTT, 4µl of 25mM $MgCl_2$, 1µl of Superscript III RT™, and 1µl of RNase OUT™ was incubated at 50°C for 1 h. PCR amplifications were carried out using a *pfx*50™ PCR kit (Invitrogen, CA), according to manufacturer's instructions. Briefly, the following mixture was used for PCR amplification: 3µ1 of cDNA, 2µl of primer mix; 5µl of 10x PCR buffer [100 mM Tris-HCl (pH 9.0), 500 mM KC1, 1% Triton X-100], 2µ1 of 25 mM $MgCl_2$, 0.5ul of *pfx*50 polymerase, and 37µ1 of DEPC water, to make a final volume of 50 µ1. Reaction was carried out in a thermal cycler (MJ Research Inc., Waltham, MA), using the following program: denaturation at 94°C for 30sec; annealing for 30sec at 60°C; and extension at 68°C for 2 min. The RT-PCR products were separated by agarose gel electrophoresis and purified using a QIAquick gel extraction kit (Qiagen, CA).

Table 6-1. Oligonucleotides used for insertion of foreign genes in the IHNV genome

Primers	Sequences
VP2R	GCTGTTTGTGT TCATGCCTTTGAGGTTGGTAGGT
VP2F	TTAACGCCACCATG AGCACATCCAAGGCAAC
VP2 5'R	CATGGTGGCGTTAATTAA TCTGTCTCTCAGGTGTCG
N Start N/PR	GTCAT GGTGGCGGTTTGAGTTGAAAAGCAC
N Start N/PF	CTCAAACCGCCACC ATGACAAGCGCACTCAGA
N/P IGF	TGAACACAAACAGCCCCCCTT
HEN/PR	GGCTGTTTGTGTTCAAGCAACAGACAGAATT
HEPstF	TCTGCAGAAACCGCCACCATGGCACGATTC
N/P PstR	TGTTGTGGTTTCTGCAGAAAAGCACTATA
N/P PstF	TATAGTGCTTTTCTGCAGAAACCACAACA
500R	CTTCACGATCGTTTCTGCTAGCTTGTTG
1F	GTATAAGAAAAGTAACTTGAC
1R	CTTCCCTCGTATTCATCCTC
900F	CCTTCTAGAGGATCTGTGCAT

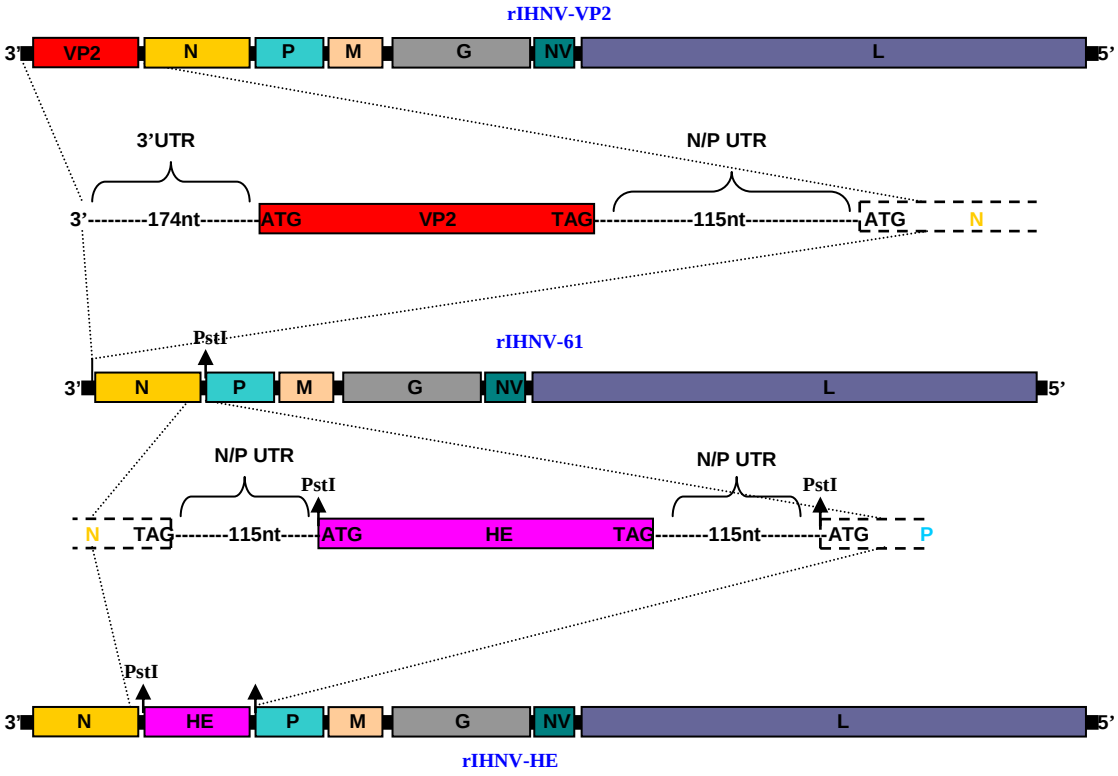

Fig. 6-1. Construction of the pIHNV-VP2 and pIHNV-HE plasmids. A 1,329 nt fragment containing a IPNV-VP2 ORF was inserted into the non-coding region immediately before the N ORF. The untranslated region (UTR) between 3' proximal of IHNV genome and N ORF (174nts) was fused at the N-terminus of the VP2 ORF. The UTR between the N and P ORFs was amplified and ligated with the VP2 ORF at the C-terminus. The pIHNV-61 was engineered by site-directed mutagenesis to introduce a *PstI* restriction enzyme site between the N and P genes, where the HE protein ORF was inserted. The UTR (115nts) between the N and P ORFs was amplified and fused with ISAV-HE ORF at the C-terminal. A *PstI* restriction enzyme site was introduced by PCR at both the ends of this cassette for cloning purpose.

Cloning and sequencing

The purified RT-PCR products were cloned into a pCR2.1 TOPO® TA vector (Invitrogen, CA). Plasmid DNA was sequenced by dideoxy chain termination method, using an automated DNA sequencer (Applied Biosystems, CA). All the DNA sequence analyses were performed by the Center of Marine Biotechnology (COMB), University of Maryland Biotechnology (UMBI) core facility, which houses an ABI 3130 XL Genetic Analyzer having 16-capillary electrophoresis channels (Applied Biosystems, CA).

Construction of full-length plasmid

The attenuated strain of IHNV 220-90, IHNV-61 was used produce recombinant viruses. Briefly, a full length cDNA copy of the IHNV RNA genome was constructed by assembling six overlapping cDNA fragments generated through RT-PCR by standard cloning techniques. The clones were ligated serially by natural or artificially created unique restriction sites as described earlier (Ammayappan and Vakharia, 2009b). Full-length clone was sequenced completely for its integrity using an automated DNA sequencer (Applied Biosystems, CA). The VP2 ORF (1,329 nt; GenBank access number, AF343573) was amplified from IPNV Buhl strain RNA by RT-PCR with specific primers (Table 6-1). An additional transcription unit comprises of untranslated regions between 3' proximal of IHNV genome and N ORF (174nts) was fused at the N-terminus of VP2 ORF. The untranslated region between the N and P ORFs was amplified and ligated with the VP2 ORF at the C-terminus. Without introducing any restriction sites, VP2 ORF was inserted with additional transcription unit at the 3'-end of the genome before N gene (Fig

6-1). This completed the full-length IHNV cDNA plasmid carrying VP2 ORF of IPNV (pIHNV-VP2).

The hemagglutinin-estrase (HE) ORF (1,185nts; GenBank access number, AY059402) was amplified from ISAV Maine-2003 strain RNA by RT-PCR with specific primers (Table 6-1). An additional transcription unit comprises of untranslated regions between the N and P ORFs (115nts) was fused at the C-terminus of HE ORF. The *PstI* restriction site was included in the PCR primers (Table 6-1), such that both ends of the HE ORF with additional transcription unit have *PstI* restriction sites. Then the HE ORF was inserted with additional transcription unit at the *PstI* restriction site which was created earlier in the full-length clone at the beginning of the P ORF ahead of start codon (Fig 6-1). This completed the full-length IHNV cDNA plasmid carrying HE ORF of ISAV (pIHNV-HE).

DNA transfection and virus recovery

The plasmids pIHNV-220-90 (1 µg), pN (0.5µg), pP (0.2 µg), pL (0.2 µg), and pNV (0.15 µg) were diluted in 500ul µl Opti-MEM® medium (Invitrogen, CA). Next, Lipofectamine™ LTX reagent (Invitrogen, CA) was added slowly, according to manufacturer's instructions, and incubated for 30 min at room temperature. The plasmid – Lipofectamine reaction mixture was added to the EPC monolayer in a six-well plate without replacing the growth medium. The transfection mixture was removed after 8 h of incubation at 28^{o} C, and the transfected cells were washed and maintained in Eagle's MEM (ATCC, VA) containing 10% fetal bovine serum at 14°C for 5 days. Cell monolayer was observed for the development of virus-induced cytopathic effect (CPE)

and also expression of EGFP (cells transfected with the EGFP plasmid). After 5 days of incubation, the cells were subjected to three cycles of freeze-thawing. Supernatant was clarified by centrifugation at 8,000 x *g* in a microcentrifuge, and used to inoculate fresh cell monolayers in T-25 flasks at 14°C. The supernatant was harvested and clarified for further processing of the recombinant viruses.

RT-PCR

RT-PCR was performed on the RNA extracted from the recovered viruses to demonstrate the presence of the VP2 and HE ORFs. Briefly, viral RNA was extracted from partially purified virus on 26% sucrose cushion, using RNeasy® Mini Kit (Qiagen) and RT-PCR was performed using primers specific for IHNV, which cover VP2 and HE ORFs respectively. The obtained RT-PCR products were then subjected to DNA sequencing to confirm the presence of VP2 and HE ORFs. Control reactions without RT were included to show that the PCR products were derived from RNA and not from transfected plasmid DNA.

Western blot analysis

To confirm expression of the recombinant proteins, Western blot analysis was performed using infected cell culture lysate. After CPE was complete (4-5days), the flasks were subjected to one cycle of freeze-thawing. The cell culture supernatant was clarified by low-speed centrifugation and the proteins were pelleted by ultra-centrifugation at 70,000 x *g* for 2 h. The pelleted materials were resuspended in TNE buffer and the expression of proteins was checked by fractionating on a 10% SDS-PAGE. The gel was transferred to nitrocellulose (NC) membrane by electroblotting. The NC

membranes were blocked with 5% skim milk and incubated at 37°C for 1 hour with polyclonal antibody against IPNV and ISAV, respectively. After washing three times with PBST buffer (PBS pH 7.4 with 0.1% Tween-20), the NC membrane was incubated with alkaline phosphatase-labeled secondary antibody (KPL, Gaithersburg, MD). Detection was obtained using the colorimetric substrate Fast red and Napthol in 0.1 M Tris-Cl buffer pH 8.0.

Virus titration

Virus titration was carried out in the EPC cells by plaque assay, as described previously with modification (Burke and Mulcahy, 1980). Briefly, the confluent monolayers of EPC cells, grown in six-well plates, were infected with serially diluted supernatants from virus stock. After a 1-h incubation at 14°C, the cells were washed once by PBS and overlaid with 0.75% methylcellulose (Difco) in Eagle MEM containing 10% FBS. After 7 days of incubation at 14°C, the overlays were removed and the cells were fixed and stained with a solution containing 25% formalin, 10% ethanol, 5% acetic acid, and 1% crystal violet for 5 min at room temperature. After rinsing the cells with distilled water, the plaques were counted.

Virus growth characteristics in cell culture

Multiple-step growth characteristics of the recombinant viruses were compared with those of the parental virus. Briefly, EPC cell monolayers were infected with 0.01 MOI of the rIHNV, rIHNV-VP2 and rIHNV-HE viruses. Supernatants were collected at indicated time points post-infection and replaced by an equivalent volume of fresh medium. The collected samples were stored at -80°C, and titrated later in parallel by

plaque assay. Each growth curve is based on the average of the virus titers from two infected monolayers.

