The Journal of Microbiology (2012) Vol. 50, No. 2, pp. 278-284
Copyright ⓒ 2012, The Microbiological Society of Korea
DOI 10.1007/s12275-012-1145-2

Shedding of Viral Hemorrhagic Septicemia Virus (Genotype IVb) by Experimentally Infected Muskellunge (*Esox masquinongy*)

Robert K. Kim[1] and Mohamed Faisal[1,2,3*]

[1]Comparative Medicine and Integrative Biology Program, [2]Department of Pathobiology and Diagnostic Investigation, College of Veterinary Medicine, Michigan State University, MI, USA
[3]Department of Fisheries and Wildlife, College of Agriculture and Natural Resources, Michigan State University, MI, USA

(Received March 22, 2011 / Accepted November 4, 2011)

Previous experimental infection demonstrated that juvenile muskellunge (*Esox masquinongy*) can survive experimental infection of viral hemorrhagic septicemia virus, Genotype IVb (VHSV IVb) at a low concentration of exposure. Herein we report that survivors of experimental infection with VHSV IVb shed the virus into the surrounding environment for an extended period of time. When muskellunge were exposed to VHSV IVb by immersion at a concentration of 1,400 plaque forming units (PFU)/ml, VHSV IVb was detected in the water of surviving fish for up to 15 weeks post-exposure (p.e.) with the highest levels of shedding occurring between weeks 1 and 5 p.e. We estimated that each juvenile muskellunge can shed upwards of 1.36×10^5 PFU/fish/h after initial exposure signifying the uptake and amplification of VHSV to several orders of magnitude above the original exposure concentration. Muskellunge surviving low concentration exposure were re-infected with VHSV IVb by immersion at week 22 p.e. at concentrations ranging from 0 to 10^6 PFU/ml. Viral shedding was detected in all re-exposed fish, including mock rechallenged controls up to 15 consecutive weeks. Rates of viral shedding were substantially higher following rechallenge in the first 5 weeks. The highest rate of viral shedding was approximately 4.6×10^6 PFU/fish/h and shedding did not necessarily correspond to the re-exposure VHSV concentration. The results of this study shed new light into the dynamics of VHSV IVb shedding in a highly susceptible host and provide useful insights to fishery managers to design effective control strategies to this deadly virus.

***Keywords*:** viral hemorrhagic septicemia virus, genotype IVb, shedding, muskellunge, rhabdovirus

Introduction

The recent emergence of viral hemorrhagic septicemia virus genotype IVb (VHSV IVb) in the Laurentian Great Lakes poses one of the greatest challenges to fisheries managers in the region (Elsayed *et al.*, 2006). This World Organization for Animal Health (OIE) - reportable virus, capable of causing widespread mortality events in a number of important fish species, has spread to all five North American Great Lakes as well as several landlocked water bodies. Further complicating the matter has been the isolation of VHSV IVb from macroinvertebrates in infected waterbodies (Faisal and Schulz, 2009; Faisal and Winters, 2011) indicating that VHSV IVb has been broadly established in the Great Lakes ecosystem. As a result, there exists a dire need to better understand the biological properties of this particular VHSV strain and decipher its interactions with susceptible hosts. Previous experimental infection studies in the authors' laboratory have demonstrated that the muskellunge are highly susceptible to VHSV IVb based on the median lethal dose of infection (LD_{50}) (Kim and Faisal, 2010a), yet can survive exposure to low virus concentrations. This has raised questions regarding the fate of VHSV IVb in survived muskellunge and whether the fish can clear their tissues of virus or continue to shed infectious virus into the surrounding environment, thereby facilitating virus transmission to other susceptible hosts.

Present information on VHSV shedding stems from the pioneering studies of Neukirch and Glass (1984) and Neukirch (1985), in which farmed rainbow trout (*Oncorhynchus mykiss*) were experimentally infected with a genotype I strain of VHSV (F1). These studies demonstrated that VHSV exists at high titers in urine samples collected from indwelling catheters. In the same context, the studies of Kocan *et al.* (1997) and Hershberger *et al.* (2010) demonstrated that juvenile Pacific herring (*Clupea pallasi*) that survived VHSV IVa experimental infections shed the virus into the surrounding water and may infect their cohorts. Since the pathogenicity and disease course varies between viral strains (Skall *et al.*, 2004) and infected hosts (Kim and Faisal, 2010b, 2010c), a study was designed to characterize the shedding dynamics and patterns in a VHSV IVb susceptible host, the muskellunge.

Benefits in characterizing the carrier state and kinetics of viral shedding in VHSV IVb infected juvenile muskellunge are twofold. First, quantifying the amount and duration of viral shedding will provide fisheries managers with the information necessary to develop control strategies and regulatory guidelines. Second, the question of whether previous exposure to VHSV infection would increase survival in re-infection may provide clues into why variable and sporadic mass mortality episodes in the field have been reported.

