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Recovery of recombinant infectious hematopoietic necrosis virus strain Sn1203 using the mammalian cell line BHK-21

Jing-Zhuang Zhao[a], Li-Ming Xu[a], Zhen-Yu Zhang[b], Miao Liu[a], Yong-Sheng Cao[a], Jia-Sheng Yin[a], Hong-Bai Liu[a], Tong-Yan Lu[a,*]

[a] Heilongjiang River Fishery Research Institute Chinese Academy of Fishery Sciences, Harbin 150070, China
[b] State Key Laboratory of Veterinary Biotechnology, Harbin Veterinary Research Institute, Chinese Academy of Agricultural Sciences, Harbin, 150001, China

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ABSTRACT

Reverse genetics systems are powerful tools for understanding the virulence mechanisms and gene functions of negative-sense RNA viruses. The reverse genetics systems commonly used for recombinant infectious hematopoietic necrosis virus (IHNV) are based on vaccinia virus infection. To avoid the potential biological safety risks associated with vaccinia virus, a recombinant IHNV virus strain Sn1203 (rIHNV-Sn1203) was rescued in this study using a mammalian cell line, BHK-21. The genome sequence authenticity of rIHNV-Sn1203 was confirmed using two silent genetic tags introduced by site-directed mutagenesis. Indirect immunofluorescence assays and transmission electron microscopy revealed that rIHNV-Sn1203 and wild-type IHNV-Sn1203 (wtIHNV-Sn1203) had identical immunogenicity and virion morphology. The virulence and pathogenicity of rIHNV-Sn1203 were assessed *in vitro* and *in vivo*. Although rIHNV-Sn1203 displayed trends toward delayed intracellular viral replication and lower virion yields compared with wtIHNV-Sn1203, statistical analyses revealed no significant differences between these two viruses. Moreover, rainbow trout challenged with rIHNV-Sn1203 and wtIHNV-Sn1203 showed indistinguishable mortality. Together, these results show that IHNV was successfully rescued using BHK-21 cells. This method is very convenient and may also be suitable for use in the recovery of other *Novirhabdoviruses*.

1. Introduction

Infectious hematopoietic necrosis (IHN) is one of the most important viral diseases of the salmon and trout aquaculture industries (Nishizawa et al., 2006; Kurath, 2005). Infectious hematopoietic necrosis virus (IHNV) is the causative agent of IHN disease, and different viral strains can result in up to 90% mortality depending on the fish species and the farming environment (Zhao et al., 2017a,b; Breyta et al., 2013; Enzmann et al., 2005). IHNV was historically endemic throughout the Pacific Northwest of North America, but it can now be found in many countries including Japan (Nishizawa et al., 2006), Iran (Ahmadivand et al., 2017), Canada (Hsu et al., 1986), Korea (Kim et al., 2007), Russia (Rudakova et al., 2007), and the Netherlands (Haenen et al., 2016). In China, the first reported IHN outbreak occurred in 1985 in a juvenile rainbow trout hatchery in Liaoning Province (Xu et al., 2016a). Subsequently, this disease has caused severe economic losses in the Chinese salmonid aquaculture industry.

IHNV is a non-segmented, single-stranded, negative-sense RNA virus that belongs to the genus *Novirhabdovirus* in the family *Rhabdoviridae* (Kurath et al., 2003). The viral genome is approximately 11 kb in length, containing six open reading frames that encode the nucleoprotein (N), phosphoprotein (P), matrix protein (M), glycoprotein (G), polymerase protein (L), and non-structural protein (NV) (Troyer et al., 2000;). The bullet-shaped IHNV virion is about 180 nm long and 75 nm in diameter (Alderman, 1989).

Reverse genetics methods for use in negative-sense RNA viruses have been well developed and used for many viruses. To date, infectious viruses have been generated from recombinant full-length cDNA of influenza A virus (Neumann et al., 1999), rabies virus (Schnell et al., 1994), Newcastle disease virus (Peeters et al., 1999), and many other viruses using the T7 RNA polymerase-expressing vaccinia virus system. The fish rhabdovirus IHNV was also recovered using a recombinant vaccinia virus expressing T7 RNA polymerase (vTF7-3) (Biacchesi et al., 2000). Using the same vTF7-3 vaccinia system, other fish rhabdoviruses, such as snakehead rhabdovirus (SHRV) (Johnson et al., 2000), and viral hemorrhagic septicemia virus (VHSV) (Biacchesi

* Corresponding author.
E-mail addresses: zhaojingzhuang1987@163.com (J.-Z. Zhao), 279463437@qq.com (L.-M. Xu), 185917893@qq.com (Z.-Y. Zhang), 446048847@qq.com (M. Liu), caoyongsheng@hrfri.ac.cn (Y.-S. Cao), xwsc20@tom.com (J.-S. Yin), 112893129@qq.com (H.-B. Liu), lutongyan@hrfri.ac.cn (T.-Y. Lu).

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et al., 2010), were also recovered successfully. Although the vaccinia system was able to recover recombinant virus effectively, it presents several biological safety risks; for example, it allows homologous recombination between the full-length plasmid and the transfected supporting plasmids (Wang et al., 2016; Garcin et al., 1995).

