Breaking the host range: mandarin fish is susceptible to a vesiculovirus derived from snakehead fish

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Members of the genus Vesiculovirus, which belongs to the family Rhabdoviridae, can cause great economic loss in fish culture. In the present report, a vesiculovirus [named snakehead fish vesiculovirus (SHVV)] was isolated from diseased hybrid snakehead fish. SHVV shared 94 % nucleotide sequence identity at the genomic level with Siniperca chuatsi rhabdovirus (SCRV), which infects mandarin fish (S. chuatsi). We showed that SHVV was able to replicate and proliferate well in SSN-1 cells, which originate from striped snakehead fish (Channa striatus). Furthermore, mandarin fish was susceptible to SHVV by bath exposure, as well as by intraperitoneal injection. The infected fish showed typical clinical signs of rhabdovirus infection, including haemorrhage and oedema. Histopathological analysis revealed that extensive inflammation and necrosis were observed in the spleen, kidney, liver, heart and brain of the moribund mandarin fish. These results will shed new light on the epidemic of vesiculovirus infections among fish.

INTRODUCTION

Members of the family Rhabdoviridae can infect various hosts, including plants, insects, fish and mammals, and cause serious diseases of the infected hosts (Lyles & Rupprecht, 2006; Kuzmin et al., 2009). To date, the family Rhabdoviridae consists of nine genera, including Novirhabdovirus, Perhabdovirus and Vesiculovirus, that can infect fish (King et al., 2012). The genome of vesiculoviruses consists of a negative-sense ssRNA encoding five viral structural proteins: the nucleoprotein (N), phosphoprotein (P), matrix protein (M), glycoprotein (G) and large protein (L) or polymerase (Lyles & Rupprecht, 2006). In 2014, a devastating disease outbreak occurred in hybrid snakehead fish cultured in a farm located in Guangdong Province, China. We isolated a vesiculovirus from the diseased snakehead fish, designated snakehead fish vesiculovirus (SHVV). It was reported that infection by Siniperca chuatsi rhabdovirus (SCRV) could cause great economic loss in mandarin fish (S. chuatsi) culture. Interestingly, SHVV shares 94 % nucleotide sequence identity with SCRV at the genomic level (Zeng et al., 2014). Mandarin fish and snakehead fish are two important commercial fish cultured in East Asian countries, and sometimes they are cultured in the same area. Understanding the transmission of viral diseases between these two fish is therefore important for developing strategies to prevent the spread of such diseases in the aquaculture industry. Thus, the susceptibility of mandarin fish to SHVV was studied in the present report. We showed that mandarin fish are susceptible to SHVV by bath exposure and intraperitoneal (i.p.) injection. SHVV was able to replicate and proliferate well in an SSN-1 cell line, which is derived from striped snakehead fish (Channa striatus). Our results will provide valuable information about the epidemic of vesiculovirus infections among fish.
RESULTS

SHVV epidemic in hybrid snakehead fish

During June and July 2014, a devastating disease outbreak occurred in hybrid snakehead fish (Channa maculata × Channa argus) cultured in a farm located in Shunde City, Guangdong Province, China. The fish were dark and lethargic, accumulated in places of low current, and swam feebly but occasionally showed hyperactivity. The gills were pale, and there was visceral pallor. There was no food in the gastrointestinal tract, which was distended with translucent fluid (Fig. 1a). Total RNAs extracted from the livers of fish with or without clinical signs were subjected to a reverse transcription (RT)-PCR assay using primers specific for fish vesiculovirus. A specific DNA fragment was amplified in the sample from diseased fish but not from the fish without clinical signs (Fig. 1c). To identify the virus species, the obtained RT-PCR product was sequenced, and

