RISKS OF THE EMERGING CORONAVIRUS, FATHEAD MINNOW NIDOVIRUS (ORDER: *NIDOVIRALES*), ON REPRESENTATIVE GREAT LAKES FISH SPECIES

By

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ABSTRACT

RISKS OF THE EMERGING CORONAVIRUS, FATHEAD MINNOW NIDOVIRUS (ORDER: *NIDOVIRALES*), ON REPRESENTATIVE GREAT LAKES FISH SPECIES

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Since the initial isolation of the fathead minnow nidovirus (FHMNV), it has continued to be found in fathead minnows from several Midwestern states including Wisconsin, Minnesota and Illinois (Batts et al, 2012). Additionally, it has also been isolated from more species including hatchery raised muskellunge (chapter 2), golden shiner (McCann, 2012) and creek chub (Warg, personal communication). Previous work determined the muskellunge FHMNV isolate (MUS-WL) to be distinctly different from the index strain (chapter 2) which raised concerns regarding its host range and pathogenicity to muskellunge. Experimental infections via intraperitoneal (IP) injection were performed to screen the susceptibility of representative Great Lakes native cyprinids and piscivores to FHMNV. Any susceptible species were then screened for susceptibility to FHMNV by immersion techniques to mimic more natural modes of infection. Additional studies were performed to assess the pathogenicity of FHMNV to muskellunge specifically, including determination of the intraperitoneal median lethal dose (IP-LD_{50}), and experimental infections by both immersion and ingestion of FHMNV infected fathead minnows. Details of the disease course including viral shedding and tissues targeted were also investigated. Clinical signs of susceptible species included petechial hemorrhages throughout the eyes, fins and skin, gill pallor, and liver pallor. Histopathologic analysis revealed lesions in the kidneys, spleen and liver of infected fish. FHMNV appears to cause major risks to native cyprinids, the baitfish industry, and muskellunge rehabilitation programs

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INTRODUCTION

Taxonomy & Background

The order *Nidovirales* is comprised of a diverse group of viruses that are enveloped and have single-stranded, positive sense RNA genomes (Siddell & Snijder, 2008). It is taxonomically divided into three families*: Coronaviridae*, *Arteriviridae* & *Roniviridae*. The family *Coronaviridae* is further divided into the genera of Coronavirus and Torovirus. Coronaviruses and toroviruses are morphologically very similar, and are believed to be the most recently evolved of the nidoviruses. One unique characteristic of nidoviruses is that they are extraordinarily genetically complex, and contain viruses with the largest known RNA genomes; 20-32kb for coronaviruses and 25-30 kb for toroviruses, as well as viruses with much smaller RNA genomes like the arteriviruses (15kb) (Ehud et al, 1999). Additionally, it has been shown that there is the potential for a high level of RNA-RNA recombination in nidoviruses, which may be important for both viral evolution and pathogenesis (Lavi et al, 1999). The genetic complexity of nidoviruses has allowed them to infect a wide range of hosts, as well as adapt to rapidly changing environmental conditions (Gorbalenya et al, 2006).

The mechanism by which nidoviruses enter host cells is through receptor-mediated viropexis. Once inside the cells, viral particles are assembled by budding into intracellular membranes of the rough endoplasmic reticulum and Golgi apparatus. The spike (S) Protein is a vitamin K-dependent plasma glycoprotein (Lundwall et al 1986) that is added to the incomplete virions before they are released from the cell through the secretory pathway. (Lavi et al, 1999).

Up until this point in time, the majority of research has been done on nidoviruses that cause readily apparent disease. In most of these cases, the disease was described long before any virus was isolated and characterized. Recently however, it has been realized that many animals may be infected with nidoviruses without showing any clinical signs. Characterization of these viruses is lagging despite its importance, and is expected to be a growing field of study (Siddell & Snijder, 2008). When the isolation of the first fish nidovirus (*White Bream Virus*) took place in 2001, scientists realized the wide host diversity of nidoviruses particularly within non-mammalian hosts. Other issues faced by nidovirologists include the fact that many nidoviruses are simply difficult to isolate and propagate, hence making them difficult to study. For example, some nidoviruses do not grow well on cell culture like the Berne virus, and even others can only be propagated in living hosts like the Gill-associated virus & Yellow head virus (Siddell & Snijder, 2008).