To avoid the potential risks associated with the vaccinia helper virus, we rescued a recombinant IHNV (rIHNV) virus using a mammalian cell line, BHK-21, which was reported to propagate IHNV at the optimal temperature for this virus (Clark and Soriano, 1974). The biological characteristics of rescued rIHNV were analyzed and compared with wild-type IHNV (wtIHNV). To the best of our knowledge, this study is the first to report the use of a mammalian cell line to rescue an IHNV virus without a helper virus. This method is very convenient and likely also suitable for the recovery of other *Novirhabdoviruses*.

2. Materials and methods

2.1. Virus strains and cell lines

The parental virus, IHNV-Sn1203 (GenBank No: KC660147.1), was isolated from moribund rainbow trout fry in eastern China. Baby hamster kidney cells (BHK-21) stably expressing T7 RNA polymerase were kindly provided by Professor Deshan Li (College of Life Science, Northeast Agriculture University, Harbin, China). Viral rescue was performed using BHK-21 cells in six-well plates. The epithelioma *papulosum cyprini* (EPC) cell line was used to propagate and identify parental and rescued recombinant viruses. EPC cells (CRL-2872, ATCC) were kindly provided by Dr. Zeng Lingbing, Yangtze River Fisheries Research Institute, Chinese Academy of Fishery Sciences, Wuhan, China. EPC cells were cultured at 25 °C using 10% fetal bovine serum with Eagle's minimum essential medium (MEM, Gibco, USA) containing 0.1 mg/ml streptomycin and penicillin as previously described (Zhao et al., 2017a), and IHNV isolates were passaged three times prior to sequencing.

2.2. Primers for rIHNV-Sn1203 construction

The full-length viral genome sequence was divided into five fragments, and each was inserted into a plasmid vector in turn. The primers used for the construction of genomic and helper plasmids are shown in Table 1.

The complete wtIHNV-Sn1203 genome sequence was divided into five fragments and amplified using primers 2–11. Primers 1 and 12 were used to add appropriate restriction enzyme recognition sequences into fragment 1 (*Not* I) and 5 (*Nar*I), as these sequences were necessary for cloning the five fragments into the vector. Primers 13–20 were used to construct helper plasmids containing the nucleoprotein (N), phosphoprotein (P), polymerase protein (L), and non-structural protein (Nv) genes, respectively. Primers 21–24 were used to amplify rIHNV-Sn1203 sequences bearing engineered genetic tags.

2.3. Construction of a full-length cDNA genome of rIHNV-Sn1203

Total IHNV-Sn1203 genomic RNA was extracted from the supernatants of IHNV-infected EPC cells using the GenElute™ Total RNA Purification Kit (RNB100, Sigma, St. Louis, MO, USA) following the manufacturer's instructions. Five fragments, together encoding the complete antisense genome of IHNV-Sn1203, were amplified by reverse transcriptase-polymerase chain reaction (RT-PCR) using a SuperScript III One-Step RT-PCR Platinum *Taq* HiFi Kit (12574030, Invitrogen) and then successively cloned into a modified pBluescript-based vector using restriction sites present in the parental virus genome. The final plasmid was named pIHNV-Sn1203 and contained two silent recognition tags (*Nhe*I and *Xho*I) engineered by site-directed mutagenesis (Fig. 1).

2.4. Construction of N, P, Nv, and L gene expression plasmids

As previously reported, co-expression of viral nucleoprotein (N), phosphoprotein (P), non-structural protein (Nv), and polymerase protein (L) is required for the production of infectious *Novirhabdovirus* virions (Thoulouze et al., 2004). The complete coding sequences of these four genes were amplified and cloned into the expression vector pTM1 using the primers shown in Table 1. These four genes were ligated into the vector using the In-Fusion HD Cloning Plus Kit (638909, Takara, Japan). The four resulting plasmids were all sequence-verified, and the final plasmids were named pIH-N, pIH-P, pIH-NV, and pIH-L.

2.5. Recovery of rIHNV-Sn1203

The recombinant plasmids pIHNV-Sn1203, pIH-N, pIH-P, pIH-NV,

Table 1
Primer sequences used for the construction and identification of rIHNV-Sn1203 plasmids.