6.4 Results

Construction of cDNA encoding IHNV antigenomic RNA containing the foreign epitopes

The full-length cDNA clone of attenuated strain of IHNV 220-90, IHNV-61 was used as a base vector to express IPNV major coat protein VP2 and also ISAV neutralizing glycoprotein HE. The VP2 protein ORF was inserted at the 3' proximal of the genome ahead of the nucleocapsid protein gene. The UTR (174nts) between 3' proximal of IHNV genome and N ORF and the UTR between the N and P ORFs was amplified and fused with IPNV VP2 ORF. This cassette was used to replace the 174 nucleotide long 3'UTR of IHNV genome and this replacement introduced an additional ORF of VP2 and N/P UTR. The insertion of this VP2- transcription cassette in between 3'terminus and N ORF, to yield the rIHNV-VP2 cDNA, increased the length of the encoded antigenome to a total of 12,578 nucleotides. While the wild-type IHNV encodes 6 major subgenomic mRNAs, the recombinant virus rIHNV-VP2 would encode 7 major subgenomic mRNAs. The strategy of cDNA construction is shown in Fig. 6-1. The HE protein ORF was inserted between the N and P genes. The UTR (115nts) between the N and P ORFs was amplified and fused with ISAV HE ORF at the C-terminal. A *PstI* restriction enzyme site was introduced by PCR at both ends of this cassette. This cassette was inserted at the *PstI* site which was created earlier in the IHNV full-engtth clone at the beginning of P ORF start codon (Fig 6-1). This insertion introduced an additional ORF

and additional N/P UTR, and increased the length of the encoded antigenome to a total of 12,434 nucleotides.

Recovery of infectious virus

The strategy for producing infectious IHNV from cDNA-encoded antigenomic RNA was described in detail in the Methods section. It involves the coexpression in EPC cells of the five cDNAs encoding the antigenomic RNA and the N, P, L and NV proteins, which are necessary and sufficient for viral RNA replication and transcription. All these plasmids are under the control of CMV promoter and the cDNA expression was driven by cellular RNA polymerase II. Transfection experiments were carried out in EPC cell monolayers in six-well plates at 28°C. Cells were transfected with a mixture of full-length and supporting plasmids and then shifted to 14°C after 8hr incubation. The recombinant viruses were amplified by few cell passages and used for further studies.

Analysis of the gene insertions by RT-PCR

RT-PCR was used to confirm the presence of the VP2 and HE genes in the predicted location of the genome of recombinant IHNV. The cell supernatant was pelleted through 26% sucrose and the genomic RNA was extracted from pelleted recombinant viruses. Two primers that flank the site of insertion of VP2 and also HE were chosen. As shown in Fig. 6-2, RT-PCR of the rIHNV virus (lane 2 and 4) yielded a single band that corresponded to the predicted fragments of ~600 and ~1200 nucleotides, which would represent approximately the first 600nts of the IHNV genome and 1200nts comprise of partial N and P genes, respectively without additional foreign sequences.

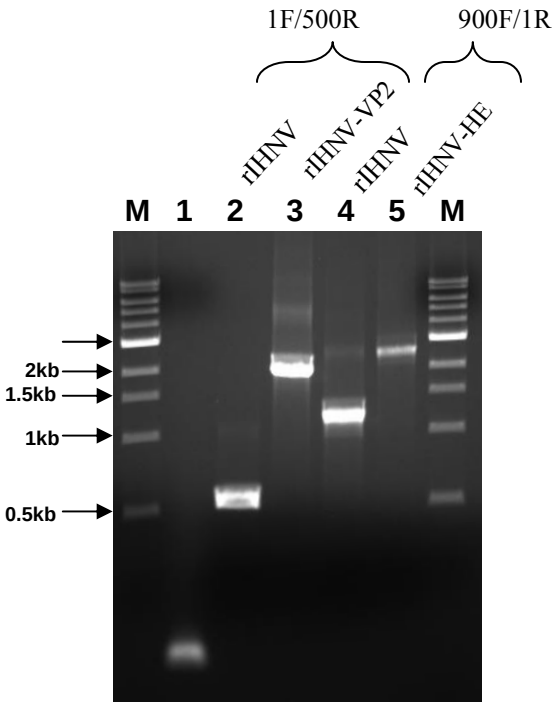

Fig. 6-2. Gel analysis of the RT-PCR products used to confirm the presence of the VP2 and the HE genes in the genome of rIHNV-VP2 and rIHNV-HE, respectively. Reverse transcriptase PCR (RT-PCR) was carried out from the RNA extracted from pelleted recombinant IHNV (rIHNV) or rIHNV expressing IPNV-VP2 protein or rIHNV expressing HE protein of ISAV. RT-PCR was performed using primers specific for IHNV, which covers VP2 and HE ORFs, respectively. The product of lanes 1 and 2 is the result of 1F and 500R primers; lanes 3 and 4 is the result of 900F and 1R primers which are specific for the IHNV genome. Lane 1, control reaction without RT. The sizes of the molecular marker are indicated on the left. The larger RT-PCR products from rIHNV-VP2 (2.1 kb) (lane 3) and rIHNV-HE (2.8 kb) (lane 5) confirmed the presence of the VP2 and HE genes, compared to the smaller RT-PCR products (0.6 kb and 1.2 kb, lanes 2 and 4, respectively) from rIHNV.

Analysis of rIHNV-VP2 viral RNA yielded a single product whose electrophoretic mobility corresponded to that of the predicted ~2100 nucleotide fragment (lane 3), which would represent the first 174 UTRs, VP2 ORF, UTR N/P ORF and ~350 nts from N ORF. At the same time, analysis of rIHNV-HE viral RNA yielded a single product with predicted ~2800 nucleotide fragment (lane 5), which would represent the last ~450nts of N ORF, N/P UTR, HE ORF, and first ~630nts of P ORF. When PCR analysis was performed without the RT step, no band was seen (lane 1), confirming that the analysis was specific to RNA. Thus, the RT-PCR analysis confirmed the presence of the VP2 ORF and also HE ORF inserts at the predicted locations of the genomic RNA of the recombinant IHNVs.

Expression of the VP2 and HE proteins by recombinant IHNVs

To examine the expression of VP2 and HE proteins by the rIHNVs, EPC cells were infected with rIHNV-VP2 and rIHNV-HE viruses at an MOI of 1. The cell culture supernatant was collected after completion of CPE and subjected to low speed clarification. The clarified supernatant was ultracentrifuged and the pellets were resuspended in TNE buffer. The viral proteins were separated on a two separate SDS-10% polyacrylamide gels. The proteins were transferred to a nitrocellulose membrane, and the membranes were treated with polyclonal antibodies against ISAV and IPNV, respectively. The supernatant of rIHNV-VP2 and rIHNV-HE infected cells showed strong bands corresponding to the VP2 and HE proteins, respectively (Fig. 6-3). In addition to the 42kDa HE protein band, two other less intense major protein bands were noticed which probably represents a tightly bound dimer or broken part of dimer that

could not be separated by the SDS-PAGE dissociation buffer, as described earlier (Krossøy *et al.,* 2001).

Growth kinetics of recombinant viruses and plaque morphology

The efficiency of replication of recombinant viruses in tissue culture was compared in a multi-step growth cycle. Duplicate monolayers of cells were infected with each virus, and samples were taken at 12-h intervals and quantitated by plaque assay. The replication efficiency of the recombinant viruses appeared to be lower than that of the wild-type virus (Fig. 6-4). This analysis showed that the production of rIHNV-VP2 and rIHNV-HE viruses (relative to that of rIHNV virus) was delayed and reached a maximum titer which was 1 log lower. EPC cells were infected with rIHNV or rIHNV-VP2 or rIHNV-HE viruses and incubated for several days until the appearance of plaques. The cells were then fixed and stained with crystal violet, and the plaque morphology was compared. The plaque sizes of the rIHNV-VP2 or rIHNV-HE viruses were comparatively smaller than that of rIHNV virus (Fig. 6-5). However, the microscopic appearance of plaques for all these viruses was approximately the same (data not shown). These results indicate that even though the biological activity of recombinant viruses carrying additional genes in cell culture was different, the change was not drastic.

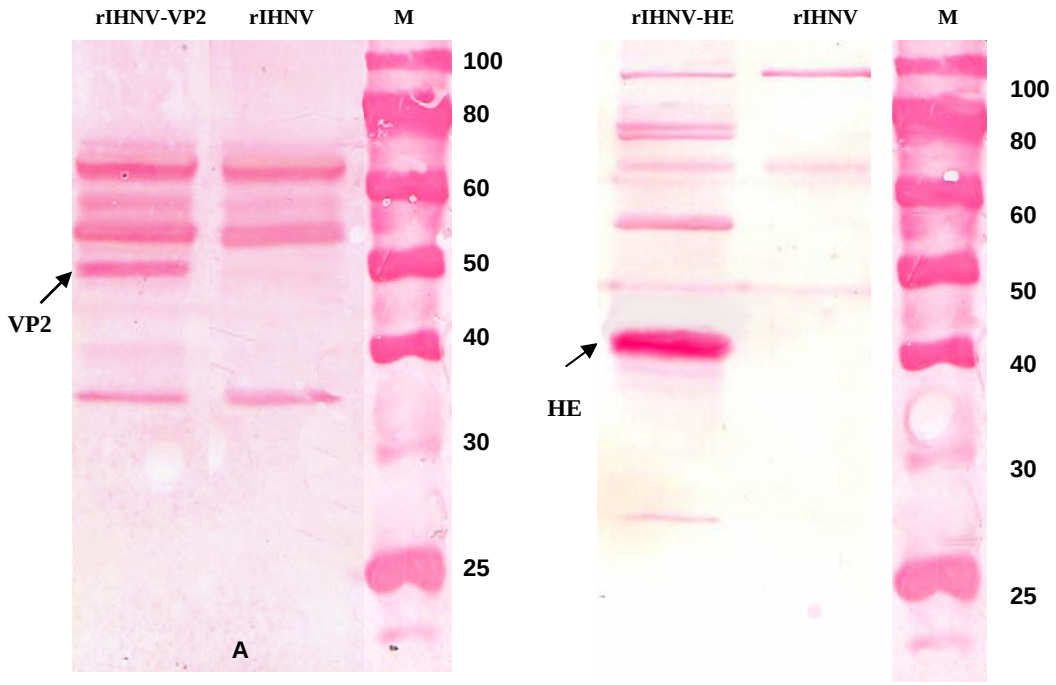

Fig. 6-3. Western blot analysis of the recombinant IHNV expressing foreign proteins**.** EPC cells were infected with the rescued rIHNV-VP2, rIHNV-HE and rIHNV viruses at an MOI of 1.0. Five days later or after completion of CPE, supernatants were collected, clarified and subjected to ultracentrifugation. The pellet was loaded onto 10% SDS-polyacrylamide gel. Expression of foreign proteins was detected by western blotting using polyclonal antibodies against wild-type IPNV and ISAV, respectively. **A)** Expression of the VP2 protein (~50kDa) of IPNV, **B)** Expression of the HE protein (~42kDa) of ISAV. Lane M: Molecular weight marker (molecular weight in kDa is shown on the right)

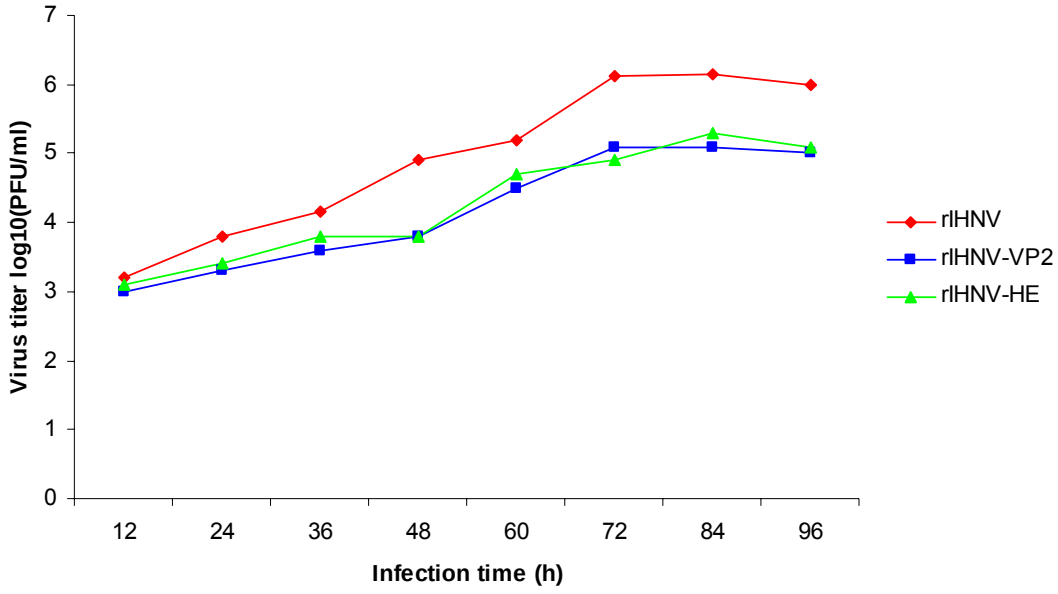

Fig .6-4. Growth kinetics of recombinant viruses in EPC cells. EPC cell line was infected (at 0.01 MOI) with the recombinant IHNV (rIHNV) (♦), recombinant IHNV expressing VP2 protein (rIHNV-VP2) (■), or recombinant IHNV expressing HE protein (rIHNV-HE) (▲). The cell culture supernatant was collected at 12hr intervals after post-infection. Supernatants were serially diluted and each dilution (100 µl) was for infection of 12 well plates. After 1 h of virus adsorption, supernatants were removed from wells and then overlaid with 0.75% methylcellulose. The infected cells were incubated at 14°C for 7 days or until the development of countable plaques.