Pathogenesis of Nidoviruses

Nidoviruses are transmitted among hosts generally through enteric or respiratory routes (Lavi et al, 1999). Once nidoviruses enter the host, they spread to additional tissues; predominately the liver, blood vessels, or the central nervous system (Lavi et al, 1999). There is also evidence that nidoviruses may have some suppressive effects on the host immune system. For example, altered cellular immune function has been reported in mice acutely infected with the coronavirus, mouse hepatitis virus (Compton et al, 1993), and some arteriviruses target and replicate in macrophages causing their demise (Snijder & Muelenberg, 1998).

Many nidoviruses in the arterivirus and coronavirus families are capable of establishing latent and persistent infections, where transcription and translation occur continuously albeit at

very low levels. These kinds of viral infections can have devastating impacts. For example, when cats are infected by a strain of feline enteric coronavirus (FCoV) which causes otherwise latent infection, a mutation can transform this virus into a highly virulent strain that can cause a lethal disease called feline infectious peritonitis (FIP) (Fehr & Perlman, 2015). Some of the clinical signs associated with this disease include fever, anorexia, depression, ascites, diffuse fibrinous peritonitis, mesothelial hyperplasia, as well as focal areas of necrosis in parenchymal organs (Horzinek et al, 1976). Another example of a virus causing latent infections is mouse hepatitis virus strain 3. Certain mice strains have intermediate susceptibility to mouse hepatitis virus 3, and experience and develop a persistent infection that that may last up to 12 months. Damage caused by these infections is typically limited to the central nervous system, but in some strains of mice the effects can be systemic (Virelizier et al, 1975).

Coronaviruses most commonly cause respiratory and enteric infections. In some cases, these viruses can be considered hepatotropic such as the mouse hepatitis virus strain 2 (Taguchi et al, 1983). On the contrary, the JHM strain of the same virus is predominately neurotropic (Stohlman et al, 1982). Infection with some coronaviruses, such as the JHM strain of mouse hepatitis virus, exhibit a bi-phasic disease pattern where the first two weeks in the disease course are characterized by acute meningoencephalitis followed by subacute or chronic disease development associated with inflammatory demyelination or vasculitis (Lavi et al, 1999).

Toroviruses produce both gastrointestinal and respiratory illnesses (Perlman et al, 2008). These viruses are not known for persistent infections affecting the central nervous system (Lavi et al, 1999), but instead target the epithelial cells of the small and large intestine leading to atrophy and necrosis of these areas (Perlman et al, 2008). In addition, depletion of lymphocytes

and activated macrophages associated with edema of the laminar propria have been observed Lavi et al, 1999)

Lastly, arterivirus infections are known to cause a variety of diseases, particularly persistent infections of the central nervous system. Equine arteritis virus (EAV) infections in horses can cause sub-clinical illness as well as systemic infections with influenza-like illness, abortion and interstitial pneumonia in pregnant individuals. This virus targets the media of small muscle arterioles, causing necrotizing arteritis. Lactate dehydrogenase-elevating virus (LDV) in mice typically causes a lifelong, persistent infection that causes immunosuppression. In some mouse strains, however, LDV can cause white matter disease and a severe motor neuron disease similar to poliomyelitis (Lavi et al, 1999).

Epidemiology and Host Range of Nidoviruses

As mentioned previously, nidoviruses are of particular interest due to their ability to cause severe disease (Siddell & Snijder, 2008). One coronavirus that has received considerable attention is SARS-CoV (order *Nidovirales*, family *Coronaviridae*, genus *Coronavirus*), which emerged relatively recently and was found to be associated with disease outbreaks of Severe Acute Respiratory Syndrome in humans (Rota et al, 2003). This virus originated in southern China, and after its initial outbreak in 2002 spread rapidly to 29 countries/regions on 5 continents. At the end of this epidemic, the total number of disease cases had reached a staggering number of 8098, with 774 deaths (Lam et al, 2003). Most of these cases occurred after exposure to SARS patients either in the household or in a healthcare setting (Rota et al, 2003). SARS-CoV and coronaviruses in general are typically fastidious in cell culture, thus making them challenging to study under laboratory conditions (Rota et al, 2003). The high infectivity

rate of this virus in combination with its association with high morbidity and mortality, lack of reliable diagnostic tests, as well as no effective treatment or vaccination options make it of utmost concern (Lam et al, 2003).

Another interesting coronavirus (FCoV) causes the disease, feline infectious peritonitis (FIP). Normally, this virus is localized and replicates in enterocytes and macrophages, either causing an asymptomatic infection or diarrhea. However, when FCoV undergoes a specific mutation it becomes highly virulent, and begins systemically infecting monocytes and macrophages leading to the FIP disease (Dewerchin et al 2005). Due to the ability of this virus to remain asymptomatic while spreading silently, there is a great deal of difficulty in assessing its true prevalence. This makes management of this disease and its spread difficult, particularly in breeding catteries and rescue environments. FIP is almost always fatal once it occurs in both domestic and wild members of the *Felidae* family (Hartmann 2004).