*For correspondence. E-mail: faisal@cvm.msu.edu; Tel.: +1-517-884-2019; Fax: +1-517-432-2310

Materials and Methods

Cell culture and virus

The Great Lakes VHSV IVb index strain MI03, originally isolated in the authors' laboratory in 2003 from muskellunge (Elsayed *et al*., 2006), was used throughout. The virus was propagated in the *Epithelioma papulosum cyprini* (EPC) cell line (Fijan *et al*., 1983) in 150 cm^2 tissue culture flasks (Corning, USA) at 15°C. Flasks were frozen at -80°C until a total of 500 ml of virus stock could be attained. Virus concentrated cell culture media was thawed, combined, and subsequently aliquoted in cryogenic vials (Corning) and refrozen at -80°C until used. Virus stock concentration was assessed by the plaque assay on EPC cells treated with polyethylene glycol and using a methylcellulose overlay as described in Batts and Winton (1989) and Batts *et al.* (1991). Tenfold serial dilutions were applied to the virus stock beginning with 1:100 to $1:10^{12}$. Plaques were allowed to form for a period of six days, and the remaining cells of each well were fixed and stained with a crystal violet (0.5% w/v) and formaldehyde (1:1 dilution with water) solution. The virus stock was calculated to have approximately 7.32×10^8 plaque forming units (PFU)/ml following one freeze-thaw cycle. Cell lines were maintained and subcultured in 150 cm^2 tissue culture flasks (Corning) at 25°C using a growth medium formulation that consisted of Earle's salt-based Minimal Essential Medium (MEM, Invitrogen, USA) supplemented with 29.2 mg/ml L-glutamine (Invitrogen), Penicillin (100 IU/ml) and Streptomycin (0.1 mg/ml; Invitrogen), 10% fetal bovine serum (Hyclone, USA), and sodium bicarbonate (7.5% w/v; Sigma-Aldrich, USA).

Fish and maintenance

Certified VHSV-free juvenile muskellunge (4 months post hatch) were obtained from the Rathbun National Fish Hatchery (Iowa, USA) in August of 2009. Briefly, kidney and spleen were aseptically removed and homogenized using a Biomaster Stomacher (Wolf Laboratories Ltd, UK) at the high speed setting for 2 min. Homogenates were diluted with Earle's salt-based minimal essential medium (MEM, Invitrogen) supplemented with 12 mM tris buffer (Sigma-Aldrich), Penicillin (100 IU/ml), Streptomycin (100 μg/ml) (Invitrogen), and Amphotericin B (250 μg/ml) (Invitrogen) to produce 1:4 dilution (w/v) of original tissues. Samples were centrifuged at 2,000×*g* and the supernatant was inoculated into individual wells of a 24-well plate containing EPC cells grown with MEM (5% fetal bovine serum). Plates were incubated at 15°C for 7 d, and observed for the formation of cytopathic effects (CPE). A second blind passage was performed and assessed for the presence of VHSV.

Fish were acclimated to temperatures from 16°C to 11°C over a three week period before the experiment was initiated. All fish were initially held in 1,900 L circular fiberglass tanks in a continuous flow-through system supplied by oxygenated well water at Michigan State University - Research Containment Facility. The fish were fed *ad lib* with 2.0 mm sinking feed (Silver Cup, Nelson and Sons, USA) and then transitioned over a 2 week period to VHSV-free certified fathead minnows (*Pimephales promelas*) purchased from Robinson Wholesale, Inc (USA).

Immersion challenge

A group of 234 fish (weight 12.8±3.1 g; total length 14.9±1.2 cm) were experimentally infected with the MI03 strain of VHSV IVb via immersion at a concentration of 1.4×10^3 PFU/ml. Fish were immersed in static virus-containing water for a period of 1.5 h, then removed, rinsed, and placed in their respective tanks. The virus immersion concentration was subsequently confirmed by completing a viral plaque assay on a subsample of the water. This concentration gave <25% mortality in juvenile muskellunge when challenged by immersion with MI03 in an earlier study (Kim and Faisal, 2010c). An additional group of 52 fish was immersed in water mixed with 1 ml of sterile maintenance-MEM and was considered the negative control group. The virus challenge and negative control tanks were divided equally so that each tank contained 26 fish. All fish were held in 74 L polyethylene tank (Aquatic Eco-Systems, USA) in continuous flow through system at a water temperature of 11±1°C and were monitored every 8-12 h for mortality.

Assessment of viral shedding

Every 7 days post-exposure (d p.e.), all of the fish from three of the nine experimental tanks and one of the negative control tanks were evaluated for viral shedding. Sampling regime was completed as follows: in the first week tanks 1, 2, and 3 were assessed, followed by testing of tanks 4, 5, and 6 in the second week, and then tanks 7, 8, and 9 in the third week. This pattern of sampling was repeated in all subsequent weeks. The fish were removed from their respective tanks and transferred to corresponding glass aquaria containing aerated static water from the same source at 500 ml/fish. The fish remained in the water for a period of 90 min and were then transferred back to their respective tanks. Approximately 50 ml of water was collected from each of the tanks. An additional 500 μl of a 1:1 mixture of Streptomycin (100 μg/ml) (Invitrogen) and Amphotericin B (250 μg/ml) was added to each water sample and subsequently centrifuged at 2,000×*g* for 10 min. After centrifugation, a viral plaque assay was performed as previously described on water samples.