Fig. 1. SHVV isolation and confirmation. (a) The symptoms of diseased hybrid snakehead fish. (b) The symptoms of infected mandarin fish. (c) A DNA fragment was amplified from the total RNA extracted from the liver of the diseased hybrid snakehead fish (lane 1) but not from fish without clinical signs (lane 2). A molecular size ladder (bp) is shown. (d) Phylogenetic tree of SHVV and other rhabdoviruses. The names and GenBank accession numbers of the virus strains are: SHVV (KP036615), HSHRV (AGI97137.1), SCRV (YP802941.1), MARV (AGZ15720.1), snakehead fish rhabdovirus (SHRV; NP050583.1), spring viremia of carp virus (SVCV; AAW47746.1), vesicular stomatitis Indiana virus (VSIV; AAA48401.1), viral hemorrhagic septicemia virus (VHSV; AGI56027.1), hirame rhabdovirus (HIRRV; NP919033.1), rabies virus (RABV; AAB30931.1), infectious hematopoietic necrosis virus (IHNV; M16023.1) and respiratory syncytial virus (RSV; U39661.1). Bar, nucleotide substitutions per site. (e) Alignment of the amino acid sequences of the G proteins of SHVV and SCRV.
the sequence was compared with other known rhabdoviral sequences. The results showed that our virus isolate was grouped in the genus Vesiculovirus together with SCRV, Monopterus albus rhabdovirus (MARV) and hybrid snakehead fish rhabdovirus (HSHRV) (Fig. 1d). It was therefore designated snakehead fish vesiculovirus (SHVV). The amino acid sequences of the G protein from SHVV and SCRV were aligned and showed 93% amino acid identity (Fig. 1e).

Growth of SHVV in cell culture

As SHVV was isolated from snakehead fish, we wondered whether SHVV was able to infect SSN-1 cells, which are derived from whole fry tissue of striped snakehead fish (Frerichs et al., 1996). When SSN-1 cells were inoculated with SHVV at an m.o.i. of 0.1, an apparent cytopathic effect (CPE) was observed at 12 h post-infection (p.i.). The TCID<sub>50</sub> was found to be 1×10<sup>6.8</sup> p.f.u. ml<sup>-1</sup> when the SSN-1 cells had been infected with SHVV for 3 days, indicating that SHVV was able to proliferate well in SSN-1 cells (Fig. 2). To monitor the multiplication of SHVV in SSN-1 cells, quantitative real-time (qRT)-PCR was performed to detect the amounts of SHVV mRNAs in SSN-1 cells. The results showed that the amounts of mRNAs of the five SHVV genes were enhanced during the progression of infection. However, only expression of the L gene continued to increase up to 36 h p.i., and the peak of expression of the other four genes was at 24 h p.i. (Fig. 3). In addition, epithelioma papulosum cyprini (EPC), fathead minnow (FHM) and embryonic zebrafish (ZF4) cells were also included to test their susceptibilities to SHVV. No CPE was observed at 2 days after inoculation with SHVV (m.o.i. 0.1) in these cells. Apparent CPE was observed only after the inoculated EPC and FHM cells had been blindly passaged for at least two generations. The SHVV titres in EPC and FHM cells were about 100 times less than that in SSN-1 cells. However, no apparent CPE was observed in ZF4 cells, even after several blind passages (data not shown).

Mandarin fish are susceptible to SHVV

Our pilot results showed that mandarin fish were susceptible to the filtrate of the liver of snakehead fish infected with SHVV by both bath exposure and i.p. injection (data not shown). We also infected the mandarin fish with SHVV generated from SSN-1 cells. Mortality occurred at 5 days after bath exposure, whilst mortality could be observed as early as 24 h after i.p. injection. The survival rate showed a negative correlation with virus concentration (Fig. 4a). As the disease progressed, fish

**Fig. 2.** CPE of SSN-1 cells infected with SHVV. SSN-1 cells were infected with SHVV at an m.o.i. of 0.1 and examined at 0 h (a), 6 h (b), 12 h (c) and 24 h (d). A CPE was apparent at 12 and 24 h p.i.