Nidoviruses in the Aquatic Environment

Nidoviruses are not just limited to the terrestrial environment. Over the last 15 years, nidoviruses have also begun appearing in aquatic hosts. One such example is that of the yellow head virus (order *Nidovirales*, family *Roniviridae*, genus *Okavirus*), a pathogen of farmed shrimp which causes yellow head disease (Sittidilokratna et al 2002). The virus was first found to infect the Asian tiger shrimp (*Penaeus monodon)* in central Thailand and is considered to be OIE reportable (World Organisation for Animal Health). Since its initial emergence in 1990, it has been reported in most Asian shrimp farming countries including India, Indonesia, Malaysia, the Phillipines, Sri Lanka, Vietnam and Taiwan. The aquaculture industry is believed to have played a considerable role in the spread of this disease (Walker & Winton 2010). Yellow head disease

occurs in juvenile shrimp, causing them to display an abnormal yellow coloration of the cephalothorax region (due to yellowing of the hepatopancreas) (Sittidilokratna et al 2002). It also causes necrosis and basophilic cytoplasmic inclusions in lymphoid organs. Yellow head virus has proven to have devastating effects on shrimp aquaculture in Asian countries, causing high mortality within a few days after exhibiting signs of the disease (Sittidilokratna et al 2002). It has been estimated that approximately 30% of yellow-head-complex viruses detected from *P. monodon* are recombinants. This evidence in combination with the geographic distribution of these viruses suggests that aquaculture and the international live shrimp trade have played a significant role in the increased emergence of viral genetic diversity (Walker & Winton 2010).

Scientists are also discovering more and more viruses which infect and cause disease in fish. In 1981, there were only 16 viruses isolated in cell culture and 11 others described by electron microscopy. By the year 1993 another 69 viruses had been identified in fish. (Granzow et al, 2001). Recently, two novel nidoviruses have emerged in fish species, and have been assigned to their own genus, *Bafinivirus* (Batts et al, 2012, Granzow et al 2001).

White Bream Virus (WBV)

The first isolation of a nidovirus in fish occurred when the White Bream Virus (WBV) was isolated from a European cyprinid species, the white bream (*Blicca bjoerkna L.*; Teleostei, order *Cypriniformes*), in the Federal State of Saxonia-Anhalt (Germany) in 2001. This isolation occurred doing a routine monitoring program of microbial pathogens in wild fish populations. The virus was detected via electron microscopy from cyprinoid cell cultures incubated with homogenized fish tissues (heart, kidney, spleen and swim bladder) (Granzow et al, 2001).

The virus had spikes presented on its outer membrane, a bacilliform shaped virion, and a rigid rod-shaped nucleocapsid; characteristics of the *Coronaviridae*, *Rhabdoviridae*, and

Baculoviridae families respectively (Granzow et al, 2001). The genome of this virus was sequenced in 2006, revealing that the WBV was actually a nidovirus in the genus, *Bafinivirus* (Schutze et al, 2006). At this point in time, there is no further information available regarding the ecology or pathogenic properties of the virus (King et al, 2012).

Fathead Minnow Nidovirus (FHMNV)

In March of 1997, a novel virus was isolated from another cyprinid species; fathead minnows (*Pimephales promelas*) of both wild-type and rosy-red phenotypic varieties on a baitfish farm in Arkansas, USA (Iwanowicz and Goodwin, 2002). These minnows were being held in two adjacent ponds at water temperatures of 15-20˚C, and had been experiencing chronic mortality for approximately two months. Both of the ponds were stocked with minnows that had already been distributed throughout the United States and were returned. Potentially, these minnows may have been mixed with fish from other regions or even from the wild before being brought back (Iwanowicz & Goodwin 2002).

FHMNV Morphology & Characterization

The virus was originally isolated from moribund fathead minnows exhibiting petechial hemorrhages in both the eyes and skin. It was found to be 130-180nm in length and 31-47nm in diameter. When the virus was grown in tissue culture on EPC (*Epithelioma Papulosum Cyprini*) cell lines, it produced characteristic cytopathic effects including cell rounding and multifocal syncytia. Optimum growth in cell culture was found to occur between 15-25℃ (Iwanowicz & Goodwin, 2002). Originally it was classified as a rhabdovirus since it was inactivated by exposure to 50℃ for 10 min, 20% ether, 2 and 50% chloroform, pH 3, and pH 10, was unaffected by 5'-iodo-2 deoxyuridine, and appeared bacilliform and occasionally bullet-shaped

by electron microscopy. However, later phylogenetic analysis revealed FHMNV to be in the genus, *Bafinivirus*, with the closest relative being the WBV.