VHSV reisolation from water samples and confirmation of isolates

The water samples were tested for the presence of VHSV using EPC cell line. For polymerase chain reaction (PCR) confirmation, total RNA was extracted using QIAamp Viral RNA Mini Kit (QIAGEN, USA), according to the manufacturer's instructions. Reverse transcription was accomplished by a two step protocol using the Affinity Script Multiple Temperature Reverse Transcriptase RT-PCR™ (Stratagene, USA) following the manufacturer's instructions. The primer set used in this assay is recommended by the World Organization for Animal Health (OIE; Manual of Diagnostic Tests for Aquatic Animals 2003) for the amplification of an 811 base pair sequence of the VHSV nucleocapsid gene: 5′-GGG-GAC-CCC-AGA-CTG-T-3′ (forward primer) and 5′-TCT-CTG-TCA-CCT-TGA-TCC-3′ (reverse

primer). Reaction mixture contained 5 µl of viral RNA, 50 pmol of each primer, 25 µl of GoTaq® Green Mastermix DNA Polymerase (Promega, USA), and DNA-ase free water to create a final volume of 50 µl in each tube. The reverse transcriptase was inactivated by subjecting the mixture to 94°C for 2 min, and 30 cycles of PCR (denaturation for 30 sec at 94°C, annealing for 30 sec at 52°C, and polymerization at 68°C for 1 min) in a Mastercycler Personal Thermal Cycler (Eppendorf, USA). The polymerization was finalized by maintaining the mixture for a period of 7 min at 68°C. The amplicons were visualized by gel electrophoresis in 1.5% agarose gels.

Rechallenge and evaluation of viral shedding in surviving fish

Fish that survived the first VHSV immersion challenge and ceased virus shedding for three consecutive weeks were pooled together and maintained for an additional three weeks. For a second immersion rechallenge with MI03-VHSV IVb, fish were divided in groups of 13 fish (seven groups) and exposed to virus concentrations that ranged from 1×10^6 PFU/ml (7.7×10^5 $TCID_{50}$) to 10 PFU/ml (7.7 $TCID_{50}$). Virus stocks were diluted and added accordingly to a glass aquarium containing 10 L of water to achieve the aforementioned concentrations, at which time the fish were immersed for 1.5 h. A negative control immersion challenge was also conducted using an additional group of 13 fish that were kept in water mixed with 1 ml of maintenance-MEM. Viral shedding was evaluated once a week in all experimentally challenged groups and the negative control by the methods described above until shedding ceased for three consecutive weeks. Unfortunately, a parallel virus exposure to naïve fish could not be performed due to the death of this group following an unexpected failure of the oxygenation pump supplying their tank.

Dead or moribund fish were collected every 8–12 h. Fish were maintained for an additional 19 weeks under the conditions detailed above. The median lethal concentration (LC_{50}) of VHSV in muskellunge that survived the first experimental infection was calculated at 28 d p.e. and the end of the observation period by the method of Reed and Muench (1938). Virus reisolation and confirmation and the viral plaque assay were completed every 7 d p.e. as described above.

Results

When juvenile muskellunge were exposed to VHSV IVb by immersion at 1,400 PFU/ml, approximately 76% of the fish survived (Fig. 1). The first mortality occurred as early as day 5 p.e., and an increased number of mortalities were observed between days 5 and 20 p.e. The number of mortalities decreased beginning at day 21 p.e. through the end of the observation period (119 d p.e.) reaching a cumulative mortality of 23% (Fig. 1). Dead and moribund fish exhibited external and internal lesions indicative of VHSV infection such as external hemorrhages on the nuchal area (Fig. 2A) and sides of the body (Fig. 2B) and severe gill palor (Fig. 2C). Internally, infected fish exhibited ecchymotic to petechial hemorrhages in the visceral organs (Fig. 2D) and

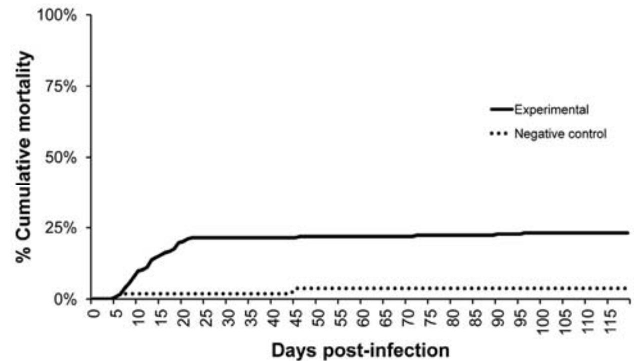

Fig. 1. Cumulative mortality of juvenile muskellunge (*Esox masquinongy*) exposed to viral hemorrhagic septicemia virus IVb (MI03) by immersion in water containing 1.4×10^3 PFU/ml. This virus concentration allowed the survival of 76% (177/234) of the virus exposed fish.

musculature (Fig. 2E). Kidney and spleen tissues from dead and moribund fish were collected and confirmed VHSV positive by cell culture and RT-PCR. In the negative control group, the cumulative mortality did not exceed 3.8% (Fig. 1), exhibited no abnormal internal or external signs of disease, and were VHSV negative by both cell culture isolation and RT-PCR.