**Fig. 3.** Expression of the five viral genes. qRT-PCR was used to analyses N, P, M, G and L mRNA expression in SHVV-infected SSN-1 cells at 3, 6, 12, 24 and 36 h p.i. Transcription values were normalized to that of β-actin. The mRNA values of the five viral genes at 3 h p.i. were all set as 1 and were used to calculate the fold changes in the mRNAs of the five genes at 6, 12, 24 and 36 h p.i. **P<0.01.
became dark and sluggish; they often swam on their side and rested in abnormal positions. Haemorrhages were observed in the swim bladder, intestine, peritoneum and muscle. The overall symptoms in mandarin fish were identical to those found in snakehead fish (Fig. 1a, b). To investigate the tissue tropism of SHVV in the mandarin fish, the inner organs of the infected mandarin fish were subjected to an RT-PCR assay. The results indicated that SHVV was detected in the liver, spleen, kidney, heart, brain and swim bladder of mandarin fish (Fig. 4b).

**Histopathology of mandarin fish infected with SHVV**

Mandarin fish from both infected and non-infected groups were sampled for histological examination. As shown in Fig. 5, PBS-injected fish did not show apparent signs of infection and inflammation; however, major changes were observed in the kidney, spleen, liver, brain and heart of the infected fish. The renal glomeruli were swollen, and inflammation was observed in the renal glomeruli and tubules (Fig. 5a, b). The splenic haematopoietic tissues were affected, with severe inflammation (Fig. 5c, d). Necrosis was common in the tissues of kidney, spleen, liver, brain and heart (Fig. 5).

**DISCUSSION**

With the rapid expansion of fish culture, new vesiculoviruses have emerged, including *Myxocyprinus asiaticus* rhabdovirus (Zhang et al., 2000), *Scophthalmus maximus* rhabdovirus (Zhang et al., 2007), SCRV (Tao et al., 2008) and MARV (Ou et al., 2013). In 1988, there was a report of snakehead fish rhabdovirus (SHRV), which belongs to the genus *Novirhabdovirus* (Ahne et al., 1988). In the present study, we report a vesiculovirus isolated from snakehead fish. The nucleotide sequence identity of the two snakehead fish viruses was only 45% at the genomic level (Zeng et al., 2014). Therefore, we named our virus isolate SHVV so that it can be clearly separated from SHRV. In this report, we showed that SHVV was able to infect mandarin fish experimentally, suggesting that there might be a potential risk for cultured mandarin fish to be infected by SHVV. This needs to be investigated in the future.

The clinical signs were closely related to the histopathological changes in mandarin fish infected with SHVV. For example, SHVV infection caused swelling and inflammation of the renal glomeruli and tubules. The kidney is one of the major organs responsible for fish osmoregulation. Therefore, SHVV infection impaired the osmoregulation of the mandarin fish, resulting in oedema of the fish. In addition, severe damage to the haematopoietic tissue of the spleen may correlate with the haemorrhage of the infected fish. In both SHVV-infected snakehead fish and mandarin fish, haemorrhage and oedema were typical clinical signs, whilst inflammation and necrosis were the most common histopathological features in the inner organs. The molecular mechanisms underlying the pathogenesis of SHVV infection remain to be addressed.

It is generally believed that the G protein plays critical roles in the early events of rhabdovirus infections, including receptor recognition and binding. It has been shown that the surface of the G protein from infectious hematopoietic necrosis virus plays a critical role in virus infection (LaPatra et al., 2008). Furthermore, the surface of the G protein of viral haemorrhagic septicaemia virus contains a specific T-cell epitope, which triggers T-cell responses during virus infection (Boudinot et al., 2004). Based on these findings, we compared the G proteins of SHVV and SCRV; the results showed that the identity was 93% at the amino acid level (Fig. 1e). It is likely that the high homology of the G protein between SHVV and SCRV might determine the outcome of SHVV infection in mandarin fish, but this remains to be elucidated.