Both of these viruses were found to be most closely related to toroviruses (a subfamily of the order: *Nidovirales*), however, these viruses lacked both an HE structural protein as well as accessory proteins. This evidence suggests members of the *Bafinivirus* genus might be the most primitive of the coronaviruses (Batts et al, 2012). Although it was found that both FHMNV and WBV were not closely related to the nidoviruses which infect other aquatic hosts like shrimp (genus *Okavirus*), they do share a similar virion morphology (Batts et al, 2012).

FHMNV Clinical Signs

Fathead minnows which had been infected with FHMNV by IP-injection and kept at a temperature of 17℃ began to show clinical signs of disease after two weeks post injection. Behavioral changes associated with FHMNV included erratic swimming in a circular pattern, followed later by listlessness and residing either at the surface or bottom of the tank. External clinical signs included but were not limited to petechial hemorrhages in the eyes, skin and muscle as well as hemorrhages in musculature. Histopathological investigation revealed infected minnows to have lesions in the spleen, kidney and liver tissues (Iwanowicz & Goodwin, 2001).

FHMNV Host Range

When FHMNV was originally isolated and believed to be a rhabdovirus, experimental challenges where fish were infected with FHMNV by IP-injection were completed with fathead

minnows, goldfish, channel catfish, rainbow trout, and golden shiners. Of these five species, the virus was only able to be re-isolated from fathead minnows (Iwanowicz & Goodwin, 2001). However, since then the virus has been isolated from both fathead minnows and golden shiners (McCann, 2012).

FHMNV Prevalence & Geographic Spread

At present, FHMNV has been detected from minnows reared in Wisconsin, Minnesota, and Illinois (Batts et al, 2012). The virus was also detected from golden shiners which were experiencing mortalities in Wisconsin (Warg, personal communication). Furthermore, FHMNV was detected in fathead minnows as well as in creek chub during a viral survey of various baitfish species (both cultured and wild) from baitfish dealers in Wisconsin (McCann, 2012). In addition, this study found that fathead minnows were statistically more likely ($p = 0.021$) to be infected with a virus than the other species tested (golden shiners and white suckers) (McCann, 2012).

Baitfish as a vehicle for spread of FHMNV

The freshwater baitfish industry is economically important, bringing in more than $170 million dollars of revenue and shipping about 10 billion fish per year. Fathead minnow and golden shiner (*Notemigonius crysoleucas*) are the most popular species used; however, there are other species in addition to these. The majority of baitfish are cultured on baitfish farms, however, there is still a significant portion of the baitfish used (about 20%) being caught from the wild. Baitfish are often shipped across state lines, and then distributed by either wholesale or retail networks to various anglers. When baitfish are used for fishing in rivers and lakes,

individuals may escape into the environment or become ingested by predators (Goodwin et al, 2011). Both of these outcomes can help facilitate disease persistence or spread.

Wild baitfish that are caught and sold in the same body of water pose no risk of disease transfer; however, it is common for wild caught baitfish to travel long distances, sometimes even across state lines. Additionally, there are few if any health inspection requirements for these live fish before transfer or sale (Goodwin et al, 2011). For the State of Michigan, fathead minnows are only required to be certified free of heterosporis and viral hemorrhagic septicemia virus (VHSV) before entering state lines (MDNR).

In a general survey of baitfish practices in Ontario, Canada nearly half of all fishermen admitted to releasing their bait buckets at their fishing destinations, and many of them actually believed they were helping the environment (Litvak & Mandrak, 2011). This just highlights the need for better education and more strict enforcement regarding baitfish use. Transferring wild baitfish between lakes/rivers can have dire consequences on both the donor and receiving water bodies (Litvak & Mandrak, 2011). This practice is an efficient way to spread disease, or potentially alter ecosystems by introducing non-native species (McCann, 2012).