As displayed in Fig. 3, detection of VHSV in water varied greatly among the fish groups and sampling events. Shedding rates observed in the three tanks of the first week p.e. ranged from 2.22×10^3 to 9.56×10^4 PFU/fish/h. By the second week, the viral shedding was low or undetectable with only one of the three tanks shedding at a rate of 1.11×10^3 PFU/fish/h. The highest rate of shedding during the observation period occurred in one fish group at 3 weeks p.e. (1.36×10^5 PFU/fish/h). Subsequent shedding rates observed in weeks 4 and 5 p.e. remained elevated in all groups, at

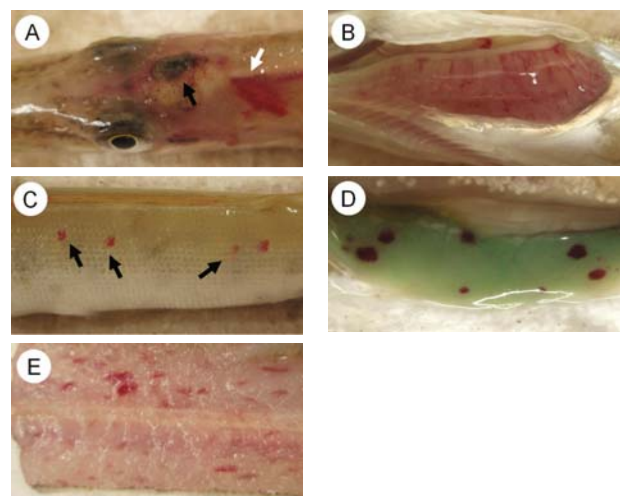

Fig. 2. Juvenile muskellunge (*Esox masquinongy*) experimentally infected with viral hemorrhagic septicemia virus IVb (MI03) at 1,400 plaque forming units/ml exhibiting external and internal signs of disease. (A) unilateral nuchal hemorrhage (black arrow) and intramuscular hemorrhages (white arrows), (B) dermal petechial hemorrhage, (C) severe gill palor, (D) petechia hemorrhages throughout liver, (E) severe intramuscular hemorrhage.

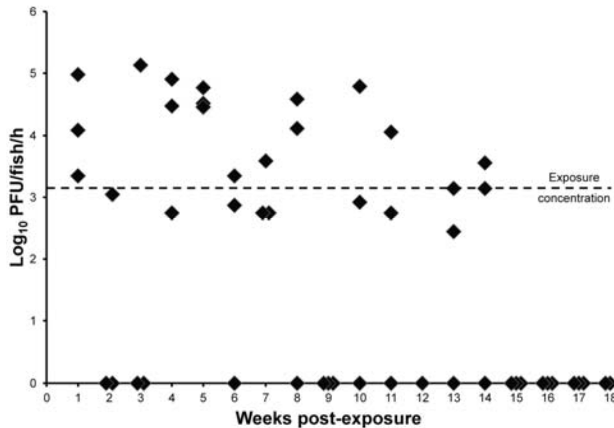

Fig. 3. **Shedding rates of juvenile muskellunge (*Esox masquinongy*) exposed to viral hemorrhagic septicemia virus IVb (MI03) at a concentration of 1,400 PFU/ml.**

which time the shedding rate steadily decreased until week 14 p.e. Viral shedding could not be detected from week 15 through week 18 (Fig. 3). Throughout the 18 weeks p.e., shedding rates fluctuated within and among the nine fish groups, but often remained above the initial exposure concentration (Fig. 3). Overall, shedding appeared to increase in the first 5 weeks p.e., which is followed by fluctuations until 15 weeks p.e. at which time shedding appeared to cease. The minimum amount of shedding detected was approximately 277 PFU/fish/h in one tank at 13 weeks post exposure.

When the VHSV-survived muskellunge were re-exposed to varying concentrations of VHSV, virus shedding resumed by all fish groups including the fish groups that were not re-exposed to VHSV. Levels of viral shedding were substantially higher following re-exposure when compared to the initial challenge (Table 1). There was, however, obvious variability in rates of VHSV shedding and cumulative mortalities that did not correspond to the concentration of re-exposure (Table 1 and Fig. 4). Interestingly, the highest rate of shedding of any of the concentrations was in the group of fish exposed at 1 PFU/ml, which was approximately 4.6×10^6 PFU/fish/h at 2 weeks p.e. The highest shedding rates of all the infected groups occurred between weeks 1 and 5 p.e. (Table 1), with the exception of fish exposed to VHSV IVb at 1×10^4 PFU/ml which had a second peak of viral shedding at 10 weeks p.e. (Table 1). Fish continued to shed virus for as long as 14 weeks p.e. (Table 1) and as short as 4 weeks p.e. (Table 1).