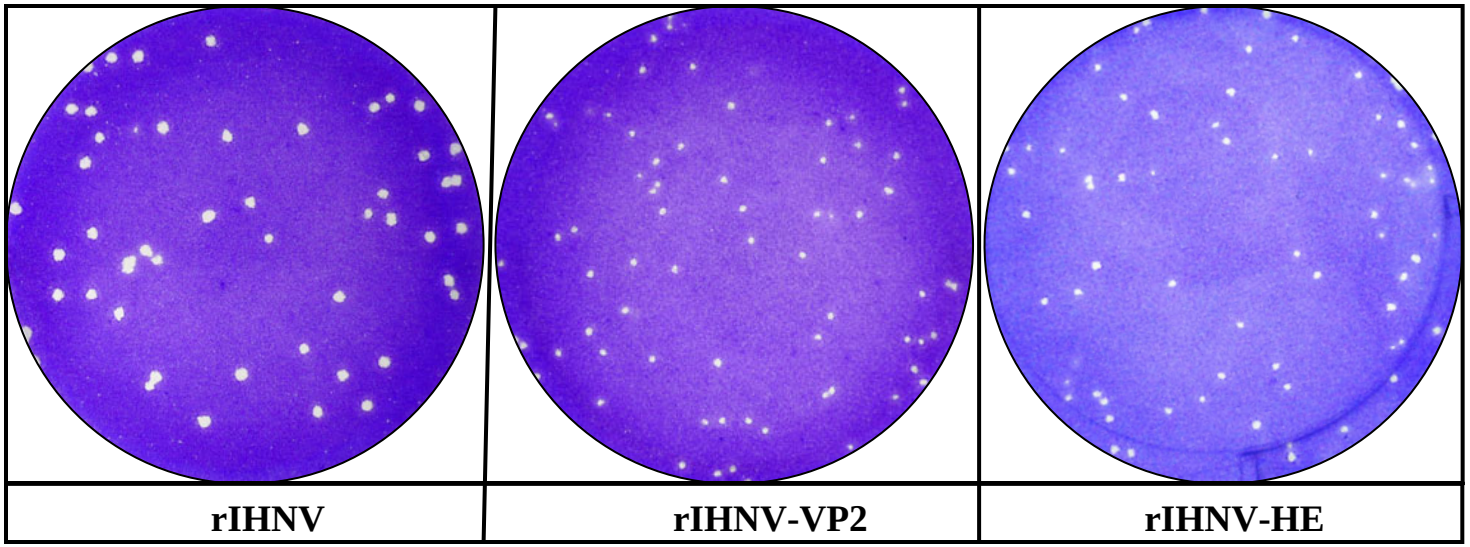

Fig. 6-5. Plaque morphology of recombinant rIHNV, rIHNV-VP2 and rIHNV-HE in EPC cells.EPC cells were infected with recombinant viruses by absorption for 1 hr. Cells were rinsed and immobilized with overlay medium containing 0.75% methylcellulose. At 9 days post-infection, the cells were fixed and stained with crystal violet.

6.5 Discussion

Vectored vaccines facilitate a live-vaccine approach that does not involve the complete pathogen. Many negative-strand RNA viruses with non-segmented genomes have potential for use as vaccine vectors. The modular nature of their genomes makes it easy to engineer foreign genes (Conzelmann *et al.,* 1998). Many researchers demonstrated that foreign genes could be expressed stably for several passages (Bukreyev *et al.,* 1996; Mebatsion *et al.,* 1996; Schnell *et al.,* 1996; Hasan *et al.,* 1998; He *et al.,* 1997). Homologous RNA recombination has not been demonstrated for non-segmented negative sense RNA viruses, which contributes to the stability of these vectors (Lamb and Kolakofsky, 1996; Palese *et al.,* 1996). Rhabdoviruses has been utilized not only to express foreign genes but also to be used as a vaccine vector (Rose *et al.,* 2001; Kapadia *et al.,* 2005; Buonocore *et al.,* 2002; Reuter *et al.,* 2002; Schlereth *et al.,* 2003; Faber *et al.,* 2005; McGettigan *et al.,* 2001a; McKenna *et al.,* 2006).

IHNV is particularly well suited to the development of a rhabdovirus vaccine vector for fish pathogens for several reasons. (i) IHNV grows to very high titers in fish cell lines especially EPC, which allows cost-effective and easy manufacture of the vaccine, (ii) IHNV recombinants expressing foreign proteins are therefore likely to grow to high titers and to produce the foreign proteins at high levels, (iii) IHNV is a rhabdovirus, its modular nature of genome which encodes only six proteins, allows easy expression of immunogeneic foreign proteins, and (iv) IHNV naturally infects trout and

salmon which are the common host for many fish viruses. Thus, IHNV would be a suitable vector for expressing immunodominant proteins of fish pathogens.

VP2 is the major outer capsid protein of IPNV and is responsible for the production of type-specific neutralizing antibody (Dobos, 1995b; Nicholson, 1993). HE protein is one of the two major envelop proteins of ISAV and produced neutralizing antibodies against ISAV (Falk *et al.,* 1998; Cunningham *et al.,* 2002; Nylund *et al.,* 2003; Plarre *et al.,* 2005). Since VP2 and HE are the major neutralizing epitopes, they should be delivered in a native conformation which is critical for correct antigen processing and presentation. The expression systems currently being used to express fish viral proteins are not efficient simply because of their higher temperature or incompatible cell line nature. This results in misfolding, conformational instability and inefficient glycosylation of proteins (Cain *et al.,* 1999; Lecocq-Xhonneux *et al*. 1994; Lorenzen and Olesen 1997; Alonso *et al.,* 2004). Research on rhabdovirus G proteins suggests that glycosylation may be critical for neutralizing antibody formation (Machamer and Rose 1988, Prehaud *et al.,* 1989). Since ISAV and IPNV grow at low temperature (15 and 20°C, respectively), the immunogenic proteins should be expressed at low temperature to maintain the epitope conformation and to stimulate appropriate immune response. IHNV is an ideal vector to express these immunogenic proteins because it grows at 14°C in EPC cells. Various attempts (Christie, 1997; Shivappa *et al.,* 2005; Allnutt *et al.,* 2007) to develop a vaccine against IPNV have not yielded consistent results. Thus, at present, no commercial vaccine is available that can be used with confidence to immunize fry of salmon and trout. In case of ISAV, although autologous vaccines are used in certain areas, no product

is currently licensed for general use. Hence, it is essential to develop a novel vaccine for mass vaccination of small fish.

In this study, we expressed VP2 protein of IPNV at the 3' proximal location of IHNV genome before the N protein ORF and HE protein of ISAV in between the N and P genes. Western blot analysis shows high level expression of VP2 and HE proteins (Fig. 6-3). At the same time, the replication kinetics shows (Fig. 6-4) that the rIHNV-VP2 and rIHNV-HE viruses grow at least one log lower than its parental virus (rIHNV). The presence of the additional gene slightly retarded the virus replication and resulted in a one-fold decrease in virus yield during multi-cycle growth. The transcriptional attenuation at the border between the IHNV genome and the additional genes could also be responsible for reduced virus replication, as demonstrated earlier (Hassan *et al.,* 1998; Bukreyev *et al.,* 1996). The viral expression of VP2 and HE proteins was stable at least for five serial passages. The results from standard passages, in each of which the virus underwent numerous rounds of replication, suggested a high degree of functional stability of the inserted gene, in accordance with other foreign gene expression systems reported for non-segmented negative-strand RNA viruses (Schnell *et al.,* 1996a, 1996b; Mebatsion *et al.,* 1996; Bukreyev *et al.,* 1996).

The recombinant IHNV viruses carrying VP2 and HE proteins (rIHNV-VP2 and rIHNV-HE) have several advantages over the existing IPNV and ISAV vaccines. The recombinant viruses will be highly economical for the aquaculture industry in terms of mass vaccination by water-borne route which would reduce the cost of vaccination. Since there is no live IPN or ISA virus is given, there is no danger of virus carriers or reversion

in virulence. Finally, it was reported that the NV protein of IHNV is associated with viral pathogenesis and essential for viral replication (Thoulouze *et al.,* 2004). It was also demonstrated that elimination of the NV protein expression in IHNV rendered the virus attenuated but still immunogenic and that the attenuated IHNV vaccine strain could be administered in water (Romero *et al.,* 2008). Therefore, the recombinant virus can be tailored with ease for live mass vaccination. Thus, the recombinant virus described here for the protection of IHNV, IPNV and ISAV will be highly beneficial to the aquaculture industry. However, their practical use still needs to be evaluated, particularly with regard to factors such as safety, route of delivery and efficacy.

Chapter 7

Conclusions and future studies

7.1 Conclusions

With the ultimate goal of producing a recombinant live-attenuated vaccine to control IHNV infections, we have established a reverse-genetics system for IHNV 220-90 strain. Information about the complete genomic sequence of IHNV-220-90 is however essential for genetic manipulation. Hence, in this study, prior to the development of the virus rescue system, we determined the unknown sequences (except G gene) of IHNV-220-90. The complete genomic sequence of 220-90 strain was determined by RT-PCR of genomic RNA from six overlapping clones. The complete genome sequence of 220-90 comprises 11,133 nucleotides (GenBank GQ413939) with the gene order of 3'-N-P-M-G-NV-L-5'. These genes are separated by conserved gene junctions, with di-nucleotide gene spacers. The nucleotides at the 3'- and 5'-termini show complementarity as the other novirhabdoviruses.

The complete genomic sequence of IHNV 220-90 determined by us was utilized to establish a reverse genetics system for IHNV. A first reverse genetics for *Novirhabdovirus* was developed by Johnson *et al.,* 2000, for snakehead rhabdovirus (SHRV) which was followed by the recovery of IHNV by Biacchesi *et al.,* 2000. These systems utilized vaccinia virus expressing T7 RNAP to rescue the recombinant viruses. Hence, there remains a need for development of methods for production of recombinant novirhabdoviruses where such methods are independent of the need for T7 RNAP and

independent of a vaccinia helper virus. The present study provides a method for production of recombinant rhabdoviruses which is completely free of vaccinia helper virus and T7 RNAP. The methods include use of a plasmid containing a full-length cDNA copy of the virulent IHNV (strain 220-90), flanked by hammerhead ribozyme (HHRz) and hepatitis delta virus ribozyme (HdvRz) sequences. The viral cDNA is under the control of cytomegalovirus (CMV) immediate-early promoter and is transcribed by cellular RNA polymerase II upon transfection. This provides a more efficient and convenient method for recovery of any *Novirhabdoviruses*.

IHNV is one of the detrimental diseases affecting salmonids and always a major threat to aquaculture. No licensed commercial vaccines aimed at mass vaccination of the juveniles are available. Fish surviving infection with IHNV are generally resistant to re-infection and often possess IHNV neutralizing antibodies. The ability of the virus to elicit an immune response in salmonids suggests that vaccine development may be feasible and provide an effective method of disease control. The development of vaccines against IHNV has focused on killed or live whole virus preparations (Nishimura *et al.,* 1985; Leong *et al.,* 1988), recombinant subunit vaccines (Gilmore *et al.,* 1988), synthetic peptide (Emmenegger *et al.,* 1977) and DNA vaccines (Anderson *et al.,* 1996). Killed and live virus vaccines provide protection but regulatory concerns, cost of production and residual virulence has inhibited commercial licensing. The situation has prompted development of recombinant subunit vaccines expressing epitopes of the glycoprotein of IHNV in bacteria (Gilmore *et al.,* 1988; Xu *et al.,* 1991, Leong and Fryer, 1993), or baculovirus (Koener and Leong, 1990; Cain *et al.,* 1999) but with limited experimental success. Therefore, it is necessary to develop a highly stable and efficient IHNV vaccine.

To achieve this goal, it is necessary to identify the role of each viral protein in pathogenesis and virulence. The development of reverse genetics techniques has made possible to investigate host interaction and pathogenicity of negative-strand RNA virus at a molecular level in greater detail. Particularly, genetic manipulation of the viral RNA genome can be performed at the cDNA level and infectious virus can be recovered and studied in greater detail for their virulence and pathogenesis. With the help of a reverse genetics system, we have investigated the role of the G protein in IHNV pathogenesis. Since the G protein of IHNV is a envelop protein which is involved in viral attachment and cell entry, studying the role of G gene is important to understand the molecular basis of the viral virulence. Using the reverse genetic system of a virulent IHNV strain 220-90, we exchanged the G gene with the attenuated strain, IHNV-61 and then studied those chimeric recombinant viruses for their replication and pathogenesis. Our studies demonstrated that the G protein of IHNV plays a major role in the virulence of IHNV.