Following the outbreak of Viral Hemorrhagic Septicemia Virus (VHSV) in the Great Lakes region in 2003, the state of Michigan put regulations into place to help prevent the spread of the virus (and others like it) as much as possible (MDNR). First, it became unlawful to stock baitfish, live fish, or roe (eggs) without a fish stocking permit. Second, fish which were caught in water bodies can only be released in that same water body. Lastly, baitfish can only be released in public water is if they are on a hook. Baitfish acquired from certified sources may be used in any body of water (VHS positive, VHS surveillance, and VHS free). Uncertified bait may only

be used in the areas it was collected from, unless it was harvested from a VHS free area in which case it can be used in any water bodies within Michigan (MDNR). All of these are good practices; however, these regulations are only based on one pathogen, VHSV. Additionally, it is very difficult to enforce these regulations when it comes to wild baitfish.

In contrast, there are many jurisdictions which regulate the importation of hatchery raised baitfish. The Great Lakes fish disease control policy and model program is one of these programs. This program was implemented to help prevent the introduction and spread of diseases in the Great Lakes basin. The aquaculture industry is one that could be severely hindered by disease occurrence. Disease outbreaks have been known to cause major losses in fish hatcheries, and have the potential to have devastating effects on feral fish populations as well. Consequences of disease outbreaks in either hatchery or feral fish populations include reduced survival of stocked fish, increases in production cost, major fish losses to the public, as well as reduced economic returns (Great Lakes Fishery Commission, special publication 93-1)

Gaps in Knowledge

Currently, there are still many unknowns regarding FHMNV. The virus is known to cause disease in fathead minnows, however, it is not known what other species this virus may be able to infect. As mentioned previously, the virus was isolated from golden shiners and creek chub; however, little work has been done to determine if FHMNV is pathogenic to these species. Creek chub have not been tested for their susceptibility to the virus, and there has been only one published attempt to experimentally infect golden shiner with FHMNV that was unsuccessful. Additionally, the virus was isolated from muskellunge in a hatchery setting in association with mortality events, but River's postulates were not fulfilled to determine if FHMNV was the

causative agent for disease. No work has been completed regarding susceptibility of any species to the muskellunge isolate of FHMNV.

In addition, the geographic spread of this virus is unknown. Bait fish are transferred frequently from state to state, and are not required to be certified free of FHMNV prior to sale. Also, different lots of bait fish are often mixed together so it is difficult to trace the origins of disease outbreaks when they do occur. So far, the virus has been detected in Arkansas, and at least six different Midwestern states (Warg et al, poster). It is not known if the increased detection of this virus has been due to a higher prevalence or due to more intensive disease testing (Warg et al, poster). More information regarding the geographic spread and host range of this virus needs to be gained in order to determine which areas and species are most at risk.

Overall Objective of the Current Study

In 2011, a syncytia producing virus was isolated from hatchery reared muskellunge at Wild Rose State Fish Hatchery (WRSFH) in Wild Rose, WI. These fish were experiencing lingering moralities and exhibited external petechial hemorrhages. A second syncytia producing virus was then isolated from the apparently healthy originating muskellunge stock at Wolf Lake State Fish Hatchery (WLSFH) in Mattawan, MI. Muskellunge from both hatcheries exhibited liver pallor, and upon histopathologic analysis muskellunge from WRSFH showed multi-focal, locally extensive, coalescing necrotizing hepatitis. These fish also exhibited tubular necrosis in the kidneys. Upon testing by RT-PCR, both of these viruses were confirmed to be FHMNV. Interestingly, FHMNV was also isolated from fathead minnows that were being fed to muskellunge at WRSFH.

Isolation of FHMNV from muskellunge alarmed fish hatchery managers, fishery biologists as well as baitfish dealers, because up until this point FHMNV was only believed to be a pathogen of cyprinids. Baitfish have a long history of being transported across state lines, and until the emergence of viral hemorrhagic septicemia (VHSV) in the Great Lakes in 2005 (ref), little attention was paid regarding their potential to be a vector for disease spread. The increased detection of FHMNV in minnows is concerning not only because it has the potential to cause devastating economic losses within the baitfish industry itself, but also because it may pose serious risks to native cyprinids and piscivorous fish species. If this virus is capable of jumping to other hosts, for instance muskellunge, this has major implications for Great Lakes rehabilitation programs of native piscivores where they are fed minnows while being raised in state fish hatcheries.

The first objective of this study is to determine how identical the two muskellunge isolates are to one another, as well as how identical they are to the original FHMNV isolate from fathead minnow through the use of phylogenetic analysis. If the muskellunge isolates are unique from the fathead minnow strain, how did this divergence occur? The large RNA genome of nidoviruses and the possibility for a high level of RNA-RNA recombination supports the idea that FHMNV could have mutated. Perhaps FHMNV is similar to FIP in the sense that it is capable of mutating into a more virulent strain; specifically one that is capable of infecting a wider host range. Additionally, I plan to perform a pilot study to fulfill River's postulates for FHMNV infection in muskellunge, by experimentally infecting individuals with known concentrations of the virus via IP-injection and attempting re isolation both by cell culture and molecular methods.