Further analysis suggested the absence of any correlation between the concentration of re-exposure and cumulative mortality. For example, fish re-challenged with 10^6 PFU/ml had less cumulative mortality than fish re-exposed to 1 PFU/ml (Fig. 4). By 40 d p.e., fish from the lowest concentration (1 PFU/ml) reached 100% mortality. Other concentrations that resulted in 100% mortality during the experiment were 1×10^5 PFU/ml, 1×10^4 PFU/ml, and 100 PFU/ml, (Fig. 4). While there were no acute mortality episodes noted in the challenged groups, fish infected at 1 and 100 PFU/ml exhibited an increased number of mortalities from 14 to 50 d p.e. (Fig. 4). Also, the mock re-challenge group had approximately 42.9% of the fish die during the study with most of the mortalities occurring between 28 and 35 d p.e. (Fig. 4).

At 28 days p.e., the LC_{50} in surviving, re-exposed muskellunge was calculated to be 1.34×10^5 PFU/ml. This value

Table 1. **Shedding rates of juvenile muskellunge (*Esox masquinongy*) re-exposed to viral hemorrhagic septicemia virus IVb (MI03) at concentrations ranging from 1 to 10^6 plaque forming units/ml and rechallenged using sterile media.** Shaded boxes indicate week(s) in which viral shedding was highest. Shaded areas indicate that all fish in that group died.

Week post re-challenge	VHSV shedding rates (pfu/fish/h)							
	Exposure concentration							
	1×10^6 PFU/ml	1×10^5 PFU/ml	1×10^4 PFU/ml	1×10^3 PFU/ml	100 PFU/ml	10 PFU/ml	1 PFU/ml	No virus
1	6.9×10^5	7.3×10^5	9.8×10^4	2.6×10^4	3.3×10^2	7.3×10^3	6.3×10^5	4.6×10^4
2	8.7×10^4	3.0×10^5	1.5×10^6	7.8×10^4	5.6×10^3	1.1×10^5	4.6×10^6	2.6×10^5
3	1.7×10^6	1.8×10^5	2.9×10^6	5.3×10^4	0	5.0×10^3	1.5×10^6	4.9×10^5
4	1.2×10^6	7.1×10^4	5.9×10^5	7.8×10^5	3.5×10^4	2.0×10^6	3.3×10^3	1.9×10^6
5	2.1×10^5	1.6×10^5	3.7×10^5	1.5×10^6	0	3.1×10^3	2.2×10^5	1.4×10^4
6	1.4×10^5	1.9×10^4	2.1×10^5	8.5×10^4	0	3.9×10^3		2.1×10^4
7	5.6×10^5	5.6×10^4	2.1×10^5	1.5×10^4	0	1.0×10^4		1.5×10^4
8	1.5×10^5	1.4×10^5	2.7×10^5	2.8×10^5	0	0		2.8×10^2
9	2.7×10^5	1.8×10^5	3.6×10^5	1.3×10^4		0		2.8×10^3
10	1.1×10^5	3.2×10^4	1.4×10^6	8.9×10^3		0		7.9×10^3
11	1.2×10^5	2.1×10^4	4.7×10^4	5.0×10^3		0		0
12	2.8×10^4	2.1×10^4	2.2×10^5	2.6×10^4		0		6.4×10^3
13	3.0×10^4	0	8.3×10^2	8.3×10^2		0		0
14	1.9×10^3		5.6×10^2	0		0		0
15	0		0	0		0		0
16	0		0	0		0		0
17	0		0	0		0		0
18	0		0	0		0		0
19	0			0		0		0

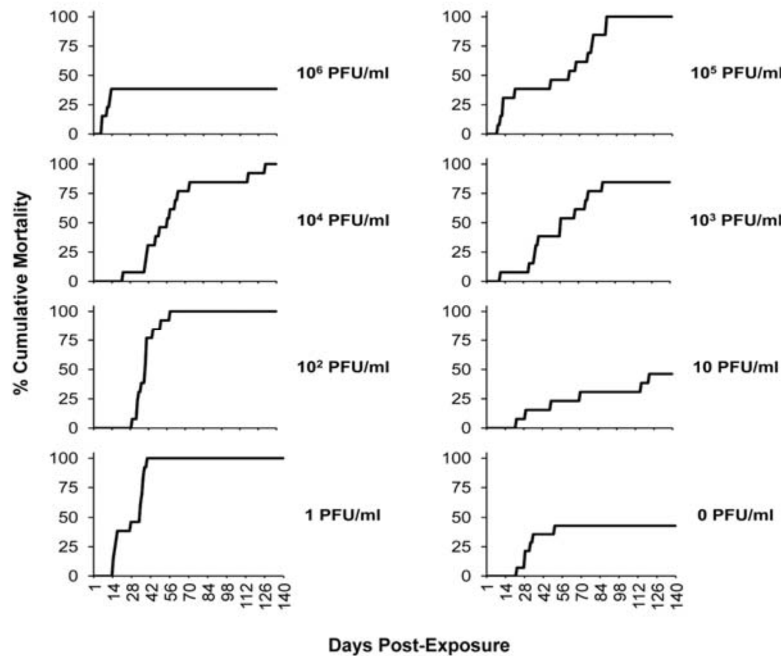

Fig. 4. **Cumulative mortality of juvenile muskellunge (*Esox masquinongy*) exposed to viral hemorrhagic septicemia virus IVb (MI03) over a 140 day period at varying concentration levels in plaque forming units (PFU)/ml.**

significantly decreased to 5.25 PFU/ml by the end of the 19 week observation period. In all mortalities, VHSV IVb was reisolated by cell culture and confirmed in homogenates of kidney and spleen tissues collected post-mortem.