Primer	Primer name	Nucleotide position	Primer sequence
1	IHN1 F Ext	1-8	AAGCGGCCGCTAATACGACTCACTATAGGGGTATAAAA
2	IHN1 F	1-23	CGACTCACTATAGGGGTATAAAAAAAGTAACTTGACTA
3	IHN1 R	2019-2050	GGCGCCTTGGGATCCTGCGGTGTCTGGGGTGA
4	IHN2 F	2030-2058	ACCGCAGGATCCCAAGAGGTGAAGAACAT
5	IHN2 R	4011-4038	GGCGCCTAGACGTCATTTATTCCGGGAT
6	IHN3 F	4018-4045	AATAAATGACGTCTACGCTATGCACAAA
7	IHN3 R	7011-7039	GGCGCCATGACGCGTTCTACCCTAAGTAA
8	IHN4 F	7015-7041	TTAGGGTAGAACGCGTCATGCAGAAAA
9	IHN4 R	9132-9159	GGCGCCATTCCATGGGCATTGAGTAGAA
10	IHN5 F	9135-9163	TACTCAATGCCCATGGAATCACAACGGCT
11	IHN5 R	11108-11131	TGGGACCATGCCGGCCGTATAAAAAAAGTAACAGAGAGAT
12	IHN5 R Ext	11125-11131	GGCGCCAGCGAGGAGGCTGGGACCATGCCGGCCGTATAAA
13	IHN N F	1-25	AAACACGATAATACCATGACAAGCGCACTCAGAGAGACGT
14	IHN N R	1148-1176	TCGGATCTTAGGTCATCAGTGGAATGAGTCGGAGTCTTCTGGCT
15	IHN P F	1-22	AAACACGATAATACCATGTCAGATGGAGAAGGAGAAC
16	IHN P R	669-693	TCGGATCTTAGGTCACTATTGACCCTGCTTCATGCGCTTC
17	IHN NV F	1-25	AAACACGATAATACCATGGACCACCGTGAAATAAACACGT
18	IHN NV R	312-336	TCGGATCTTAGGTCACTATCTGGGATAAGCAAGAAATTCT
19	IHN L F	1-25	AAACACGATAATACCATGGACTTCTTCGATCTCGACATAG
20	IHN L R	5937-5961	TCGGATCTTAGGTCACTATTGTTCGCCTAGTGGAAAGAAG
21	Fra 1 F	347-371	AGGGAACGAGAAGGGCATTGGGCCT
22	Fra 1 R	1619-1644	TTCTTGTCGGGTCTCCTGGTGGGTTT
23	Fra 2 F	2820-2846	AAAAACGGGGGAAGGAAAAATAGGGGT
24	Fra 2 R	4324-4350	TCCAATTTCCTGATGGAGATCCCCGAT

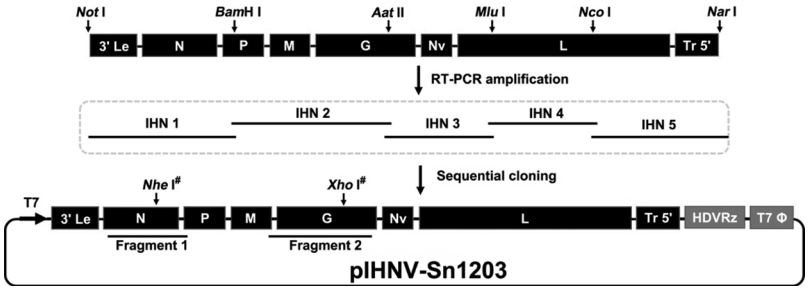

Fig. 1. Schematic representation of the cloning strategy for the complete IHNV-Sn1203 genome. Five overlapping fragments were amplified and cloned into the vector backbone. The vector contains the hepatitis delta virus ribozyme sequence (HDVRz), which can cleave the T7 polymerase promoter-transcribed RNA molecule into the exact length of the IHNV RNA genome. Two additional unique restriction sites (*Nhe*I and *Xho*I) were added to the viral genome sequence using site-directed mutagenesis, and they served as molecular tags to differentiate rIHNV-Sn1203 from wtIHNV-Sn1203.

Table 2
Primer sequences used in qRT-PCR.

Primer name	Primer sequence
IHNV-N Forward	GCTCACCAAGGCTGTTTAT
IHNV-N Reverse	CATCAGTCTTACAATGCGTCTA
IHNV-P Forward	AAGGACTTCGGAGGTCTAAT
IHNV-P Reverse	TTTCTCGTGCTCTTGCTG
IHNV-M Forward	GATGGAGTTCGGAAGCAC
IHNV-M Reverse	AGGCTGGGTCTGAAGGTA
IHNV-G Forward	CATCAGTCTTACAATGCGTCTA
IHNV-G Reverse	TGGAGGCGGGATAAGAAA
IHNV-NV Forward	ACGGAGACCTGGTATGGC
IHNV-NV Reverse	CTCGTCCTTGGTGATGCT
IHNV-L Forward	TGGGAGCCATTGGTGATT
IHNV-L Reverse	GGTTGAGCGTCGGTTTGC

and pIH-L were purified using the PureLink HiPure Plasmid Filter Maxiprep Kit (00464302, Invitrogen). BHK-21 cells were seeded in six-well plates and cultured at 37 °C in an atmosphere containing 5% CO_2. When the cell density reached 70–80%, the cells were washed twice with minimal essential medium (Opti-MEM®) and transfected with a mixture of pIHNV-Sn1203, pIH-N, pIH-P, pIH-NV, and pIH-L using Lipofectamine 2000 Reagent (1857332, Invitrogen) following the manufacturer's instructions. The transfected cells were incubated at 15 °C for 5 days, then lysed using two freeze-thaw cycles. The supernatant was centrifuged at 7000 × *g* for 10 min at 4 °C and then used to inoculate fresh EPC cell monolayers at 15 °C. After 5 days, the supernatant was collected and passaged once more. The final supernatant was then used as a stock of rIHNV-Sn1203.

2.6. Identification of rIHNV-Sn1203

2.6.1. Sequence analysis of rIHNV-Sn1203

Viral RNA of wtIHNV-Sn1203 and rIHNV-Sn1203 were extracted from the virus-containing cell culture supernatants using TRIzol Reagent. Fragment 1 (shown in Fig. 1) was amplified by RT-PCR using Fra1F and Fra1R as primers (Table 1) and then purified and digested with *Nhe*I. Fragment 2 was amplified using Fra2F and Fra2R and then purified and digested with *Xho*I.