To demonstrate the vector potential of rIHNV-220-90, we recovered recombinant IHNV expressing EGFP (rIHNV-EGFP) as a foreign protein through our improved reverse genetics technique. The recovered recombinant virus showed growth properties similar to that of the wild-type virus in tissue culture, but the titer of the EGFP-expressing virus was slightly reduced when compared to rIHNV. The rIHNV-EGFP virus expressed EGFP stably for at least ten serial passages in cell culture. These results demonstrated that the recovered recombinant IHNV in this study could be used as a vector to stably express foreign proteins. Therefore, we further explored the vector potential of IHNV 220-90 by expressing immunogenic proteins of two important fish viruses. The infectious pancreatic necrosis virus (IPNV) and the infectious salmon anemia virus (ISAV) are two

of the notifiable fish diseases listed by OIE. We expressed VP2 protein, which is the major coat protein of IPNV and HE protein, which is the immunodominant envelop protein of ISAV using recombinant IHNV. The VP2 protein was expressed from the very 3' proximal of the IHNV genome and HE protein was expressed between the N and P genes. The growth properties of these recombinant viruses showed that they grew one log titer lower and produced smaller size plaques than their parental recombinant virus. The rIHNV-VP2 and rIHNV-HE viruses expressed VP2 and HE proteins stably for at least for five serial passages in cell culture, which demonstrated that IHNV could be used as a vaccine vector to express immunodominant proteins of other fish pathogens.

The major findings of this research are:

1. The complete sequence of IHNV 220-90 strain is determined. It consists of 11,133 nucleotide long single-stranded negative-sense genome and contains six genes in the order: 3'-N-P-M-G-NV-L-5'.
2. The complete sequence of attenuated strain of IHNV 220-90 (IHNV-61) is also determined and compared with the virulent strain. The glycoprotein gene has majority of differences and contains 17 amino acid substitutions, and M protein has one amino acid substitution.
3. Recombinant IHNV 220-90 was successfully recovered entirely from cDNA by reverse genetics approach. An efficient method of virus recovery for IHNV is established by rescuing recombinant virus using vaccinia virus-free system. This system utilized cellular polymerase II to drive cytomegalovirus (CMV) promoter plasmids.

4. The chimeric viruses produced by swapping of the M and G protein genes and 5' trailer between the virulent and attenuated strain, demonstrated that molecular determinants of virulence resides mainly in the glycoprotein gene.
5. The vector potential of IHNV was explored by expressing VP2 protein, which is the major coat protein of IPNV and HE protein, which is the immunodominant envelop protein of ISAV, and demonstrated that IHNV could be used as a vaccine vector to express immunodominant proteins of various fish pathogens.

7.2 Future studies

The role of an individual viral gene can be easily studied in the context of infectious virus, as opposed to studying the gene function in isolation. Desired mutations or deletions can be introduced in the IHNV genome with relative ease and the role of viral promoters, transcription signals, non-coding regions, and the intergenic sequences can be investigated in detail.

In tissue culture, IHNV infection causes the shutdown of host protein synthesis (Hsu *et al.,* 1986; Leong *et al.,* 1983) and cytopathology characterized by cell rounding and cell death. Persistent infection has also been established in fish cells infected with IHNV (Engelking and Leong, 1981). It was demonstrated that over-expression of matrix protein alone causes inhibition of host transcription and down regulation of host protein synthesis which are common functions of the matrix proteins of the *Rhabdoviridae* family (Chiou *et al.,* 2000). Mutations in viral genes required for cytopathic effects are often involved in the establishment of persistent infections (Black and Lyles, 1992). Similar mechanisms may also apply to the many negative-strand RNA viruses in which matrix

protein is known to establish persistent infections (Ayata *et al.,* 1989; Cattaneo *et al.,* 1988; Roux and Waldvogel, 1982). One of the major concerns in IHNV infection is the survivors are long-term carriers of the virus and no determination has been made on whether vaccination also prevents the formation of a virus-carrier state in the survivors. Therfore, it is of paramount importance to develop a vaccine that will prevent carrier formation. It is an attractive hypothesis that the establishment of persistence could be circumvented by mutating the M protein of IHNV and it could be achieved by reverse genetic technology established for IHNV.

Additional studies using N, P, NV and L genes of other virulent and avirulent strains will also provide more insight towards the critical role of these genes in IHNV pathogenesis. This information can be applied to attenuate the virulence of a virus to a less pathogenic form without altering its immunogenicity that can be used as a novel efficient vaccine against IHNV.

The in vivo challenge studies should be done for rIHNV-VP2 and rIHNV-HE to investigate efficiency of these viruses in protecting fish against IPNV and ISAV, respectively. This will demonstrate the efficieny of recombinant IHNV in terms of vaccine vector for fish pathogens. The vector potential of IHNV should be explored for not only fish pathogens but also for mammalian pathogens as a non-replicating vector vaccine. The IHNV vector potential could be further explored in the sense of protein expression system. When mammalian or baculovirus or yeast expression systems are used to express proteins at low temperature, there would be no proper folding or glycosylation, which makes the protein of interest inefficient in terms of function and

yield. This could be easily avoided by IHNV expression system, which is very efficient and convenient to express temperature-sensitive proteins for vaccination, diagnostic or other experimental purposes.

IHNV belongs to genus *Novirhabdovirus* which contain an additional gene coding for a small non-virion (NV) protein of unassigned function localized between the G and L genes, which is specific for this genus (Hoffmann *et al.,* 2005). It was demonstrated that NV gene deleted rIHNV replicated very poorly in cell culture and also non-pathogenic in fish (Thoulouze *et al.,* 2004). This earlier study shed light on the essential role of NV gene in replication and pathogenesis. However, the exact mechanism of NV gene is yet to be demonstrated. There are many possible functions for this protein; may control the rate of transcription and replication; may be helpful in release of virion from the cell surface; may be involved in apoptosis pathways; or may be involved in the modulation of immune system in host. All these questions have to be answered and thoroughly investigated to understand molecular mechanism of this protein in IHNV replication and pathogenesis and to develop better vaccine for infectious hematopoietic necrosis disease.

With the help of structural information of IHNV, improvements in efficiency of reverse genetic system for IHNV, and functional genomic study in fish and fish cells, further knowledge of the viral disease process, virus-host interaction and host protection will be gained. Based on these future studies, safe and stable live attenuated vaccines would be developed.

Chapter 8

References

1. Ammayappan, A., and Vakharia, V.N. 2009a. Molecular characterization of the Great Lakes viral hemorrhagic septicemia virus (VHSV) isolate from USA. Virol. J. 6:171.
2. Ammayappan, A., and Vakharia, V.N. 2009b. Efficient recovery of infectious hematopoietic necrosis virus (IHNV) from cloned cDNA using a vaccinia virus-free reverse genetics system. Submitted for publication.
3. Ammayappan, A., and Vakharia, V.N. 2009c. Molecular characterization of the virulent infectious hematopoietic necrosis virus (IHNV) strain 220-90. Submitted for publication.
4. Ahmed, M., and Lyles, D.S. 1998. Effect of vesicular stomatitis virus matrix protein on transcription directed by host RNA polymerases I, II, and III. J. Virol. 72:8413-8419.
5. Allnutt, F.C., Bowers, R.M., Rowe, C.G., Vakharia, V.N., LaPatra, S.E., Dhar, A.K. 2007. Antigenicity of infectious pancreatic necrosis virus VP2 subviral particles expressed in yeast.Vaccine. 25:4880-4888.
6. Alonso, M., Kim, C., Johnson, H., Marc, C., Pressley, M., Leong, J.C. 2004. The NV gene of snakehead rhabdovirus (SHRV) is not required for pathogenesis, and a heterologous glycoprotein can be incorporated into the SHRV envelope. J. Virol. 78: 5875-5882.

7. Amend, D.F., and Smith, L. 1975. Pathophysiology of infectious hematopoietic necrosis virus disease in rainbow trout: hematological and blood chemical changes in moribund fish. Infect. Immun. 11:171-179.

8. Amend, D.F. 1975. Detection and transmission of infectious hematopoietic necrosis virus in rainbow trout. J. Wildl. Dis. 11:471-480.

9. Amend, D.F., Yasutake, W.T., Fryer, J.L., Pilcher, K.S., Wingfield, W.H. 1973. Epizootiology of infectious hematopoietic necrosis virus in salmonids. Pages 80-98 *in* A. Dill (ed.). Symposium on the major communicable fish diseases in Europe and their control. European Inland Fisheries Advisory Commission, Food and Agriculture Organization of the United Nations, FAO Technical Paper 17, Supp. 2. pp225.

10. Amend, D. F. 1970. Control of infectious hematopoietic necrosis virus disease by elevating the water temperature. J. Fish. Res. Board Can. 27:265-270.

11. Amend, D.F. 1976. Prevention and control of viral diseases of salmonids. J. Fish. Res. Board Can. 33:1059-1066.

12. Amend, D. F., Yasutake, W. T., and Mead, R. W. 1969. A hematopoietic virus disease of rainbow trout and sockeye salmon. Trans. Amer. Fish. Soc. 98:796-804.

13. Amend, D. F., and Chambers, V. C. 1970. Morphology of certain viruses of salmonid fishes. I. In vivo studies of some viruses causing hematopoietic necrosis. J. Fish. Res. Board Can. 27:1285-1293.

14. Amos, K.H. 1985. Procedures for the detection and identification of certain fish pathogens. 3rd edition, Fish Health Section, American Fisheries Society, Corvallis, 6-21.

15. Anderson, E.D., Mourich, D.V., Fahrenkrug, S.C., LaPatra, S., Shepherd, J., Leong, J.A., 1996. Genetic immunization of rainbow trout (*Oncorhynchus mykiss*) against infectious hematopoietic necrosis virus. Mol. Mar. Biol. Biotechnol. 5:114-122.

16. Ayata, M., Hirano, A., and Wong, T. C. 1989. Structural defect linked to nonrandom mutations in the matrix gene of Biken strain subacute sclerosing panencephalitis virus defined by cDNA cloning and expression of chimeric genes. J. Virol. 63:1162-1173.

17. Banerjee, A.K. 1987. Transcription and replication of rhabdoviruses. Microbiol. Rev. 51:66-87.

18. Barr, J.N., Whelan, S.P., Wertz, G.W. 1997. cis-Acting signals involved in termination of vesicular stomatitis virus mRNA synthesis include the conserved AUAC and the U7 signal for polyadenylation. J. Virol. 71:8718-8725.

19. Barr, J.N., and Wertz, G.W. 2001. Polymerase slippage at vesicular stomatitis virus gene junctions to generate poly (A) is regulated by the upstream 3'-AUAC-5' tetranucleotide: implications for the mechanism of transcription termination. J. Virol. 75:6901-6913.

20. Barge, A., Gaudin, Y., Coulon, P., and Ruigrok, R. W. 1993. Vesicular stomatitis virus M protein may be inside the ribonucleocapsid coil. J. Virol. 67:7246-7253.

21. Bearzotti, M., Delmas, B., Lamoureux, A., Loustau, A.M., Chilmonczyk, S., and Bremont. M. 1999. Fish rhabdovirus cell entry is mediated by fibronectin. J. Virol. 73:7703-7709.

22. Bearzotti, M., Monnier, A.F., Vende, P., Grosclaude, J., de Kinkelin, P., Benmansour, A., 1995. The glycoprotein of viral hemorrhagic septicemia virus (VHSV): antigenicity and role in virulence. Vet. Res. 26:413-422.

23. Benmansour, A., Brahimi, M., Tuffereau, C., Coulon, P., Lafay, F., and Flamand, A. 1992. Rapid sequence evolution of street rabies glycoprotein is related to the highly heterogeneous nature of the viral population. Virol. 187:33-45.

24. Benmansour, A., Paubert, G., Bernard, J., and De Kinkelin, P. 1994. The polymerase-associated protein (M1) and the matrix protein (M2) from a virulent and an avirulent strain of viral hemorrhagic septicemia virus (VHSV), a fish rhabdovirus. Virol. 198:602-612.

25. Benmansour, A., Basurco, B., Monnier, A.F., Vende, P., Winton, J.R., and de Kinkelin, P. 1997. Sequence variation of the glycoprotein gene identifies three distinct lineages within field isolates of viral haemorrhagic septicemia virus, a fish rhabdovirus. J. Gen. Virol. 78:2837-2846.

26. Bergmann, S., Ariel, E., Skall, H. F., Fichtner, D., Schlotfeld, H. J., and Olesen, N. J. 2002. Comparison of diagnostic methods for detection of an

infection with different isolates of infectious haematopoietic necrosis virus (IHNV). Berl. Munch. Tierarztl. Wschr. 115:385-389. (In German.)