Discussion

This study provided evidence that muskellunge surviving VHSV infection shed the virus into surrounding water at high titers and for a relatively extended period of time. The importance of this finding is multifold. Given that muskellunge are a species of high susceptibility to VHSV IVb (Kim and Faisal, 2010a), a relatively large portion of a population may be prone to contract the infection when exposure concentrations are low leading to survivors that will contribute to spreading the virus. Moreover, the virus titers shed exceeded the original exposure concentration, signifying that VHSV has been amplified within the muskellunge, thereby increasing the virus load in the surrounding water to levels that can initiate infection in less susceptible fish species such as salmonids and percids (Kim and Faisal, 2010a, 2010b) inhabiting the same watershed. Considering the long virus shedding period and the elevated virus concentrations shed, surviving hosts for VHSV should be deemed a major contributor for the spread and persistence of virus in a waterbody.

The fact that muskellunge shed VHSV for up to 105 days following the first challenge seems to be unique for both the emerging VHSV sublineage and the fish species. When Hershberger *et al.* (2010) followed the shedding of VHSV IVa in the highly susceptible Pacific herring, they failed to detect the virus in water after 20 d p.e. In the same context, Neukirch and Glass (1984) followed the shedding of VHSV genotype I strain (F1) in its susceptible host, the rainbow trout, and were able to detect VHSV in the urine of infected fish for an upwards of 40 d p.e. Therefore, the relatively high-titered and long VHSV shedding by muskellunge, compared to other fish species and virus genotypes/sublineages can be considered unusual and requires additional investigation. Other studies, however, have demonstrated that the outcome of VHSV infection relies not only on the fish host species (or strain) but also on the genotype or the sublineage within the genotype (Skall *et al*., 2004; Kim and Faisal, 2010b). Therefore, observed differences in the duration of viral shedding should be assessed in light of a number of factors which include virus genotype, sublineage (or strain), age of the host, and the prevailing environmental conditions, water temperature in particular. It was clear, however, that the disease course did vary among individual fish exposed to the same virus concentration, as some died within the first week and exhibited major pathology, while others were apparently healthy and survived the infection.

The fact that VHSV is shed into the water from infected fish is not surprising, given that cohabitation has been used in experimental infection studies (de Kinkelin and Castric, 1982; Snow *et al*., 2000; Bowden, 2003; Muroga *et al*., 2004; López-Vázquez *et al*., 2007). However, the specific tissue origin(s) from which the virus particles are released into the environment remains a subject of discussion. The early studies of Neukirch and Glass (1984) strongly suggested that the kidney and urinary tract, but not feces, were the major routes by which VHSV is shed since the virus could be reisolated from urine collected by indwelling catheters. The idea that the kidneys may be a major source of virus is corroborated by the studies of Brudeseth *et al.* (2005) and Al-Hussinee *et al.* (2010), when histologic sections visualized by immunohistochemistry (IHC) revealed the presence of virus in the interstitium and tubular epithelia of the kidneys. Other tissues in which VHSV particles have been identified by IHC include the gills (Al-Hussinee *et al*., 2010), while others surmise that the postmortem degradation of VHSV

infected fish tissues may also be a source (Wolf, 1988). As a result, a multitude of tissues are likely involved in the amplification and shedding of VHSV. Although infected fish may be primary contributors in spreading VHSV in the ecosystem, other aquatic organisms can also play a role in the spread of VHSV such as piscicolid leeches (Faisal and Schulz, 2009) and *Diporeia* sp. (Faisal and Winters, 2011).

Repeated exposure of muskellunge to VHSV IVb generated interesting observations related to VHSV shedding dynamics and potential protection conferred after surviving an initial infection. When VHSV shedding rates of individual tanks were evaluated in light of the re-exposure concentrations, there were no observable correlations, a finding that underscores the high variability among individual fish in their interactions with VHSV. Despite the increased mortalities noted in fish infected at lower concentrations, higher concentration infected fish either survived or succumbed to mortality albeit at lower rates. This information in combination with an increased LC_{50} value (1.34×10^5 PFU/ml), suggests that protection can occur due to previous exposure and may lessen the severity or time to death in subsequent exposures to VHSV. Conceivably, the increased LC_{50} seen in the rechallenged group at 28 days, compared to the LC_{50} performed on naive muskellunge obtained from the same source (Kim and Faisal, 2010a) may imply increased protection in VHSV survivors due to an adaptive immune response. Because of the age and size difference in the two fish groups, one cannot claim that surviving a VHSV infection may confer full protection against reinfection. On the other hand, protection following an exposure to VHSV was demonstrated in a study by Hershberger *et al.* (2007) in which larval Pacific herring, previously exposed to VHSV IVa, exhibited increased survival following a second exposure as juveniles when compared to naïve groups of fish. The increased survival of two highly susceptible, yet biologically dissimilar, species provides relevance to events that may be occurring under field conditions. Given that single exposure to VHSV in the wild populations cannot be circumvented, especially in VHSV endemic waters, wild fish may become less apt to succumb to infection following subsequent exposure. On the other hand, since our studies prove that re-exposure can reinitiate episodes of viral shedding at higher quantities, infected survivors may be a critical element to sustaining VHSV endemicity.