The viral mRNAs are transcribed sequentially in the order that they appear in the genome of rhabdovirus (N-P-M-G-L).
Fig. 5. Haematoxylin and eosin staining of the inner organs of mandarin fish infected with SHVV: infected kidney (a), non-infected kidney (b), infected spleen (c), non-infected spleen (d), infected liver (e), non-infected liver (f), infected brain (g), non-infected brain (h), infected heart (i) and non-infected heart (j). Black arrowheads indicate necrosis, whilst white arrowheads indicate inflammation of the tissues. Bars, 50 μm.
Approximately 20–30% attenuation of expression of the downstream genes occurs at each gene junction (Villarreal et al., 1976; Iverson & Rose, 1981; Wertz et al., 1998). This transcription attenuation results in a gradient of mRNA and protein expression (i.e. N>P>M>G>L). By comparison with the amount of mRNA at 3 h p.i., we showed that the mRNAs of the N, P, M and G genes reached their peak at 24 h p.i.; by contrast, the mRNA of the L gene increased continually up to 36 h p.i. The reasons for this difference are unknown; one possibility is that the expression levels of the N, P, M and G proteins at 24 h p.i. were enough for viral particle assembly, but more polymerase (L protein) was still required during the progress of the infection. Because the amplification efficiencies of the qRT-PCR might be different, it was difficult for us to compare the absolute amounts of mRNAs of the five viral genes expressed in SSN-1 cells. The temporal expression of the viral genes remains to be investigated.

Six cell lines derived from cyprinid fish, EPC, FHM, Koi proboscis cells, grass carp swim bladder (GSB) cells, Ctenopharyngodon idella kidney (CIK) cells and Ctenopharyngodon idella brain (CIB) cells, have been tested for their susceptibility to SHVV (Zeng et al., 2013). The results showed that Koi proboscis cells were not susceptible to the virus. CPE was observed only in the SHVV-inoculated EPC, FHM, GSB, CIK and CIB cells, which had been blind passaged for at least two generations. These results are identical to our current report. There were several disadvantages when these cell lines were used for the studies of SHVV. First, blind passage was required for the efficient multiplication of SHVV in these cells. Secondly, the appearance of CPE in these cells was at 2 days p.i., which was much slower than that in SSN-1 cells (12 h p.i.). Thirdly, the yields of SHVV in these cells were much lower (around 100 times) than that in SSN-1 cells. The reasons behind these differences are not known. However, these cell lines were all derived from cyprinid fish, which are evolutionarily far away from the species of snakehead fish, which belongs to the Perciformes. Obviously, SSN-1 will be valuable for the studies of SHVV–host interactions in the future.

**METHODS**

**Cell and virus.** SSN-1 cells were kindly provided by Dr Hong Liu, Shenzhen Animal & Plant Inspection and Quarantine Technology Center. EPC, FHM and ZF4 cells were stored in our laboratory. SHVV was isolated from diseased hybrid snakehead fish by members of our laboratory.

**SSN-1 cell infection.** SSN-1 cells were grown in minimal essential medium supplemented with 10% FBS (Gibco). Propagation of SHVV was achieved by infecting 75–80% confluent cells at an m.o.i. of 0.1. At 3 days p.i., the supernatant and SSN-1 cells were harvested and centrifuged at 2000 g for 10 min at 4 °C, and the supernatants were stored at −80 °C until use. Virus titres were measured by an end-point dilution assay in SSN-1 cells at least in triplicate. TCID$_{50}$ was calculated by the method of Reed & Muench (1938).

**Mandarin fish maintenance and infection.** Mandarin fish (mean body weight 12.91 g) were purchased from a farm located in Wuhan city, Hubei Province, China. Fish were maintained at 25–26 °C in a recirculating freshwater system and were fed with forage fish for at least 2 weeks so that they acclimated to the laboratory conditions before experiments. Forage fish were fingerlings of silver carp (Hypophthalmichthys molitrix), which were free of SHVV, as tested by RT-PCR. Mandarin fish were anaesthetized by tricaine methanesulfonate (Syndel, MS-222) according to the standard protocol recommended by the manufacturer before challenge. Experiments were approved by the guidelines of Institutional Animal and Care and Use Committees (IACUC) of Huazhong Agricultural University. The mandarin fish were divided randomly into different groups, each group containing 25 fish. For the i.p. experiments, the virus titre in the stock solution was $1.0 \times 10^8$ c.f.u. ml$^{-1}$, and it was diluted in PBS at dilutions of 1:1, 1:10, 1:100, 1:1000, 1:10000, 1:100000. The mandarin fish