27. Biacchesi, S., Thoulouze, M. I., Bearzotti, M., Yu, Y. X., and Bremont, M. 2000. Recovery of NV knockout infectious hematopoietic necrosis virus expressing foreign genes. J. Virol. 74:11247-11253.

28. Blondel, D., Harmison, G. G., and Schubert, M. 1990. Role of matrix protein in cytopathogenesis of vesicular stomatitis virus. J. Virol. 64:1716-1725.

29. Blumberg, B. M., Leppert, M., and Kolakofsky, D. 1981. Interaction of VSV leader RNA and nucleocapsid protein may control VSV genome replication. Cell. 23:837-845.

30. Bootland, L.M., and Leong, J.C. 1999. Infectious hematopoietic necrosis virus. Fish diseases and disorders. Edited by Woo PTK, Bruno DW. CAB International, New York, 3: 57-121.

31. Bovo, G.G., Giorgetti, P.E., Jorgensen, V., Olesen, N.J. 1987. Infectious hematopoetic necrosis: first detection in Italy. Bull. Europ. Assoc. Fish. Pathol. 7: 124.

32. Boudinot, P., Blanco, M., de Kinkelin, P., and Benmansour, A. 1998. Combined DNA immunization with the glycoprotein gene of viral hemorrhagic septicemia virus and infectious hematopoietic necrosis virus induces double-specific protective immunity and nonspecific response in rainbow trout. Virol. 249:297-306.

33. Bukreyev, A., Camargo, E., Collins, P.L., 1996. Recovery of infectious respiratory syncytial virus expressing an additional, foreign gene. J. Virol. 70:6634-6641.

34. Buonocore, L., Blight, K. J., Rice, C. M., and Rose, J. K. 2002. Characterization of vesicular stomatitis virus recombinants that express and incorporate high levels of hepatitis C virus glycoproteins. J. Virol. 76:6865-6872.

35. Burke, J.A., and Mulcahy, D. 1980. Plaquing procedure for infectious hematopoietic necrosis virus. Appl. Environ. Microbiol. 39:872-876.

36. Busch, R.A. 1983. Viral disease considerations in the commercial trout industry in Idaho. In: J.C. Leong and T.Y. Barila (Eds.), Workshop on Viral Diseases of Salmonid Fishes in the Columbia River Basin, Bonneville Power Administration, Portland, pp. 84-100.

37. Cain, K. D., LaPatra S. E., Shewmaker, B. J., Jones, K., Byrne, M., and Ristow, S. S. 1999. Immunogenicity of a recombinant infectious hematopoietic necrosis virus glycoprotein produced in insect cells. Dis. Aquat. Org. 36:67-72.

38. Carneiro, F. A., Bianconi, M. L., Weissmuller, G., Stauffer, F., and Da Poian, A. T. 2002. Membrane recognition by vesicular stomatitis virus involves enthalpy-driven protein-lipid interactions. J. Virol. 76:3756-3764.

39. Cattaneo, R., Rebman, G., Schmid, A. K., Baczko ter Meulen, V., and Billeter. M. A. 1988. Biased hypermutation and other genetic changes in defective measles virus in human brain infections. Cell. 55:255-265.

40. Chen, S.N., Kou, G.H., Hedrick, R.P., Fryer, J.L. 1983. The occurrence of viral infections of fish in Taiwan. In: Ellis AE (ed), Fish and shellfish pathology. Academic Press, New York,pp 313–319

41. Chilmonczyk, S., and Monge, D. 1980. Rainbow trout gill pillar cells: demonstration of inert particle phagocytosis and involvement in viral infection. J. Reticuloendothel. Soc. 28:327-332.

42. Chiou, P. P., Kim, C. H., Ormonde, P., and Leong, J. C. 2000. Infectious hematopoietic necrosis virus matrix protein inhibits host-directed gene expression and induces morphological changes of apoptosis in cell cultures. J. Virol. 74:7619-7627.

43. Christie, K.E. 1997. Immunization with viral antigens: infectious pancreatic necrosis. In Fish vaccinology (R. Gudding, A. Lillehaug., P.J. Midtlyng and F. Brown, eds). Dev. biol. Standard. 90:191-199.

44. Clarke, David K., Nasar, F., Lee, M., Johnson, J., Erik, W., Kevin, C. et al. 2007. Synergistic Attenuation of Vesicular Stomatitis Virus by Combination of Specific G Gene Truncations and N Gene Translocations. J. Virol. 81: 2056-2064

45. Coll, J. 1997. Synthetic peptides from the heptad repeats of the glycoproteins of rabies, vesicular stomatitis and fish rhabdoviruses bind phosphatidylserine. Arch. Virol. 142:2089-2097.

46. Coll, J. M. 1999. Early steps in rhabdoviral infection. Recent Res. Develop. Virol. 1:75-83.

47. Coll, J.M., 1995a. The glycoprotein G of rhabdoviruses. Arch. Virol. 140, 827-851.

48. Coll, J. 1995b. Low-pH increases the binding of haemorrhagic septicemia rhabdovirus to membrane phospholipids. J. Fish Dis. 18:519-527.

49. Cunningham, C. O., Gregory, A., Black, J., Simpson, I. and Raynard, R. S. 2002. A novel variant of infectious salmon anaemia virus (ISAV) haemagglutinin gene suggests mechanisms for virus diversity. Bull Eur Ass Fish Pathol. 22:366-374.

50. Dietzschold, B., Wiktor, T. J., Trojanowski, J. Q., Macfarlan, R. I., Wunner, W. H., Torres-Anjel, M. J. and Koprowski, H.. 1985. Differences in cell-to-cell spread of pathogenic and apathogenic rabies virus in vivo and in vitro. J. Virol. 56:12-18.

51. Dietzschold, B., Wunner, W. H., Wiktor, T. J., Lopes, A. D., Lafon, M., Smith, C. L., and Koprowski, H. 1983. Characterization of an antigenic determinant of the glycoprotein that correlates with pathogenicity of rabies virus. Proc. Natl. Acad. Sci. USA 80:70-74.

52. Ding, H., Green, T. J., Lu, S., and Luo. M. 2006. Crystal structure of the oligomerization domain of the phosphoprotein of vesicular stomatitis virus. J. Virol. 80:2808-2814.

53. Dobos, P. 1995a. Protein-primed RNA synthesis *in vitro* by the virion associated RNA polymerase of infectious pancreatic necrosis virus. Virol. 208:19-25.

54. Dobos, P. 1995b. The molecular biology of infectious pancreatic necrosis virus (IPNV). Annu. Rev. Fish Dis. 5:24-54.

55. Dobos, P. 1976. Size and structure of the genome of infectious pancreatic necrosis virus. Nucleic Acids Res. 3:1903-1919.

56. Dobos, P. 1977. Virus-specific protein synthesis in cells infected by infectious pancreatic necrosis virus. J. Virol. 21:242-258

57. Duncan, R., Nagy, E., Krell, P. J. and Dobos, P. 1987. Synthesis of the infectious pancreatic necrosis virus polyprotein, detection of a virus-encoded protease, and fine structure mapping of genome segment A coding regions. J. Virol. 61:3655-3664.

58. Duncan, R., Mason, C. L., Nagy, E., Leong, J. A., and Dobos, P. 1991. Sequence analysis of infectious pancreatic necrosis virus genome segment B and its encoded VP1 protein: a putative RNA-dependent RNA polymerase lacking the Gly-Asp-Asp motif. Virol. 181:541-552.

59. Durrer, P., Gaudin, Y., Ruigrok, R. W. H., Graf, R. and Brunner, J. 1995. Photolabeling identifies a putative fusion domain in the envelope glycoprotein of rabies and vesicular stomatitis viruses. J. Biol. Chem. 270:17575-17581.

60. Eliassen, T. M., Frøystad, M. K., Dannevig, B. H., Jankowska, M., Brech, A., Falk, K., Romøren, K. and Gjøen, T. 2000. Initial events in infectious salmon anaemia virus infection: evidence for the requirement of a low-pH step. J.Virol. 74:218-227.

61. Emerson, S.U., and Schubert, M. 1987. Location of the binding domains for the RNA polymerase L and the ribonucleocapsid template within different

halves of the NS phosphoprotein of vesicular stomatitis virus. Proc. Natl. Acad. Sci. USA. 84:5655-5659.

62. Emmenegger, E. J., Meyers, T. R., Burton, T. O., and Kurath. G. 2000. Genetic diversity and epidemiology of infectious hematopoietic necrosis virus in Alaska. Dis. Aquat. Organ. 40:163-176.

63. Emmenegger, E. J., and Kurath, G. 2002. Genetic characterisation of infectious hematopoietic necrosis virus of costal salmonid stocks in Washington State. J. Aquat. Anim. Health 14:25-34.

64. Emmenegger, E. J., Troyer, R. M., and Kurath. G. 2003. Characterisation of the mutant spectra of a fish RNA virus within individual hosts during natural infections. Virus Res. 96:15-25.

65. Enzmann, P.-J., Dangschat, H., Feneis, B., Schmitt, D., Wizigmann, G., and Schlotfeld, H.-J. 1992. Demonstration of IHN virus in Germany. Bull. Eur. Assoc. Fish Pathol. 12:185.

66. Enzmann, P.-J., Kurath, G., Fichtner, D., and Bergmann. S. M. 2005. Infectious hematopoietic necrosis virus: monophyletic origin of European isolates from North American genogroup M. Dis. Aquat. Organ. 66:187-195.

67. Estepa, A., and Coll, J. 1996a. Pepscan mapping and fusion-related properties of the major phosphatidylserine-binding domain of the glycoprotein of viral hemorrhagic septicemia virus, a salmonid rhabdovirus. Virol. 216:60-70.

68. Estepa, A., and Coll, J. 1996b. Phosphatidylserine binding to solid-phase rhabdoviral peptides: a new method to study phospholipid/viral protein interactions. J. Virol. Methods 61:37-45.

69. Estepa, A., Fernandez-Alonso, M., and Coll, J. 1999. Structure, binding and neutralization of VHSV with synthetic peptides. Virus Res. 63:27-34.

70. Engelking, H. M., and Leong, J. C. 1981. IHNV persistently infects Chinook salmon embryo cells. Virol. 109:47-58.

71. Engelking, H. M., and Leong, J. C. 1989. Glycoprotein from infectious hematopoietic necrosis virus (IHNV) induces protective immunity against five IHNV types. J. Aquat. Anim. Health 1:291-300.

72. Faber, M., Lamirande, E. W., Roberts, A., Rice, A. B., Koprowski, H., Dietzschold, B., and Schnell, M. J. 2005. A single immunization with a rhabdovirus-based vector expressing severe acute respiratory syndrome coronavirus (SARS-CoV) S protein results in the production of high levels of SARS-CoV-neutralizing antibodies. J. Gen. Virol. 86:1435-1440.

73. Fukuda, H., et al. 1989. Attenuation of infectious hematopoietic necrosis virus (IHNV) and selection of low virulent clone by successive passage and plaquing. Bull. Japanese Society of Sci. Fish. 55:261-265.

74. Fallahi, R., Soltani, M., Kargar, R., Zorriehzahra, J., Shchelkunov, I., Hemmatzadeh, F., Nouri, A. 2003. Isolation and Identification of the infectious Haematopoietic Necrosis Virus (IHNV) - Like Agent from Farmed Rainbow Trout (*Oncorhynchus mykiss*) from Iran. Arch. Razi. Ins. 56:37-45

75. Falk, K., Namork, E., Rimstad, E., Mjaaland, S., and Dannevig, B. H. 1997. Characterization of infectious salmon anaemia virus, an orthomyxo-like virus isolated from Atlantic salmon (*Salmo salar L.).* J. Virol. 71:9016-9023.

76. Falk, K., Namork, E., Dannevig, B.H. 1998. Characterization and applications of a monoclonal antibody against infectious salmon anaemia virus. Dis. Aquat. Organ. 34:77-85.

77. Fellowes, O. N., Dimopoullos, G. T., and Callis, J. J. 1955. Isolation of vesicular stomatitis virus from an infected laboratory worker. Am. J. Vet. Res. 16:623-626.

78. Fijan, N., Sulimanovic, D., Bearzotti, M., Muzinlc, D., Zwillenberg, L. D., Chilmonczyk, S., Vautherot, J. F., and de knkelin, P. 1983. Some properties of the Epitheliorna papulosurn cyprini (EPC) cell line from carp Cyprinus carpio. Ann. Virol. (Inst. Pasteur) 134E:207-220.

79. Flanagan, E., Brian, B., Andrew, L., and Wertz, GW. 2000. Moving the glycoprotein gene of vesicular stomatitis virus to promoter-proximal positions accelerates and enhances the protective immune response J. Virol. 74: 7895-7902.