2.6.2. Indirect immunofluorescence assay

EPC cells were infected with rIHNV-Sn1203 (MOI = 0.1), wtIHNV-Sn1203 (MOI = 0.1), or phosphate-buffered saline (PBS) (mock infection). The infected cells were cultured in MEM medium containing 2% (v/v) FBS at 15 °C for 24 h, then fixed with 4% (w/v) paraformaldehyde. After permeabilization with 0.5% (v/v) Triton X-100, the cells were successively incubated with rabbit anti-IHNV antibody and Cy3-labeled goat anti-rabbit IgG antibody as previously described (Zhao et al., 2017a; Xu et al.,2016b). After being washed three times with PBS, the cells were observed using a fluorescence microscope (Leica, DMi8, Germany).

2.6.3. Transmission electron microscopy

EPC cells were treated with rIHNV-Sn1203 (MOI = 0.1) or wtIHNV-Sn1203 (MOI = 0.1). After being incubated for 24 h, the treated cells were washed with PBS and collected. The cells were then fixed with 2.5% (v/v) glutaraldehyde for 24 h and post-fixed in 1% (w/v) osmium tetroxide for 90 min. After dehydration using an ethanol concentration gradient (50%, 70%, 80%, and 90%), the cells were washed with acetone for 20 min, then embedded in resin for 24 h and sectioned. The sections were stained with uranyl acetate and then observed using a transmission electron microscope (Hitachi, 7650, Japan).

2.7. Virulence assay

2.7.1. Quantification of viral replication

EPC cells (2×10^6) were collected at 12-h intervals from 12 to 96 h after virus infection, and total mRNA was extracted. Quantitative real-time polymerase chain reaction (qRT-PCR) was used to quantify IHNV genomic material using a qRT–PCR kit (RR096 A, Takara, Japan). Target IHNV genes were amplified using the primers listed in Table 2, and their expression levels were calculated using the $2^{-\Delta\Delta CT}$ method (Zhao et al., 2017b). The β-actin gene was used as a reference.

2.7.2. Extracellular IHNV virion yields

Supernatants of IHNV-treated cells were collected at 12-h intervals from 12 to 96 h after virus infection for titer determination. The supernatants were serially diluted 10-fold and assayed to determine the 50% tissue culture infective dose ($TCID_{50}$). EPC cells were plated in 96-well plates and then treated with 100 µl of diluted IHNV. After being incubated for 1 h, the cells were washed with PBS and then cultured in MEM medium containing 2% FBS. Infected cells were observed to assess the presence of cytopathic effect, and the results were recorded daily for 10 days.

2.7.3. Pathogenicity assay

In vivo infection was used to assess the pathogenicity of rIHNV-Sn1203. Rainbow trout (5 ± 1 g mean bodyweight) were divided into three groups. Each group contained 50 trout in separate tanks (two tanks in parallel) maintained at 15 °C. After two weeks in these tanks, the rainbow trout were challenged by intraperitoneal injection and immersion. Rainbow trout were injected with 2.0×10^2 pfu indicated virus (wtIHNV-Sn1203 and rIHNV-Sn1203) in the intraperitoneal injection experiment. For the immersion challenge experiment, rainbow trout were held statically for 1 h with air added to the tank, and the concentration of virus was 1.0×10^5 pfu/ml. Control groups were mock infected with PBS. These groups were held in separate tanks and cumulative mortality was recorded for 25 days.

2.8. Statistical analyses

Differences in viral gene expression between wtIHNV-Sn1203 group and rIHNV-Sn1203 were analyzed using ANOVAs with the GraphPad Prism version 6 for multiple comparisons, and Student's *t*-tests were used to assess differences between these two groups at a single time point. To estimate the expected mortality profile of wtIHNV-Sn1203 group and rIHNV-Sn1203 group, Kaplan-Meier survival curves with pointwise 95% confidence bands were computed and plotted. Statistical

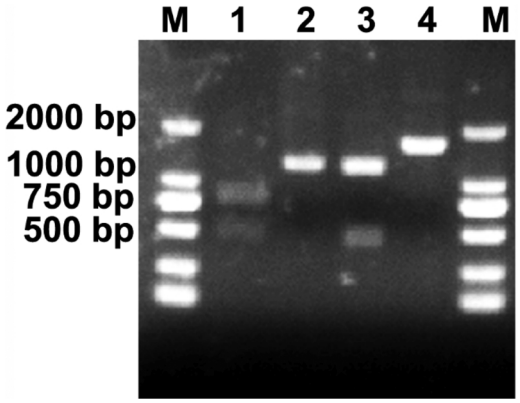

Fig. 2. Identification of rIHNV-Sn1203 using silent genetic tags. The viral RNA of wtIHNV-Sn1203 and rIHNV-Sn1203 were extracted from virus-infected cell culture supernatants. Fragments 1 and 2 were both amplified by RT-PCR. Lane M shows DNA molecular weight standards. Lanes 1 and 2 show fragment 1 amplified from rIHNV-Sn1203 and wtIHNV-Sn1203, respectively, and then digested with *Nhe*I. Lanes 3 and 4 show fragment 2 amplified from rIHNV-Sn1203 and wtIHNV-Sn1203, respectively, and then digested with *Xho*I.

significance was assumed at $p < 0.05$.