In order to understand the true extent and damage this virus could cause to the Great Lakes ecosystem, it is necessary to determine which species could be negatively impacted and to what extent. It is already known that this virus is highly pathogenic to the fathead minnow; however, the 2011 isolation from muskellunge in Michigan and Wisconsin hints the virus may have a wider host range than previously believed. The second objective is to determine the comparative susceptibility of nine different indigenous fish species present in the Great Lakes to FHMNV. Firstly, since FHMNV is already known to be a pathogen of fathead minnows, the susceptibility of other commonly used native cyprinids to this virus will be determined/screened. The cyprinid species which will be included in this study are fathead minnow (to be used as a positive control), golden shiner (*Notemigonus crysoleucas*), spotfin shiner (*Cyprinella spiloptera*) and creek chub (*Semotilus atromaculatus*). Finally, I will be testing the susceptibility of economically and ecologically important piscivorous fish in the Great Lakes that are at risk to FHMNV including muskellunge, rainbow trout (*Oncorhynchus mykiss*), largemouth bass (*Micropterus salmoides*) and walleye (*Sander vitreus*).

Of the nine species screened for susceptibility to the virus by IP-injection, any that exhibit mortalities will be screened for susceptibility to FHMNV by an experimental immersion challenge to mimic more natural modes of infection. Additionally, if any piscivorous species prove to be susceptible to FHMNV by IP-injection and immersion, further studies will be completed to determine the pathogenicity of the virus, and if they can become infected through ingestion of infected fathead minnows. Viral shedding as well as which tissues are targeted throughout the course of infection will be investigated to better understand the disease course.

The results from these studies will shed light on the host range of FHMNV as well as the potential mechanisms for infection, and will assist in determining management strategies for control of this dangerous pathogen.

CHAPTER 1

Isolation of the Fathead Minnow Nidovirus (FHMNV) from Spotted Muskellunge (*Esox masquinongy*) suffering lingering mortalities

Abstract

In 2011, the fathead minnow nidovirus (FHMNV, Genus *Bafinivirus*, Family *Coronaviridae,* Order *Nidovirales*) was isolated from pond-raised juvenile muskellunge suffering from lingering mortalities at the Wild Rose Fish Hatchery (WR) in Wild Rose, Wisconsin,. Moribund muskellunge exhibited tubular necrosis in the kidneys as well as multifocal coalescing necrotizing hepatitis. FHMNV was also isolated from apparently healthy juvenile muskellunge at the Wolf Lake (WL) State Fish Hatchery in Mattawan, Michigan. The identity of the two syncytia-forming viruses (designated MUS-WR and MUS-WL) as strains of FHMNV was determined based on multiple gene sequencing and phylogenetic analyses. The pathogenicity of the MUS-WL FHMNV strain was determined by experimentally infecting naïve juvenile muskellunge through intraperitoneal injection with two viral concentrations (~ 6.31×10^3 and $6.31 \times 10^1 TCID_{50}$/fish). Both doses resulted in 100% mortality in experimentally infected fish which exhibited severely pale gills and petechial hemorrhaging in eyes, fins, and skin. Histopathological alterations in experimentally infected fish were observed mainly in the hematopoietic tissues in the form of focal areas of necrosis. Phylogenetic analysis of concatenated partial spike glycoprotein and helicase gene sequences demonstrated that the MUS-WL FHMNV isolate is distinct from MUS-WR and two other FHMNV originally isolated from moribund fathead minnows including the index FHMNV strain (GU002364). Based on a partial helicase gene sequence, two RT-PCR assays were developed; one is able to differentiate

Bafiniviruses from other coronaviruses and the other discriminated between the two bafiniviruses; the white bream virus and FHMNV. The risks posed to muskellunge by FHMNV seem to be more serious than originally thought.

Introduction

Muskellunge (*Esox masquinongy*) is one of the most important sportfish in North America. In order to sustain muskellunge stocks in the Laurentian Great Lakes, fishery regulatory agencies have initiated rehabilitation programs whereby gametes are collected from wild fish and the offspring raised to the fingerling stage in state fish hatcheries before stocking in major inland lakes. During the rearing phase in hatcheries, muskellunge are fed first *Artemia* and then live fathead minnow (*Pimephales promelas*) (Wahl 1999). This widely used practice has raised major concerns regarding the pathogens that baitfish may introduce to captive muskellunge fingerlings. This is particularly alarming since fish health inspection of baitfish is not a common practice (Faisal 2007).