The magnification of viral shedding rates after rechallenge introduces new information into the persistence of VHSV infected fish. Previous studies using other VHSV genotypes demonstrate that VHSV can persist in fish tissues for up to 421 d p.e. (Neukirch, 1986), without leading to clinical signs or mortality. The detection of shedding in the rechallenge group not exposed to VHSV (0 PFU/ml; Table 1), suggests that a stressful stimulus, such as handling, may be sufficient to reignite viral shedding. Such findings must be interpreted with caution since the impact of stress was not a measurable factor in this study and therefore warrants a more detailed study to clarify this matter. Furthermore, how this observation relates to VHSV ecology in wild fish stocks requires further investigation into the pathological mechanisms governing the disease course and host defense mechanisms.

The information presented in our studies not only confirms that VHSV is shed in juvenile muskellunge, but provides novel information into the overall course of shedding of this emerging VHSV strain. Not only are juvenile muskellunge highly susceptible to VHSV infection, but may play an important role in the amplification and subsequent spread of the virus to other more resistant fish species. More importantly, the study demonstrates that viral shedding is a complex event and should be considered a major factor in the expansion and spread of VHSV throughout the Great Lakes basin. The prolonged course of shedding and the ability to reinitiate shedding following a stressful event or re-exposure imposes new challenges for hatchery managers and aquaculturists in eliminating the persistence of virus in production facilities. Alternatively, these studies provide promising data that fish have the capacity to survive infection. However, this could be due to a number of factors such as the full development of host defense mechanisms and/or that more repeated exposure is needed to achieve full protection, a matter that should be considered in vaccination strategies. In the end, the findings of this study reinforce the importance of regulatory guidelines to restrict the movement of fish and water from VHSV endemic areas.

Acknowledgements

The authors would like to thank the Great Lakes Fishery Trust (Grant #08WRGR0006, 2007-883) and the United States Department of Agriculture – Cooperative State Research, Education, and Extension Service (Grant #2007-37610-18383).

References

Al-Hussinee, L., Huber, P., Russell, S., LePage, V., Reid, A., Young, K.M., Nagy, E., Stevenson, R.M.W., and Lumsden, J.S. 2010. Viral haemorrhagic septicaemia virus IVb experimental infection of rainbow trout, *Oncorhynchus mykiss* (Walbaum), and fathead minnow, *Pimephales promelas* (Rafinesque). *J. Fish. Dis.* **33**, 347–360.

Batts, W.N., Traxler, G.S., and Winton, J.R. 1991. Factors affecting the efficiency of plating for selected fish rhabdoviruses, pp. 17–24. *In* Fryer, J.L. (ed.). Proceedings of the Second International Symposium on Viruses of Lower Vertebrates, Oregon State University Press, Corvallis, OR, USA.

Batts, W.N. and Winton, J.R. 1989. Enhanced detection of infectious hematopoietic necrosis virus and other fish viruses by pretreatment of cell monolayers with polyethylene glycol. *J. Aquat. Anim. Health* **1**, 284–290.

Bowden, T.J. 2003. A study of the susceptibility of Atlantic halibut, *Hippoglossus hippoglossus* (L.), to viral haemorrhagic septicaemia virus isolated from turbot, *Scophthalmus maximus* (L.). *J. Fish. Dis.* **26**, 207–212.

Brudeseth, B.E., Raynard, R.S., King, J.A., and Evensen, Ø. 2005. Sequential pathology after experimental infection with marine viral hemorrhagic septicemia virus isolates of low and high virulence in turbot (*Scophthalmus maximus* L.) *Vet. Pathol.* **42**, 9–18.

de Kinkelin, P. and Castric, J. 1982. An experimental study of the susceptibility of Atlantic salmon fry, *Salmo salar* L., to viral haemorrhagic septicaemia. *J. Fish. Dis.* **5**, 57–65.

Elsayed, E., Faisal, M., Thomas, M., Whelan, G., Batts, W., and

Winton, J. 2006. Isolation of viral haemorrhagic septicaemia virus from muskellunge, *Esox masquinongy* (Mitchill), in Lake St. Clair, Michigan, USA reveals a new sublineage of the North American genotype. *J. Fish. Dis.* **29**, 611–619.

Faisal, M. and Schulz, C.A. 2009. Detection of viral hemorrhagic septicemia virus (VHSV) from the leech *Myzobdella lugubris* Leidy, 1851. *Parasit. Vectors* **2**, 45.