3. Results

3.1. Identification of rIHNV-Sn1203 using silent genetic tags

To differentiate rIHNV-Sn1203 from wtIHNV-Sn1203, we added two silent genetic tags (*Nhe*I and *Xho*I sites) to the rIHNV-Sn1203 genome using site-directed mutagenesis. After three passages of rIHNV-Sn1203, the total viral genomic RNA was extracted from the supernatants of IHNV-infected EPC cells and used for virus identification. Fragment 1 (1298 bp) and fragment 2 (1531 bp) were both amplified from the extracted RNA by RT-PCR. After purification, fragment 1 was digested with *Nhe*I and fragment 2 was digested with *Xho*I. The results show that fragments 1 and 2 from rIHNV-Sn1203 could be digested using *Nhe*I and *Xho*I, but fragments from wtIHNV-Sn1203 could not be digested with these enzymes (Fig. 2). The full genome sequence identity of the recombinant infectious clone was also determined, and no genetic mutation was found compared with wild-type IHNV except for the two introduced silent genetic tags (*Nhe*I and *Xho*I sites) (data not shown).

3.2. Indirect immunofluorescence analysis of rIHNV-Sn1203

After inoculation with wtIHNV-Sn1203 and rIHNV-Sn1203, EPC cells were assayed by indirect immunofluorescence. The results show specific red fluorescence in both wtIHNV-Sn1203- and rIHNV-Sn1203-infected cells but no fluorescence in PBS-treated cells (Fig. 3).

3.3. Transmission electron microscopy of rIHNV-Sn1203

To investigate whether the rIHNV-Sn1203 had the same virion morphology as wtIHNV-Sn1203, the ultrastructures of rIHNV-Sn1203 and wtIHNV-Sn1203 were visualized using transmission electron microscopy. As shown in Fig. 4, typical bullet-shaped virions were observed for both wtIHNV-Sn1203 and rIHNV-Sn1203, with an average virion size of approximately 80 nm × 160 nm. We observed both immature virions undergoing assembly in the cytoplasm as well as mature virions budding from the cell membrane.

3.4. Virulence analyses of rIHNV-Sn1203

3.4.1. Growth curves of rIHNV-Sn1203

The growth kinetics of rIHNV-Sn1203 were determined by

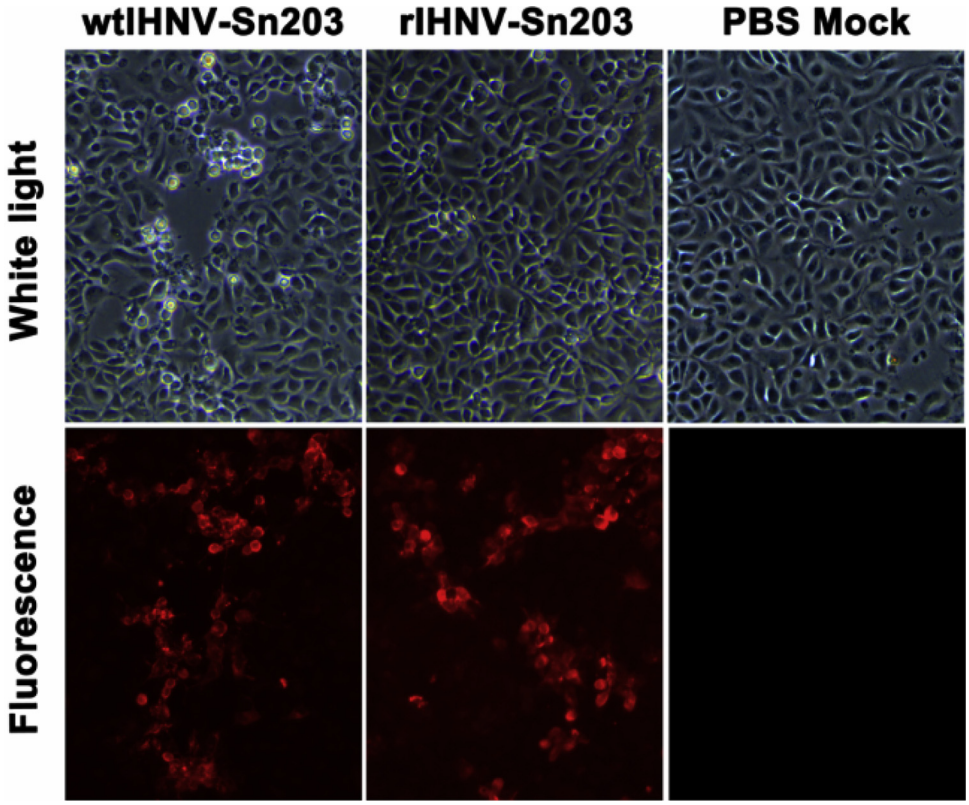

Fig. 3. Indirect immunofluorescence analysis of rIHNV-Sn1203. After being infected with rIHNV-Sn1203 or wtIHNV-Sn1203 for 24 h, the cells were incubated sequentially with rabbit anti-IHNV antibody and Cy3-labeled goat anti-rabbit IgG antibody. The cells were then observed using a fluorescence microscope.