80. Finke, S., and Conzelmann, K.K. 2005. Replication strategies of rabies virus. Virus Res. 111: 120-131.

81. Follet, J.E., Thomas, J.B., and Hauck, A.K. 1987. Infectious hemaotpoietic necrosis virus in moribund and dead juvenile chum, *Oncorhychus keta*, (Walbaum) salmon, and spawning chinhook *O. tshawytscha* (Walbaum) and adult chum salmon at an Alaskan hatchery. J. Fish. Dis. 10: 309-314

82. Follet, J.E., Meyers, T.R., Burton, T.O., Geesin, J.L. 1997. Comparative susceptibilities of salmonid species in Alaska to infectious hematopoietic

necrosis virus (IHNV) and North American viral hemorrhagic septicemia (VHSV). J. Aquat. Anim. Health 9:34-40.

83. Fynan, E.F., Webster, R. G., Fuller, D. H., Haynes, J. R., Santoro, J. C., and Robinson, H. L. 1995. DNA Vaccines: Protective immunizations by parenteral, mucosal and gene gun inoculations. Proc. Natl. Acad. Sci. U.S.A. 90:11478-11482.

84. Garver, K. A., Troyer, R. M., and Kurath, G. 2003. Two distinct phylogenetic clades of infectious hematopoietic necrosis virus overlap within the Columbia River basin. Dis. Aquat. Organ. 55:187-203.

85. Gaudin, Y., Ruigrok, R. W., Tuffereau, C., Knossow, M. and Flamand, A. 1992. Rabies virus glycoprotein is a trimer. Virol. 187:627-632.

86. Gaudin, Y., Ruigrok, R. W. H., and Brunner, J. 1995. Low-pH induced conformational changes in viral fusion proteins: implications for the fusion mechanism. J. Gen. Virol. 76:1541-1556.

87. Gaudin, Y., Tuffereau, C., Durrer, P., Brunner, J., Flamand, A. and Ruigrok, R. 1999. Rabies virus-induced membrane fusion. Mol. Mem. Biol. 16:21-31.

88. Granzow, H., Weiland, F., Fichtner, D., and Enzmann. P. J. 1997. Studies of the ultrastructure and morphogenesis of fish pathogenic viruses grown in cell culture. J. Fish. Dis. 20:1-10.

89. Guenther, R.W., Watson, S.W., Rucker, R.R. 1959. Etiology of sockeye salmon "virus" disease. US Fish Wildl. Serv. Spec. Sci. Rep. Fish. 296:1-10.

90. Hanson, R. P., Rasmussen, A. F., Brandly, C. A., and Brown, J. W. 1950. Human infection with the virus of vesicular stomatitis. J. Lab. Clin. Med. 36:754-758.

91. Harty, R.N., Brown, M.E., Hayes, F.P., Wright, N.T., and Schnell, M.J.J. 2001 Vaccinia virus-free recovery of vesicular stomatitis virus. Mol. Microbiol. Biotechnol. 3:513-517.

92. Hasan, M.K., Kato, A., Shioda, T., Sakai, Y., Yu, D., and Nagai Y. 1998. Creation of an infectious recombinant Sendai virus expressing the firefly luciferase gene from the 3' proximal first locus. J. Gen. Virol. 78:2813-2820.

93. Howard, M., and Wertz, G. 1989. Vesicular stomatitis virus RNA replication: a role for the NS protein. J. Gen. Virol. 70:2683-2694.

94. Hoffman, M. A., and Banerjee, A. K. 1997. An infectious clone of human parainfluenza virus type 3. J. Virol. 71:4272-4277.

95. Hoffmann, E., Neumann, G., Kawaoka, Y., Hobom, G., and Webster, R.G. 2000. A DNA transfection system for generation of influenza A virus from eight plasmids. Proc. Natl. Acad. Sci. U. S. A. 97:6108-6113.

96. Hoffmann, B., Beer, M., Schutze, H. and Mettenleiter, T. C. 2005. Fish rhabdoviruses: molecular epidemiology and evolution. Curr. Top. Microbiol. Immunol. 292:81-117.

97. Hill, B.J. 1975. Physico-chemical and serological characterization of five rhabdoviruses infecting fish. J. Gen. Virol. 27:369-378.

98. Hsu, Y., Engelking, H. and Leong, J. 1985. Analysis of quantity and synthesis of the virion proteins of infectious hematopoietic necrosis virus. Fish Pathol. 20:331-338.

99. Hsu, Y. L., Engelking, H. M., and Leong, J. C. 1986. Occurrence of different types of infectious hematopoietic necrosis virus in fish. Appl. Environ. Microbiol. 52:1353-1361.

100. Huang, C. 1993. Mapping of antigenic sites of infectious hematopoietic necrosis virus glycoprotein. PhD thesis, University of Washington, Seattle, USA.

101. Huang, C., Chien, M.-S., Landolt, M., Batts, W., and Winton. J. 1996. Mapping the neutralizing epitopes on the glycoprotein of infectious haematopoietic necrosis virus, a fish rhabdovirus. J. Gen. Virol. 77:3033-3040.

102. Hughes, M.K., and Hughes, A.L. 1995. Natural selection on Plasmod-ium surface proteins. Mol. Biochem. Parasitol. 71:99-113.

103. Inoue, K., Shoji, Y., Kurane, I., Iijima, T., Sakai, T., and Morimoto, K. J. 2003. An improved method for recovering rabies virus from cloned cDNA. Virol Methods. 107:229-236.

104. Ito, N., Takayama, M., Yamada, K., Sugiyama, M., and Minamoto, N. 2001. Rescue of rabies virus from cloned cdna and identification of the pathogenicity-related gene: glycoprotein gene is associated with virulence for adult mice. J. Virol. 75:9121-9128.

105. Johnson, M. C., Simon, B. E., Kim, C. H., and Leong, J. A. 2000. Production of recombinant snakehead rhabdovirus: the NV protein is not required for viral replication. J. Virol. 74:2343-2350.

106. Johnston, S.A., and Tang, D. C. 1994. Gene gun transfection of animal cells and genetic immunization. Methods Cell. Biol. 43:353-365.

107. Kahn, J. S., Roberts, A., Weibel, C., Buonocore, L., and Rose, J. K. 2001. Replication-competent or attenuated, nonpropagating vesicular stomatitis viruses expressing respiratory syncytial virus (RSV) antigens protect mice against RSV challenge. J. Virol. 75:11079-11087.

108. Kapadia, S. U., Rose, J. K., Lamirande, E., Vogel, L., Subbarao, K., and Roberts, A. 2005. Long-term protection from SARS coronavirus infection conferred by a single immunization with an attenuated VSV-based vaccine. Virol. 340:174-182.

109. Kassis, R., Larrous, F., Estaquier, J., and Bourhy, H. 2004. Lyssavirus matrix protein induces apoptosis by a TRAIL-dependent mechanism involving caspase-8 activation. J. Virol. 78:6543-6555.

110. Kibenge, F.S., Godoy, M.G., Wang, Y., Kibenge, M.J., Gherardelli, V., Mansilla, S., Lisperger, A., Jarpa. M., Larroquete, G., Avendaño, F., Lara, M., and Gallardo, A. 2009. Infectious salmon anaemia virus (ISAV) isolated from the ISA disease outbreaks in Chile diverged from ISAV isolates from Norway around 1996 and was disseminated around 2005, based on surface glycoprotein gene sequences. Virol. J. 6:88.

111. Kibenge, F.S.B., Kibenge, M.J.T., Joseph, T., and McDougall, J. 2003. The development of infectious salmon anemia virus vaccines in Canada. In Miller, Otis; Cipriano, Rocco C., tech. coords. International response to infectious salmon anemia: prevention, control, and eradication: proceedings of a symposium; 3-4 September 2002; New Orleans, LA. Tech. Bull. 1902. Washington, DC. 2003:39-49.

112. Kibenge, F.S.B, Gárate, O.N., Johnson, G., Arriagada, R., Kibenge, M.J.T., and Wadowska, D. 2001. Isolation and identification of infectious salmon anaemia virus (ISAV) from coho salmon in Chile. Dis. Aqua.Org. 45: 9-18.

113. Kielian, M., and Jungerwirth, S. 1990. Mechanisms of enveloped virus entry into cells. Mol. Biol. Med. 7:17-31.

114. Kim, C. H., Winton, J. R. and Leong, J. C. 1994. Neutralization-resistant variants of infectious hematopoietic necrosis virus have altered virulence and tissue tropism. J. Virol. 68:8447-8453.

115. Kim, W.S., Oh, M.J., Nishizawa, T., Park, J.W., Kurath, G., and Yoshimizu, M. 2007. Genotyping of Korean isolates of infectious hematopoietic necrosis virus (IHNV) based on the glycoprotein gene. Arch. Virol. 152:2119-2124.

116. Kimura, T., and Yoshimizu, M. 1991. Viral diseases of fish in Japan. Ann. Rev. Fish dis. 1:67-82.

117. Kingsford, L., and Emerson, S.U. 1980. Transcriptional activities of different phosphorylated species of NS protein purified from vesicular stomatitis virions and cytoplasm of infected cells. J. Virol. 33:1097-1105.

118. Knuesel, R., Segner, H., and Wahli, T. 2003. A survey of viral diseases in farmed and feral salmonids in Switzerland. J. Fish Dis. 26:167-182.

119. Koener, J., C. Passavant, G., Kurath, and J. Leong. 1987. Nucleotide sequence of a cDNA clone carrying the glycoprotein G gene of infectious hematopoietic necrosis virus, a fish rhabdovirus. J. Virol. 61:1342–1349

120. Koener, J.F., Leong, J.C. 1990. Expression of the glycoprotein gene from a fish rhabdovirus by using baculovirus vectors. J. Virol.64:428-430.

121. Kuznar, J., Soler, M., Farias, G., and Espinoza, J. C. 1995. Attachment and entry of infectious pancreatic necrosis virus (IPNV) into CHSE-214 cells. Arch. Virol. 140:1833-1840.

122. Kolodziejek, J., Schachner, O., Dürrwald, R., Latif, M., and Nowotny, N. 2008. "Mid-G" Region Sequences of the Glycoprotein Gene of Austrian Infectious Hematopoietic Necrosis Virus Isolates Form Two Lineages within European Isolates and Are Distinct from American and Asian Lineages. J. Clin. Microbiol. 46:22-30.

123. Krossøy, B., Devold, M., Saunders, L., Knappskog, P. M., Aspehaug, V., Falk, K., Nylund, A., Koumans, S., Endresen, C. and Biering, E. 2001. Cloning and identification of the infectious salmon anaemia virus haemagglutinin. J. Gen. Virol. 82:1757-1765.

124. Kucera, P., Dolivo, M., Coulon, P., and Flamand, A. 1985. Pathways of the early propagation of virulent and avirulent rabies strains from the eye to the brain. J. Virol. 55:158-162.

125. Kurath, G., and Leong, J.C. 1985. Characterization of infectious hematopoietic necrosis virus mRNA species demonstrates a nonvirion rhabdovirus protein. J. Virol. 53:462-468.

126. Kurath, G., Ahern, K. G., Pearson, G. D., and Leong, J.C. 1985. Molecular cloning of the six mRNA species of infectious hematopoietic necrosis virus, a fish rhabdovirus, and gene order determination by R-loop mapping. J. Virol. 53:469-476.

127. Kurath, G., and Leong, J. C. 1987. Transcription *in vitro* of infectious haematopoietic necrosis virus, a fish rhabdovirus. J. Virol. 68:1767-1771.

128. Kurath, G., Garver, K. A., and Troyer, R. M., Emmenegger, E. J. Einer-Jensen, K., and Anderson, E. D. 2003. Phylogeography of infectious haematopoietic necrosis virus in North America. J. Gen. Virol. 84:803-814.

129. Lafay, F., Coulon, P., Astic, L., Saucier, D., Riche, D., Holley, A., and Flamand, A. 1991. Spread of the CVS strain of rabies virus and of the avirulent mutant AvOl along the olfactory pathways of the mouse after intranasal inoculation. Virol. 183:320-330.

130. LaPatra, S.E., and Fryer, J.L. 1990. Susceptibility of brown trout (*Salmo trutta*) to two strains of infectious hematopoietic necrosis virus. Dis. Aquat. Org. 16:115-120.

131. LaPatra, S. E., Lauda, K. A., and Morton, A. W. 1991. Antigenic and virulence comparisons of eight isolates of infectious hematopoietic necrosis virus from the Hagerman Valley, Idaho, USA. In Proceedings of the Second International

Symposium on Viruses of Lower Vertebrates, pp. 125–132. Corvallis, OR: Oregon State University.

132. LaPatra, S.E., Parsons, J.E., Jones, G.R., and McRoberts, W.O. 1993. Early life stage survival and susceptibility of brook trout, coho salmon, rainbow trout and their reciprocal hybrids to infectious hematopoietic necrosis virus. J. Aquat. Animal Health 5:270-274.