Faisal, M. and Winters, A.D. 2011. Detection of viral hemorrhagic septicemia virus (VHSV) from *Diporeia* spp. (*Pontoporeiidae, Amphipoda*) in the Laurentian Great Lakes, USA. *Parasit. Vectors* **4**, 2.

Fijan, N., Sulimanovic, D., Bearzotti, M., Muzinic, D., Zwillenberg, L.O., Chilmonczyk, S., Vautherot, J.F., and de Kinkelin, P. 1983. Some properties of the *epithelioma papulosum cyprini* (EPC) cell line from carp (*Cyprinus carpio*). *Ann. Inst. Pasteur Virol.* **134**, 207–220.

Hershberger, P., Gregg, J., Grady, C., Collins, R., and Winton, J. 2010. Kinetics of viral shedding provide insights into the epidemiology of viral hemorrhagic septicemia in Pacific herring. *Mar. Ecol. Prog. Ser.* **400**, 187–193.

Hershberger, P.K., Gregg, J., Pacheco, C., Winton, J., Richard, J., and Traxler, G. 2007. Larval Pacific herring, *Clupea pallasii* (Valenceinnes) are highly susceptible to viral haemorrhagic septicaemia and survivors are partially protected after their metamorphosis to juvenile. *J. Fish. Dis.* **30**, 445–458.

Kim, R.K. and Faisal, M. 2010a. The Laurentian Great Lakes strain (MI03) of the viral haemorrhagic septicaemia virus is highly pathogenic for juvenile muskellunge, *Esox masquinongy* (Mitchill). *J. Fish. Dis.* **33**, 513–527.

Kim, R. and Faisal, M. 2010b. Experimental studies confirm the wide host range of the Great Lakes viral haemorrhagic septicaemia virus genotype IVb. *J. Fish. Dis.* **33**, 83–88.

Kim, R. and Faisal, M. 2010c. Comparative susceptibility of representative Great Lakes fish species to the North American viral hemorrhagic septicemia virus Sublineage IVb. *Dis. Aquat. Org.* **91**, 23–34.

Kocan, R., Bradley, M., Elder, N., Meyers, T., Batts, B., and Winton, J. 1997. North American strain of viral haemorrhagic septicaemia virus is highly pathogenic for laboratory-reared Pacific herring. *J. Aquat. Anim. Health* **9**, 279–290.

López-Vázquez, C., Dopazo, C.P., Barja, J.L., and Bandín, I. 2007. Experimental infection of turbot, *Psetta maxima* (L.), with strains of viral haemorrhagic septicaemia virus isolated from wild and farmed marine fish. *J. Fish. Dis.* **30**, 303–312.

Muroga, K., Iida, H., Mori, K., Nishizawa, T., and Arimoto, M. 2004. Experimental horizontal transmission of viral hemorrhagic septicemia virus (VHSV) in Japanese flounder *Paralichthys olivaceus*. *Dis. Aquat. Org.* **58**, 111–115.

Neukirch, M. and Glass, B. 1984. Some aspects of virus shedding by rainbow trout (*Salmo gairdneri* Rich.) after waterborne infection with viral haemorrhagic septicaemia (VHS) virus. *Zentralbl. Bakteriol. Mikrobiol. Hyg. A* **257**, 433–438.

Neukirch, M. 1985. Uptake, multiplication and excretion of viral hemorrhagic septicemia virus in trout (*Salmo gairdneri*), pp. 295–300. *In* Ellis, A.E. (ed.). Fish and Shellfish Pathology, Academic Press, London, UK.

Neukirch, M. 1986. Demonstration of persistent viral haemorrhagic septcaemia (VHS) virus in rainbow trout after experimental waterborne infection. *Zentralbl. Veterinarmed. B* **33**, 471–476.

Reed, L.J. and Muench, H. 1938. A simple method of estimating fifty per cent endpoints. *Am. J. Hyg.* **27**, 493–497.

Skall, H.F., Slierendrecht, W.J., King, J.A., and Olesen, N.J. 2004. Experimental infection of rainbow trout *Oncorhynchus mykiss* with viral haemorrhagic septicaemia virus isolates from European marine and farmed fish. *Dis. Aquat. Org.* **58**, 99–110.

Snow, M., Cunningham, C.O., and Bricknell, I.R. 2000. Susceptibility of juvenile Atlantic cod *Gadus morhua* to viral haemorrhagic septicaemia virus isolated from wild-caught Atlantic cod. *Dis. Aquat. Org.* **41**, 225–229.

Wolf, K. 1988. Viral hemorrhagic septicemia, pp. 217–249. *In* Wolf, K. (ed.), Fish Viruses and Fish Viral Diseases, Comstock Publishing Associates, Cornell University Press, Ithaca, N.Y., USA.

World, Organization for Animal Health (OIE). 2003. *In* Vallat, B. (ed.), Chapter 2.1.5, Viral Haemorrhagic Septicaemia. Manual of Diagnostic Tests for Aquatic Animals. Office International des Epizooties, Paris, France.