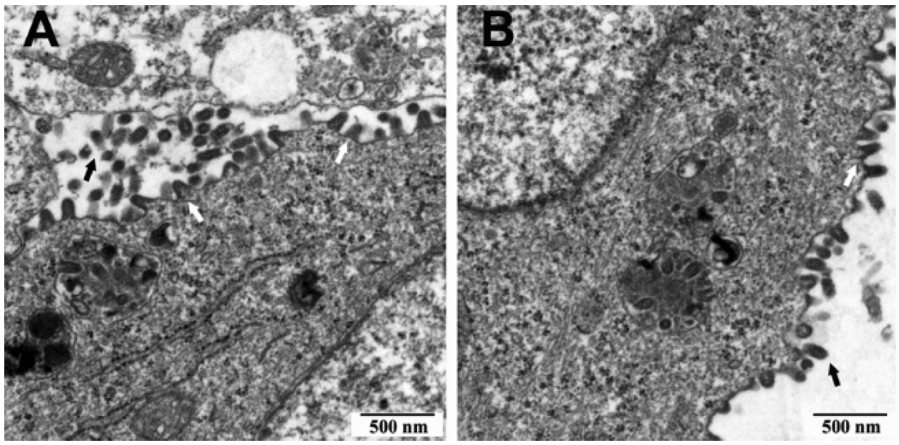

Fig. 4. Transmission electron microscopy of rIHNV-Sn1203. A–B EPC cells were infected with wtIHNV-Sn1203 (A) or rIHNV-Sn1203 (B) at an MOI of 0.1 for 24 h, and samples were then fixed with glutaraldehyde, embedded in resin for 24 h and sectioned. After staining with uranyl acetate, virion morphology was observed using transmission electron microscopy. White arrows indicate immature virions assembling and budding from the cell membrane, and black arrows indicate mature virus.

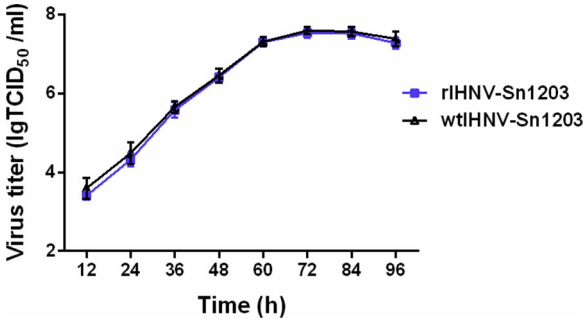

Fig. 5. Growth kinetics of rIHNV-Sn1203. EPC cells were infected with wtIHNV-Sn1203 or rIHNV-Sn1203 (MOI = 0.1). Cell supernatants were harvested in triplicate in two independent experiments at 12-h intervals post-infection. Virus titers were measured by determining the $TCID_{50}$ on EPC cells and are expressed at the mean $\log_{10}$ $TCID_{50}$/ml.

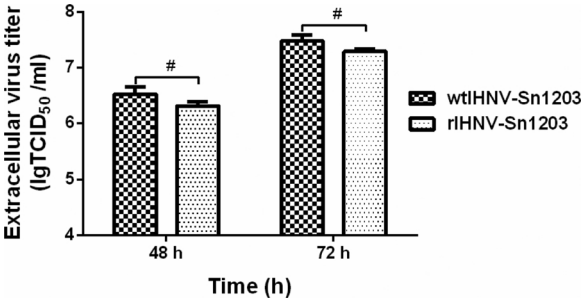

Fig. 7. Extracellular viral yields of rIHNV-Sn1203. Supernatants of rIHNV-Sn1203- or wtIHNV-Sn1203-infected cells were collected 48 h and 72 h post-infection. $TCID_{50}$ assays were performed to measure extracellular virion yields. The standard deviations of the means are shown by error bars (n = 3). # indicates no statistically significant difference ($p > 0.05$).

measuring viral titers at 12-h intervals from 12 to 96 h post-infection. As expected, rIHNV-Sn1203 had similar growth kinetics compared with wtIHNV-Sn1203, and both viruses reached a plateau stage at 72 h post-infection (wtIHNV-Sn1203, $1 \times 10^{7.59}$; rIHNV-Sn1203, $1 \times 10^{7.53}$) (Fig. 5).

3.4.2. Quantification of viral replication

To quantify the intracellular viral replication of rIHNV-Sn1203 and wtIHNV-Sn1203, the viral mRNA abundance of N, P, M, G, Nv, and L transcripts were measured by qRT-PCR. As shown in Fig. 6, there were no significant differences in the N, P, M, G, Nv, and L mRNA levels between rIHNV-Sn1203 and wtIHNV-Sn1203.