In the past few years a number of microbial pathogens and parasites have been identified in baitfish (Phelps et al., 2014, Goodwin et al, 2010). One such pathogen is the fathead minnow nidovirus (FHMNV), a positive sense RNA virus that was isolated from moribund fathead minnows exhibiting external hemorrhages (Iwanowicz & Goodwin, 2002). FHMNV was believed to be a rhabdovirus (Iwanowicz & Goodwin 2002), but later genetic analysis identified FHMNV as a member of the genus *Bafinivirus,* family *Coronaviridae, order* Nidovirales (Batts et al 2012). While FHMNV has been recognized as a fathead minnow pathogen; its pathogenicity to other fish species, especially piscivorous fish, has never been ascertained.

In 2011, an episode of mortalities among cultured juvenile muskellunge was reported from Wild Rose State Fish Hatchery, WI. FHMNV was isolated from these fish. Shortly

thereafter, FHMNV was also isolated from apparently health juvenile muskellunge raised at Wolf Lake State Fish Hatchery, MI. This muskellunge isolate from Wolf Lake State Fish Hatchery, MI was determined to be distinctly different from previous FHMNV strains. Herein, we report on the identification of the two muskellunge isolates and ascertain their pathogenicity.

Methods

Initial virus isolation

In August of 2011, 800 muskellunge fingerlings reared intensively in raceways and fed commercial pellets at Wolf Lake State Fish Hatchery (WLSFH), Mattawan, MI were transferred to the Wild Rose State Fish Hatchery (WRSFH), Wild Rose, WI and were immediately stocked in a ½ acre outside pond. This pond was pre-stocked with fathead minnows which were obtained from a commercial baitfish supplier in order to be used as a forage source. Within three weeks of arrival, lingering mortality was observed that reached up to 10-20 fish/day within 60 days. In early October, a sample of 8 moribund muskellunge from WRSFH exhibiting pale gills and external petechial hemorrhages were submitted for analysis and were subjected to clinical and laboratory health assessment.

In late November of the same year, 60 muskellunge from the originating stock at WLSFH, following seven weeks of being fed on commercial minnows, were analyzed for the presence of microbial pathogens or parasites that may have been associated with the observed mortality in Wisconsin. These fish had not been experiencing any mortality and were apparently healthy.

Fish Health Examination, tissue processing and virus testing

Fish were examined for the presence lesions or external parasites. Skin and gill smears were made and examined microscopically for the presence of ectoparasites. Fish were dissected

under aseptic conditions, abdominal cavity and internal organs examined for the presence of lesions or abnormalities. Bacterial isolation was attempted from the kidneys by streaking on multiple bacterial media as detailed in the American Fishery Society Blue Book (AFS-FHS 2012). For virus testing, kidneys, spleen, and heart were aseptically collected in whirlpaks and pooled in groups of 4-5 individual fish.

Tissue processing for viral isolation

Tissue samples were diluted with Earle's salt-based minimal essential medium (MEM), supplemented with 12 mM Tris buffer (Sigma Chemical Co, St. Louis, MO, USA), penicillin (100 IU ml^{-1}) (Invitrogen Life Technologies, Carlsbad, CA), and amphotericin B (250 μg ml^{-1}, Invitrogen) to produce a 1:4 dilution of original tissues w/v. Samples were then homogenized using a Biomaster Stomacher (Wolf Laboratories, York, UK) at the high speed setting for two minutes. Following homogenization, samples were centrifuged at 5000 rpm for 30 minutes, and the supernatant from each individual sample was inoculated into triplicate wells of a 96-well plate containing *Epithelioma papulosum cyprini* (EPC; Fijan et al. 1983) cells grown with MEM supplemented with 5% fetal bovine serum (Hyclone). Infected plates were incubated at 15° C for up to 21 days, and observed for the formation of cytopathic effects (CPE).

Cell culture and virus propagation

Stocks of EPC cell line were grown and maintained in 150cm^2 tissue culture flasks (Corning Inc.) at 25° C using MEM (Invitrogen) supplemented with 29.2mg ml^{-1} L-glutamine (Invitrogen), penicillin (100 IU ml^{-1}) and streptomycin (0.1 mg ml^{-1}) (Invitrogen), 10% fetal bovine serum (Hyclone), and sodium bicarbonate (7.5% w/v; Sigma). Virus stocks were produced in EPC and harvested when CPE, predominated the cell sheet, supernatant aliquoted, and stored -80° C until used. To determine the virus concentration, the median tissue culture

infectious dose ($TCID_{50}$) assay was performed on EPC and calculated as described by Reed & Muench (1938).