133. LaPatra, S. E., Lauda, K. A. and Jones, G. R. 1994. Antigenic variants of infectious hematopoietic necrosis virus and implications for vaccine development. Dis. Aquat. Org. 20:119-126.

134. Laurencin, F.B. 1987. IHN in France. Bull. Europ. Assoc. Fish Pathol. 7: 104.

135. Lawson, N.D., Stillman, E.A, Whitt, M.A., and Rose, J.K. 1995. Recombinant vesicular stomatitis viruses from DNA. Proc. Natl. Acad. Sci. USA. 92: 4477-4481.

136. Leong, J. C., Hsu, Y. L., Engelking, H. M,, and Mulcahy, D. 1981. Strains of infectious haematopoietic necrosis virus may be identified by structural protein differences. Dev. biol. Standard. 49:43-55.

137. Leong, J. C., Hsu, Y. L., and Engelking, H. M. 1983. Synthesis of the structural proteins of infectious hematopoietic necrosis virus. In J. Crosa (ed.), Bacterial and viral diseases of fish. Molecular studies. University of Washington Press, Seattle. p. 61-70.

138. Leong, J.C., Fryer, J.L., and Winton, J.R. 1988. Vaccination against infectious hematopoietic necrosis virus. In: Ellis AE (ed.), Fish vaccination. Academic Press, New York, p 193-203.

139. Leong, J. C., and Fryer, J. L. 1993. Viral vaccines for aquaculture. Annu. Revi. Fish Dis. 4: 225-240.

140. Letchworth, G. J., Rodriguez, L. L., and Del Cbarrera, J. 1999. Vesicular stomatitis. Vet. J. 157:239-260.

141. Lorenzen, N., Cupit, P. M., Einer-Jensen, K., Lorenzen, E., Ahrens, P., Secombes, C. J., and Cunningham, C. 2000. Immunoprophylaxis in fish by injection of mouse antibody genes. Nat. Biotechnol. 18:1177-1180.

142. Lorenzen, N., Olesen, N. J., and Jorgensen, P. E. 1990. Neutralization of Egtved virus pathogenicity to cell cultures and fish by monoclonal antibodies to the viral G protein. J. Gen. Virol. 71:561-567.

143. Lorenzen, N., Olesen, N. and Koch, C. 1999. Immunity to VHS virus in rainbow trout. Aquaculture 172:41-61.

144. Lorenzen, N., and Olesen, N.J. 1997. Immunization with viral antigens: viral haemorrhagic septicaemia. Dev. Biol. Stand. 90:201-209.

145. Lorenzen N. 1999. Recombinant vaccines: experimental and applied aspects. Fish Shellfish Immunol. 9:361-365.

146. Lorenzen, N., Lorenzen, E., and Einer-Jensen, K. 1999. Genetic vaccination of rainbow trout against VHS virus: small amounts of DNA protect against a heterologous serotype. Virus Res. 63:19-25.

147. Lorenzen, E., Einer-Jensen, K., Martinussen, T., LaPatra, S.E., and Lorenzen, N. 2000. DNA vaccination of rainbow trout against viral hemorrhagic septicemia virus: a dose–response and time-course study. J. Aquat. Anim. Health. 12:167-180.

148. Lovely, J.E., Dannewig, B.H., Falk, K., Hutchin, L., MacKinnon, A.M., Melvill, K.J., Rimstad, E., and Griffiths, S.G. 1999. First identification of infectious salmon anemia virus in North America with haemorrhagic kidney syndrome. Dis. Aquat. Org. 35: 145-148.

149. Li, T., and Pattnaik, A.K., 1999. Overlapping signals for transcription and replication at the 3' terminus of the vesicular stomatitis virus genome. J. Virol. 73:444-452.

150. Liu, X. and Collodi, P. 2002. Novel form of fibronectin from zebrafish mediates infectious hematopoietic necrosis virus infection. J. Virol. 76,492-498.

151. Lyles, D. S., McKenzie, M. O., Kaptur, P. E., Grant, K. W., and Jerome, W. G. 1996. Complementation of M Gene Mutants of Vesicular Stomatitis Virus by Plasmid-Derived M Protein Converts Spherical Extracellular Particles into Native Bullet Shapes. Virol. 217:76-87.

152. Manning, D.S., and Leong, J.C. 1990. Expression in Escherichia coli of the large genomic segment of infectious pancreatic necrosis virus. Virol. 1990 179:16-25.

153. Masters, P. S., and Banerjee, A. K. 1988. Complex formation with vesicular stomatitis virus phosphoprotein NS prevents binding of nucleocapsid protein N to nonspecific RNA. J. Virol. 62:2658-2664.

154. Mcallister, P.E. 1979. Fish viruses and viral infections. In Comprehensive Virology, (ed.) Fraenkel-Conrat H., and Wagner, R. R. New York and London: Plenum Press. vol. 14, pp. 401-470.

155. McAllister, P.E., and Wagner, R.R. 1975. Structural proteins of two salmonid rhabdoviruses. Virol. 15:733-738.

156. McAllister, P. E., Owens, W. J. and Ruppenthal, T. M. 1987. Detection of infectious pancreatic necrosis virus in pelleted cell and particulate components from ovarian fluid of brook trout (*Salvilimus fontindis*). Dis. Aquat. Org. 2:235-237.

157. McGettigan, J. P., Foley, H. D., Belyakov, I. M., Berzofsky, J. A., Pomerantz, R. J., and Schnell. M. J. 2001a. Rabies virus-based vectors expressing human immunodeficiency virus type 1 (HIV-1) envelope protein induce a strong, cross-reactive cytotoxic T-lymphocyte response against envelope proteins from different HIV-1 isolates. J. Virol. 75:4430-4434.

158. McGettigan, J. P., Sarma, S., Orenstein, J. M., Pomerantz, R. J., and Schnell, M. J. 2001b. Expression and immunogenicity of human immunodeficiency virus type 1 Gag expressed by a replication-competent rhabdovirus-based vaccine vector. J. Virol. 75:8724-8732.

159. McKenna, P., Koser, M., Carlson K. R., *et al.,* 2006. Abstr. 13th Int. Conf. on Negative Strand Viruses, abstr. 297.

160. Mebatsion, T., Weiland, F., and Conzelmann. K. K. 1999. Matrix protein of rabies virus is responsible for the assembly and budding of bullet-shaped particles and interacts with the transmembrane spike glycoprotein G. J. Virol. 73:242-250.

161. Mebatsion, T., Schnell, M.J., Cox, J.H., Finke, S., and Conzelmann, K.K. 1996. Highly stable expression of a foreign gene from rabies virus vectors. Proc. Natl. Acad. Sci. USA 93:7310-7314.

162. Meyers, T., and Winton, J. 1996. Viral hemorrhagic septicemia virus in North America. Annu. Rev. Fish Dis. 5:3-24.

163. Mikalsen, A.B., Sindre, H., Torgersen, J., Rimstad, E. 2005. Protective effects of a DNA vaccine expressing the infectious salmon anaemia virus hemagglutinin-esterase in Atlantic salmon. Vaccine. 23:4895-4905.

164. Mjaaland, S., Rimstad, E., Falk, K., and Dannevig, B. H. 1997. Genomic characterization of the virus causing infectious salmon anaemia in Atlantic salmon (*Salmo salar L.*): an orthomyxo-like virus in a teleost. J. Virol. 71: 7681-7686.

165. Mulcahy, D., and Wood, J. 1986. A natural epizootic of infectious hematopoetic necrosis in imported Atlantic salmon, *Salmo salar* L., reared in the enzootic region. J. Fish. Dis. 9:173-175.

166. Morzunov, S.P., Winton, J.R., Nichol, S.T. 1995. The complete genome structure and phylogenetic relationship of infectious hematopoietic necrosis virus. Virus Res. 38:175-192.

167. Mullins, J.E., Groman, D., and Wadowska, D., 1998. Infectious salmon anaemia in salt water Atlantic salmon (*Salmo salar L.)* in New Brunswick, Canada. Bull. Eur. Assoc. Fish Pathol. 18:110-114.

168. Nei, M., and Gojobori, T. 1986. Simple methods for estimating the number of synonymous and nonsynonymous nucleotide substitutions. Mol. Biol. Evol. 3:418-426.

169. Newcomb, W. W., and Brown, J. C. 1981. Role of the vesicular stomatitis virus matrix protein in maintaining the viral nucleocapsid in the condensed form found in native virions. J. Virol. 39:295-299.

170. Newcomb, W. W., Tobin, G. J., McGowan, J. J., and Brown, J. C. 1982. In vitro reassembly of vesicular stomatitis virus skeletons. J. Virol. 41:1055-1062.

171. Nichol, S.T., Rowe, J. E., and Winton, J. R. 1995. Molecular epizootiology and evolution of the glycoprotein and non–virion protein genes of infectious hematopoietic necrosis virus, a fish rhabdovirus. Virus Res. 38:159-173.

172. Nicholas, K.B., Nicholas, H.B.J., and Deerfield, D.W. 1997. GeneDoc: analysis and visualization of genetic variation. EMBNEW NEWS. 4:14.

173. Nicholson, B. L. 1993. Use of monoclonal antibodies in identification and characterization of fish viruses. Annu. Rev. Fish Dis. 3:241-257.

174. Nishizawa, T., Kinoshita, S., Kim, W.S., Higashi, S., and Yoshimizu, M. 2006. Nucleotide diversity of Japanese isolates of infectious hematopoietic necrosis virus (IHNV) based on the glycoprotein gene. Dis Aquat. Organ. 71:267-272.

175. Nunez, E., Fernandez, A., Estepa, A., Gonzalez-Ros, J., Gavilanes, F., and Coll. J. 1998. Phospholipid interactions of a peptide from the fusion-related domain of the glycoprotein of VHSV, a fish rhabdovirus. Virol. 243:322-330.

176. Nylund, A., Devold, M., Plarre, H., Isdal, E., and Aarseth, M. 2003. Emergence and maintenance of infectious salmon anemia virus (ISAV) in Europe: a new hypothesis. Dis. Aquat. Organ. 56:11-24.

177. Park, M.A., Sohn, S.G., Lee, S.D., Chun, S.K., Park, J.W., Fryer, J.L., Hah, Y.C. 1993. Infectious haematopoietic necrosis virus from salmonids cultured in Korea. J. Fish Dis. 16:471-478.

178. Pearlman, S. M., and Huang, A. S. 1973. RNA synthesis of vesicular stomatitis virus. V. Interaction between transcription and replication. J. Virol. 12:1395-1400.

179. Pilcher, K.S., and Fryer, J.L. 1980. The viral diseases of fish: a review through 1978. CRC Crit. Rev. Microbiol. 7: 287-364.

180. Plarre, H., Devold, M., Snow, M., And Nylund, A. 2005. Prevalence of infectious salmon anaemia virus (ISAV) in wild salmonids in Western Norway. Dis. Aquat. Organ. 66:71-79.

181. Prehaud, C., Coulon, P. Lafay, F. Thiers, C., and Flamand. A. 1989. Antigenic site II of the rabies virus glycoprotein: structure and role in viral virulence. J. Virol. 62:1-7.

182. Pulmanausahakul, R., Li, J., Schnell, M.J., and Dietzschold, B. 2008. The Glycoprotein and the Matrix Protein of Rabies Virus Affect Pathogenicity by Regulating Viral Replication and Facilitating Cell-to-Cell Spread. J. Virol. 82: 2330-2338

183. Purcell, M.K., Nichols, K.M., Winton, J.R., Kurath, G., Thorgaard, G.H., Wheeler, P., Hansen, J.D., Herwig, R.P., and Park, L.K. 2006. Comprehensive

gene expression profiling following DNA vaccination of rainbow trout against infectious hematopoietic necrosis virus. Mol Immunol. 43:2089-2106.

184. Qanungo, K. R., Shaji, D., Mathur, M., and Banerjee. A. K. 2004. Two RNA polymerase complexes from vesicular stomatitis virus-infected cells that carry out transcription and replication of genome RNA. Proc. Natl. Acad. Sci. USA 101:5952-5957.

185. Rabinovich, N. R., McInnes, P. Klein, D. L., and Hall, B. F. 1994. Vaccine technologies: View to the future. Science 265:1401-1404.

186. Reuter, J. D., Vivas-Gonzalez, B. E., Gomez, D., Wilson, J. H., Brandsma, J. L., Greenstone, H. L., Rose, J. K., and Roberts. A. 2002. Intranasal vaccination with a recombinant vesicular stomatitis virus expressing cottontail rabbit papillomavirus L1 protein provides complete protection against papillomavirus-induced disease. J. Virol. 76:8900-8909.

187. Rodger, H.D., and Richards, R.H., 1998. Haemorrhagic smolt syndrome: a severe anemic condition in farmed salmon in Scotland. Vet. Rec., 538-541.