Supernatants from rIHNV-Sn1203- and wtIHNV-Sn1203-infected cells were collected at 48 h and 72 h post-infection, and the $TCID_{50}$ was calculated to determine the extracellular viral yields. The results show that at 48 h post-infection, the rIHNV-Sn1203 titer was $10^{6.32}$ $TCID_{50}$/ml and the wtIHNV-Sn1203 titer was $10^{6.53}$ $TCID_{50}$/ml, while at 72 h

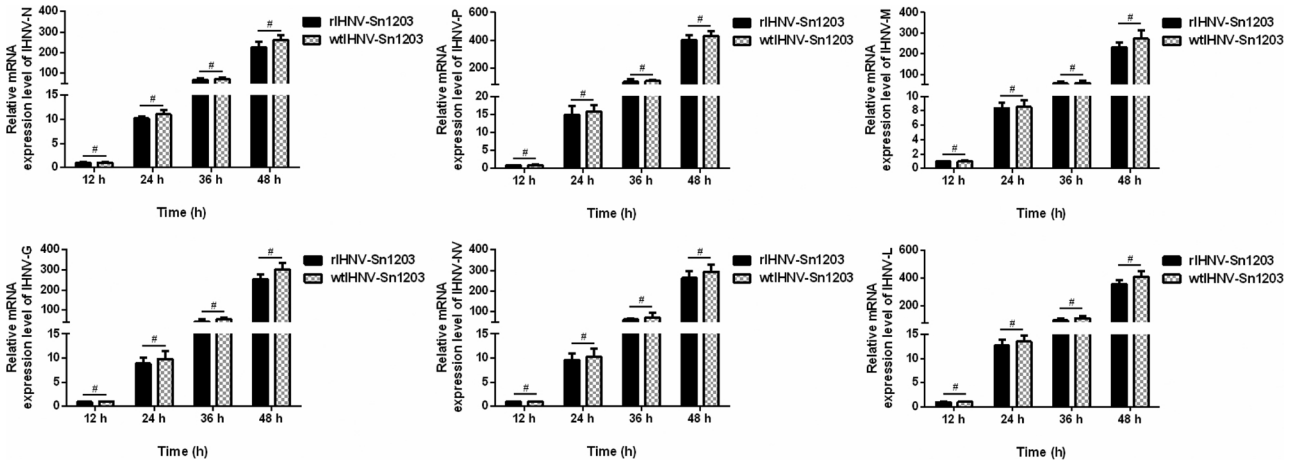

Fig. 6. Intracellular mRNA abundance of rIHNV-Sn1203. Cells were infected with rIHNV-Sn1203 or wtIHNV-Sn1203 (MOI = 0.1). The N, P, M, G, Nv, and L mRNA levels of rIHNV or wtIHNV in infected cells were determined by qRT-PCR at 12-h intervals. The β-actin gene was used as a reference, and target IHNV mRNA levels were calculated with the $2-^{\Delta\Delta CT}$ method. The standard deviations of the means are represented by error bars (n = 3). # indicates no statistically significant difference ($p > 0.05$).

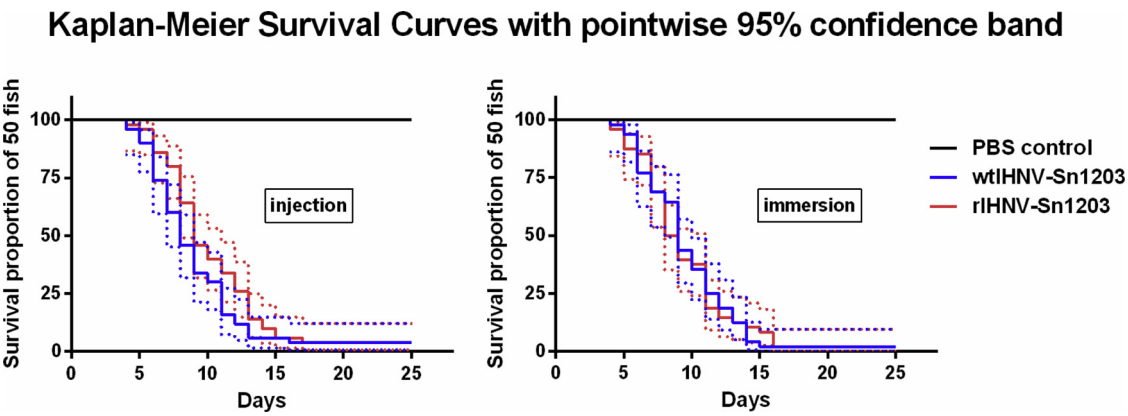

Fig. 8. Kaplan-Meier survival curves of rainbow trout challenged with different IHNVs. Rainbow trout were challenged by intraperitoneal injection or immersion with PBS (mock challenge), wtIHNV-Sn1203, or rIHNV-Sn1203 as described in Materials and methods. Deaths caused by IHNV were recorded daily for 25 days.

post-infection, the rIHNV-Sn1203 titer was $10^{7.29}$ $TCID_{50}$/ml and the wtIHNV-Sn1203 titer was $10^{7.48}$ $TCID_{50}$/ml. These results demonstrat that there were no differences between the extracellular virion yields of rIHNV-Sn1203 and wtIHNV-Sn1203 (Fig. 7).

3.5. Pathogenicity analyses of rIHNV-Sn1203

A virus challenge (injection or immersion) of rainbow trout was used to assess the relative pathogenicity of rIHNV-Sn1203 and wtIHNV-Sn1203. After infection with IHNV by injection for 3 days, rainbow trout in both treatment groups began to die, with peak mortality occurring after 6–8 days. The cumulative mortality of injection after 25 days was 94% in rIHNV-Sn1203-infected trout and 96% in wtIHNV-Sn1203-infected trout (Fig. 8). The rainbow trout challenged with IHNV by immersion began to die after 4 days, and peak mortality occurring after 6–11 days. The cumulative mortalities of immersion were both 94% in rIHNV-Sn1203-infected and wtIHNV-Sn1203-infected trout (Fig. 8). Although the rIHNV-Sn1203-challenged trout showed a trend toward lower mortality between 5 and 14 days post-infection in injection challenge, the cumulative mortality in both rIHNV-Sn1203- and wtIHNV-Sn1203-infected trout showed no significant differences. These results suggest that there were no significant differences in virus pathogenicity between wtIHNV-Sn1203 and rIHNV-Sn1203.