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were injected i.p. with 50 μl of the above dilutions, whereas control fish were injected with an equal volume of PBS. For the bath exposure experiments, fish were immersed in water containing or not containing SHVV at a concentration of 1.0 × 10^{5} c.f.u. ml^{-1} for 5 h. Thereafter, the above-treated fish were maintained in the recirculating freshwater system and fed with forage fish. Mortalities were tallied daily. The experimental fish were routinely sampled for isolation of the virus and histological analysis. The inner organs of the infected fish were either fixed in 4% paraformaldehyde for haematoxylin and eosin staining or frozen immediately in liquid nitrogen until use.

**Virus RT-PCR and qRT-PCR assays.** Virus was detected by RT-PCR or qRT-PCR using the primers listed in Table 1. For the RT-PCR assay, fish vesiculovirus-specific primers were used as described previously (Tao et al., 2008). Other primers were designed using PrimerBlast (http://www.ncbi.nlm.nih.gov/tools/primer-blast/). Total RNA was isolated from tissues or cells using TRIzol reagent (Invitrogen). cDNAs were synthesized using a PrimeScript RT reagent kit with gDNA Eraser (Takara) following the manufacturer’s protocol. PCRs were carried out in a volume of 20 μl containing 1 μl Premix Ex Taq (Takara), 1 μl each forward and reverse primers (10 μM), 7 μl nuclease-free water and 1 μl cDNA. Cycling parameters were 95 °C for 5 min, followed by 35 cycles of 94 °C for 30 s, 55 °C for 30 s and 72 °C for 30 s, one cycle at 72 °C for 10 min and finally incubation at 4 °C for 10 min. The RT-PCR products were subjected to electrophoresis in 1% agarose gel. The purified DNA fragments were subcloned into a pGEM-T Easy vector and transformed into E.coli Top 10. For the qRT-PCR assay, virus RNA was reverse transcribed into cDNA using a PrimeScript RT reagent kit with gDNA Eraser (Takara) following the manufacturer’s protocol. PCRs were carried out in triplicate. Dissociation curve analysis was performed using a LightCycle480 System (Roche). Cycling parameters were 95 °C for 10 s, 58 °C for 15 s and ended one cycle at 72 °C for 30 s, one cycle at 72 °C for 10 min and finally incubation at 4 °C for 10 min. The RT-PCR products were subjected to electrophoresis in 1% agarose gel. The purified DNA fragments were subcloned into a pGEM-T Easy vector (Promega) for subsequent DNA sequencing (Qingke). qRT-PCR was performed using a LightCycle480 System (Roche). Reactions were performed in a 10 μl volume comprising 1 μl cDNA, 5 μl 2× SYBR Green Master Mix (Roche) and 250 nM final concentrations of each forward and reverse primers (10 μM), 7 μl nuclease-free water and 1 μl cDNA. Cycling parameters were 95 °C for 5 min, followed by 45 cycles of 95 °C for 10 s, 58 °C for 10 s and 72 °C for 15 s, and ended with an increase to 95 °C at 5 °C s^{-1} to determine the melting curve. All reactions were done in triplicate. Dissociation curve analysis was performed after each assay to determine the melting temperature. β-Actin was stably expressed throughout the experiments and was used as the internal control. The relative expression ratio of the target genes versus the β-actin gene was calculated using the 2^{ΔΔC_{t}} method, and all data were given in terms of relative mRNA expression. The mRNA levels at 3 h p.i. were set at a value of 1, and the relative fold changes of the five SHVV mRNAs were calculated by comparison with the amounts of mRNAs at 3 h p.i.

**Virus phylogenetic analysis.** The G protein nucleotide sequences from various species of rhabdoviruses were obtained from GenBank. Multiple sequence alignment was performed with the DNAMAN program. A phylogenetic tree was reconstructed using the neighbor-joining method in the MEGA 4.0 package. The topological stability of the trees was evaluated by 1000 bootstrap replications.

**REFERENCES**


**ACKNOWLEDGEMENTS**

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