188. Romero A, Figueras A, Thoulouze, M.I., Bremont, M., and Novoa, B. 2008. Recombinant infectious hematopoietic necrosis viruses induce protection for rainbow trout Oncorhynchus mykiss. Dis. Aquat. Organ. 80:123-135.

189. Roberts, A., Kretzschmar, E., Perkins, A. S., Forman, J., Price, R., Buonocore, L., Kawaoka, Y., and Rose. J. K. 1998. Vaccination with a recombinant vesicular stomatitis virus expressing an influenza virus hemagglutinin provides complete protection from influenza virus challenge. J. Virol. 72:4704-4711.

190. Rose, J.K. 1980. Complete intergenic and flanking gene sequences from the genome of vesicular stomatitis virus. Cell 19:415-421.

191. Rose, N. F., Marx, P. A., Luckay, A., Nixon, D. F., Moretto, W. J., Donahoe, S. M., Montefiori, D., Roberts, A., Buonocore, L., and Rose. J. K. 2001. An effective AIDS vaccine based on live attenuated vesicular stomatitis virus recombinants. Cell 106:539-549.

192. Roux, L., and Waldvogel, F. A. 1982. Instability of the viral M protein in BHK-21 cells persistently infected with Sendai virus. Cell 28:293-302.

193. Rucker, R. R., Whipple, W.J., Parvin, J.R., and Evans, C. A. 1953. A contagious disease of salmon possibly of virus origin. U.S. Fish and Wild life Service, Fish. Bull. 76:35-46.

194. Sano, T., Nishimura, T., Yamazaki, N., Hanada, H., and Watanabe. Y. 1977. Studies of viral diseases of Japanese fish. VI. Infectious hematopoietic necrosis (IHN) of salmonids in the mainland of Japan. J. Tokyo Univ. Fish. 63:81-85.

195. Schlegal, R., Tralka, T., Willingham, M., and Pastan, I. 1983. Inhibition of VSV binding and infectivity by phosphatidylserine: is phosphatidylserine a VSV-binding site? Cell 32:639-646.

196. Schlereth, B., Buonocore, L., Tietz, A., ter Meulen, V., Rose, J. K., and Niewiesk, S. 2003. Successful mucosal immunization of cotton rats in the presence of measles virus-specific antibodies depends on degree of attenuation of vaccine vector and virus dose. J. Gen. Virol. 84:2145-2151.

197. Schnell, M. J., Mebastsion, T. and Conzelmann, K. K. 1994. Infectious rabies viruses from cloned cDNA. EMBO J. 13:4195-4203.

198. Schnell, M.J., Buonocore, L., Kretzschmar, E., Johnson, E., Rose, J.K. 1996. Foreign glycoproteins expressed from recombinant vesicular stomatitis viruses are incorporated efficiently into virus particles. Proc. Natl. Acad. Sci. USA 93:11359-11365.

199. Schnell, M.J., Buonocore, L., Whitt, M.A., and Rose, J.K. 1996. The minimal conserved transcription stop-start signal promotes stable expression of a foreign gene in vesicular stomatitis virus. J. Virol. 70:2318-2323.

200. Schütze, H., Enzmann, P.J., Kuchling, R., Mundt, E., Niemann, H., and Mettenleiter, T.C. 1995. Complete genomic sequence of the fish rhabdovirus infectious haematopoietic necrosis virus. J. Gen. Virol. 76:2519-2527.

201. Schyth, B.D., Olesen, N.J., Østerg°ard, P., Falk, K., 2003. Laboratory diagnosis of infectious salmon anemia (ISA): experience gained from the outbreaks on the Faroe Islands 2000–2003. In: Diseases of Fish and Shellfish, 11th International Conference of the EAFP, vol. 11, p. O-79.

202. Seibert, S.A., Howell, C.Y., Hughes, M.K., and Hughes, A.L. 1995. Natural selection on the gag, pol and env genes of human immunodeficiency virus 1 (HIV-1). Mol. Biol. Evol. 12: 803-813.

203. Seif, I., Coulon, P., Rollin, P. E., and Flamand. A. 1985. Rabies virulence: effect on pathogenicity and sequence characterization of rabies virus mutations affecting antigenic site III of the glycoprotein. J. Virol. 53:926-934.

204. Shivappa, R.B., McAllister, P.E., Edwards, G.H., Santi, N., Evensen, O., and Vakharia, V.N. 2005. Development of a subunit vaccine for infectious pancreatic necrosis virus using a baculovirus insect/larvae system. Dev. Biol. (Basel). 121:165-174.

205. Shors, S.T., and Winston, V. 1989. Detection of infectious hematopoietic necrosis virus in an invertebrate (Callibaetis sp). Am. J. Vet. Res. 50:1307-1309.

206. Soria, M., Little, S. P., and Huang, A.S. 1973. Characterization of vesicular stomatitis virus nucleocapsids. I. Complementary 40 S RNA molecules in nucleocapsids. Virol. 61:270-280.

207. Stillman, E.A., Rose, J.K., and Whitt, M.A., 1995. Replication and amplification of novel vesicular stomatitis virus minigenomes encoding viral structural proteins. J. Virol. 69:2946-2953.

208. Smallwood, S., and Moyer, S.A., 1993. Promoter analysis of the vesicular stomatitis virus RNA polymerase. Virol. 192:254-263.

209. Tamura. K., Dudley. J., Nei. M., and Kumar. S. 2007. MEGA4: Molecular evolutionary genetics analysis (MEGA) software version 4.0. Mol. Biol. Evol. 24:1596-1599.

210. Thoulouze, M. I., Bouguyon, E., Carpentier, C., and Bremont. M. 2004. Essential role of the NV protein of *Novirhabdovirus* for pathogenicity in rainbow trout. J. Virol. 78:4098-4107.

211. Thorud, K., and Djupvik, H.O. 1988. Infectious salmon anaemia in Atlantic salmon (*Salmo salar L.*). Bull. Eur. Assoc. Fish Pathol. 8, 109-111.

212. Tordo, N., Poch, O., Ermine, A., Keith, G. and Rougeon, F. 1986. Walking along the rabies genome: Is the large G-L intergenic region a remnant gene? Proc. Natl. Acad. Sci. USA 83:3914-3918.

213. Tordo, N., Benmansour, A., Calisher, C., Dietzgen, R. G., Fang, R.-X., Jackson, A. O., Kurath, G., Nadin-Davis, S., Tesh, R. B., and Walker, P. J. 2005. Family Rhabdoviridae. In Virus Taxonomy: Eighth Report of the International Committee on Taxonomy of Viruses, (Ed.) Fauquet, C., Mayo, M. A., Maniloff, J., Desselberger, U., and Ball, L. A. San Diego, CA: Academic Press. pp. 623-644.

214. Tordo, N., Poch, O., Ermine, A., Keith, G., and Rougeon, F. 1988. Completion of the rabies virus genome sequence determination: highly conserved domains among the L (polymerase) proteins of unsegmented negative-strand RNA viruses. Virol. 165:565-576.

215. Tordo, N., De Haan, P., Goldbach, R., and Poch, O. 1992. Evolution of negative stranded RNA genomes. In: E. Koonin (Ed.), Seminars in Virology, Vol. 3, Saunders, London, pp. 341-357.

216. Troyer, R. M., LaPatra, S. E., and Kurath, G. 2000. Genetic analyses reveal unusually high diversity of infectious hematopoietic necrosis virus in rainbow trout aquaculture. J. Gen. Virol. 81:2823-2832.

217. Troyer, R. M., and G. Kurath. 2003. Molecular epidemiology of infectious hematopoietic necrosis virus reveals complex virus traffic and evolution within southern Idaho aquaculture. Dis. Aquat. Organ. 55:175-185.

218. Traxler, G.C. 1986. An epizootic of infectious hematopoietic necrosis in 2-year old kokanee, *Oncorhynch nerka* (Walbaum), at lake Cowichan, British Columbia. J. Fish Dis. 9:545-549.

219. Thomas, D., Newcomb, W. W., Brown, J. C., Wall, J. S., Hainfeld, J. F., Trus, B. L., and Steven, A. C. 1985. Mass and molecular composition of vesicular stomatitis virus: a scanning transmission electron microscopy analysis. J. Virol. 54:598-607.

220. Ulmer, J. B., Donnelly, J. J., Parker, S. E., Rhodes, G. H., Felgner, P. L., Dwarki, V. J., Gromkowski, S. H., Deck, R. R., DeWitt, C. M., Friedman, A., et al. 1993. Heterologous protection against influenza by injection of DNA encoding a viral protein. Science 259:1745-1749.

221. Vardic, I., Kapetanovic, D., Teskeredzic, Z., and Teskeredzic, E. 2007. First record of infectious hematopoietic necrosis virus in Croatia. Acta. Vet. Brno. 76:75-70.

222. Vogel, F.R., and Sarver, N. 1995. Nucleic acid vaccines. Clin. Microbiol. Rev. 8:406-410.

223. Walker, P. J., Benmansour, A., Calisher, C. H., Dietzgen, R., Fang, R. X., Jackson, A. O., Kurath, G., Leong, J. C., Nadin-Davies, S., Tesh, R. B., and Tordo, N. 2000. Family *Rhabdoviridae*, *In* M. H. V. van Regenmortel, C. M. Fauquet, and D. H. L. Bishop (ed.), Virus taxonomy: classification and nomenclature of viruses. Seventh report of the International Committee on Taxonomy of Viruses. Springer, Berlin, Germany. p. 563-583.

224. Wagner, E., Engelhardt, O. G., Gruber, S., Haller, O., and Kochs, G. 2001. Rescue of recombinant Thogoto virus from cloned cDNA. J. Virol. 75:9282-9286.

225. Wang, B. *et al.,* 1993. Gene inoculation generates immune responses against human immunodeficiency virus. Proc. Natl. Acad. Sci. U.S.A. 90:4156-4160.

226. Watson, S. W., Guenther, R. W., and Rucker, R. R. 1954. A virus disease of sockeye salmon: interim reports U.S. Dept. of Interior. Special Scientitic Report fisheries no. 138.

227. Weissenhorn, W., Dessen, A., Calder, L. J., Harrison, S. C., Skehel, J. J., and Wiley, D. C. 1999. Structural basis for membrane fusion by enveloped viruses. Mol. Membr. Biol. 16:3-9.

228. Wertz, G. W., Davis, N. L., and Patton, J. 1987. in The Rhabdoviruses, Wagner, R. R., ed. (Plenum, New York.), pp 271-296.

229. Wertz, G.W., Whelan, S., LeGrone, A., and Ball, L.A. 1994. Extent of terminal complementarity modulates the balance between transcription and replication of vesicular stomatitis virus RNA. Proc. Natl. Acad. Sci. 91:8587-8591.

230. Wertz, G.W., Moudy, R., and Ball, L.A. 2002.Adding genes to the RNA genome of vesicular stomatitis virus: positional effects on stability of expression. J. Virol. 76:7642-7650.

231. Whelan, S. P. J., Ball, L. A., Barr, J. N., and Wertz, G. T. W. 1995. Efficient recovery of infectious vesicular stomatitis virus entirely from cDNA clones. Proc. Natl. Acad. Sci. USA 92:8388-8392.

232. Whelan, S.P., and Wertz, G.W. 1999. Regulation of RNA synthesis by the genomic termini of vesicular stomatitis virus: identification of distinct sequences essential for transcription but not replication. J. Virol. 73:297-306.

233. Wingfield, W. H., J. L. Fryer, and K. S. Pilcher. 1969. Properties of sockeye salmon virus (Oregon strain). Proc. Soc. Exp. Biol. Med. 30:1055-1059.

234. Winton, J. R., Batts, W. N., Nishizawa, T., and Stehr, C. 1989. Characterization of the first North American isolates of viral hemorrhagic septicemia virus. American Fisheries Society, Fish Health Section Newsletter 17:2-3.

235. Winton, J.R. 1991. Recent advances in detection and control of infectious hematopoietic necrosis virus in aquaculture. Ann. Rev. Fish Dis. 1:83-93.

236. Winton, J.R., 1997. Immunization with viral antigens: infectious haematopoietic necrosis. Dev. Biol. Stand. 90:211-220.

237. Wolf, K. 1988. Fish viruses and fish viral diseases. Cornell University Press, Ithaca, NewYork.

238. Wolf, K., Quimby, M.C., Pettijohn, L.L., and Landolt, M.L. 1973. Fish viruses: Isolation and identification of infectious hematopoietic necrosis in eastern North America journal of the fisheries research board of Canada. 30:1625-1627.

239. Xiang, Z.Q., Knowles, B. B., McCarrick, J. W. and Ertl, H. C. J. 1995. Immune effector mechanisms required for protection in rabies virus. Virol. 214:398-403.