4. Discussion

Reverse genetics technology is a powerful platform by to study the roles of specific genes in a virus genome sequence, and it can also be used to design viral-vectored vaccines. The first reverse genetics system used to recover rIHNV was the vaccinia virus pre-infection system (Biacchesi et al., 2000). There, the vaccinia virus was used as a helper virus to express T7 RNA polymerase and support the rescue of rIHNV. Using the vaccinia virus system, one can successfully obtain rescued rIHNV, but the use of helper virus may confer some biological safety risks. A study of rescued Sendai virus created *via* helper vaccinia virus showed that the resulting recombinant Sendai virus removed deleterious mutations in the L gene. This finding confirms that vaccinia virus may cause uncertain mutations in genomic sequences *via* homologous recombination during pre-infection (Garcin et al., 1995).

The optimal temperature for vaccinia virus infection and T7 RNA polymerase expression is 37 °C, but this temperature is not optimal for IHNV replication (optimal temperature: 15 °C). Thus, to use this platform for rescuing IHNV, one would need to incubate EPC cells with vaccinia virus at 37 °C for at least 8 h to allow the expression of T7 RNA polymerase and then drop the temperature to 15 °C for virus replication. This higher temperature incubation has shown some deleterious effects on EPC cells and may hinder the recovery and titer of rIHNV (Lopez et al., 2001). To circumvent the risks associated with vaccinia virus, Alonoso et al. established an EPC cell line that stably expresses T7 RNA polymerase to rescue SHRV (Alonso et al., 2004). Another vaccinia-virus-free reverse genetics system was later established using a CMV promoter-driven rescue plasmid that can be transfected into EPC cells (Ammayappan et al., 2010). Although virus rescue is feasible using T7 RNA polymerase-expressing EPC cells or a CMV promoter-driven rescue plasmid, the relatively lower transfection efficiency of EPC cell lines may require more time and higher expenses to obtain recombinant viruses for commercial use.

To overcome these problems, we rescued a rIHNV-Sn1203 virus using the mammalian cell line, BHK-21. BHK-21 cells are baby hamster kidney cells; they are widely used for virus proliferation and veterinary vaccine production because of their higher transfection efficiency and well-studied genetic background. A previous study reported that BHK-21 cells could grow at a wide range of temperatures, and three fish rhabdoviruses (IHNV, VHSV, and spring viremia of carp virus (SVCV)) were able grow and replicate in BHK-21 cells at their optimal replication temperatures (Clark and Soriano, 1974). These results indicate that IHNV id capable of using the cellular and nuclear machinery of BHK-21 to produce viral RNA segments and functional viral proteins.

Knowing that BHK-21 cells were suitable for IHNV replication, the recovery of recombinant IHNV was performed by co-transfecting recombinant plasmids containing pIHNV-Sn1203 sequences and four helper plasmids. During plasmid construction, different restriction sites were employed to obtain pIHNV-Sn1203. Silent mutations do not change the amino acid sequence, but they may affect post-transcriptional processing (Cartegni et al., 2002). Taking this into consideration, we used six restriction sites that naturally exist in the viral genome sequence to construct the plasmid pIHNV-Sn1203, and we introduced only two silent genetic tags (*Nhe*I and *Xho*I) to differentiate rIHNV-Sn1203 from wtIHNV-Sn1203. After identification by its genetic tags, rIHNV-Sn1203 was detected by indirect immunofluorescence assay and transmission electron microscopy. The rescued virus, rIHNV-Sn1203, showed identical immunogenicity and morphology compared with wtIHNV-Sn1203. Virulence analyses showed that rIHNV-Sn1203 reached a growth plateau stage at 72 h post-infection, and there were no significant differences between the intracellular viral replication or extracellular viral yields of rIHNV-Sn1203 and wtIHNV-Sn1203. Rainbow trout infected with either rIHNV-Sn1203 or wtIHNV-Sn1203 exhibited 90% cumulative mortality, which indicates that the recombinant and wild-type viruses were equivalently pathogenic.

In conclusion, rIHNV-Sn1203 was successfully rescued using BHK-21 cells, and the resulting recombinant virus had the same biological characteristics as wtIHNV-Sn1203. Given that IHNV is a fish rhabdovirus, BHK-21 cells can likely be used to rescue other related viruses, such as VHSV and SVCV. Although a previously reported study

established a vaccinia-virus-free reverse genetics system using a CMV promoter-driven rescue plasmid, our result may provide another choice for recombinant virus rescue without the construction another CMV-driven rescue plasmid, and this method could also be used to study the virulence-related genes and molecular pathogenic mechanisms of the studied virus.

Conflict of interest

The authors declare that they have no conflict of interest related to this work.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.jviromet.2019.01.002.

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