Virus identification

EPC exhibiting cytopathic effects were subjected to further identification using the reverse transcriptase (RT) PCR. Total RNA was extracted from fish tissue using the Qiagen RNeasy mini kit (Qiagen, Inc., Valencia, CA, USA). Viral RNA was extracted from infected EPC cells using a QIAamp viral RNA kit (Qiagen, Hilden, Germany) following the manufacturer's protocol. Total cDNA was synthesized by the Reverse Transcription System using a random hexamer primer (Promega, Madison, WI, USA), and then used as a template for RT-PCR amplification.

Experimental infection of naïve muskellunge with the FHMNV Wolf Lake isolate (MUS-WL)

Fish and maintenance

Certified disease free raceway-raised, feed-pellet adapted juvenile (approximately 6 months post hatch), muskellunge were obtained from the Chautauqua Fish Hatchery, Chautauqua, NY. Prior to use in experimental infection, five fish were euthanized using an overdose of tricaine methanesulfonate (MS-222; Argent Chemical Laboratories) (25 mg ml^{-1}) and tested for the presence of pathogens including the FHMNV as described above.

Experimental fish were allowed to acclimate to laboratory conditions for 3 weeks, during which time the temperature was progressively lowered to $11 \pm 1^{\circ}C$ before the experiment was initiated. All fish were initially held in 420 L fiberglass tanks in a continuous flow-through system supplied with oxygenated and facility-chilled well water at the Michigan State University-Research Containment Facility, East Lansing, MI in accordance with the Institution

Animal Care and Use Committee (IACUC) guidelines (AUF Approval #: RC102183). Muskellunge were fed certified disease free fathead minnows.

Experimental infection

In order to fulfill River's postulates, juvenile, naïve muskellunge were challenged with Wolf Lake FHMNV isolate using a standard intraperitoneal (IP) injection protocol to ensure all fish were infected with a known dose of the virus. The virus was quantitated using the quantitative reverse transcription loop-mediated isothermal amplification assay (LAMP) developed in our laboratory for the FHMNV (Zhang et al., 2014). Three groups of fish (n=10) were used in this experiment, the first group received 450 viral copies/µL per fish (low dose group) while the second group received 120,000 viral copies/µL (high dose group) suspended in 100 µl/fish. The third group received the same volume of sterile MEM (control group). All fish were monitored daily for an observation period of 30 d post-infection (p.i.). The inflowing water was supplied by an underground well and chilled to 11 $\pm$ 1° C throughout the duration of the experiment. The outflow was united into a common tract entering an ultraviolet sterilization unit (Aquafine Corporation, Valencia, CA). Tanks were monitored every 8 to 12 h daily for moribund or dead fish.

Virus re-isolation

Kidneys and spleen were collected from dead/moribund fish and processed as above. Half of the fish tissues from each dose were tested for the presence of virus by the FHMNV-specific RT-PCR and the Loop Mediated Isothermal Amplification (LAMP) (Zhang et al., 2014) and the other half were processed, diluted and tested for FHMNV by cell culture as described previously. To assess the tissue alterations induced by FHMNV, kidney/spleen sampled from moribund fish as well as external and internal lesions were fixed in 10% formalin and embedded

in paraffin prior to sectioning. Tissue sections (5 μm) were stained with hematoxylin and eosin (H&E) as detailed in Prophet et al. (1992).

RNA extractions and RT-PCR

When CPE was observed, the samples were subjected to further testing using reverse transcriptase (RT) PCR. Total RNA was extracted from fish tissue using the Qiagen RNeasy mini kit (Qiagen, Inc., Valencia, CA, USA). Viral RNA was extracted from infected EPC cells using a QIAamp viral RNA kit (Qiagen, Hilden, Germany) following the manufacturer's protocol. Total cDNA was synthesized by the Reverse Transcription System using a random hexamer primer (Promega, Madison, WI, USA), and then used as a template for RT-PCR amplification. RT-PCR was performed as detailed by Batts et al (2012).

Results

Clinical and laboratory examination

Moribund muskellunge from WRSFH exhibited gill pallor and multifocal external petechial hemorrhages. Internally, muskellunge exhibited mottled liver appearance and firm consistency of the posterior kidney. One of the fish harbored larval nematodes. Motile aeromonads were isolated from seven out of eight fish. Muskellunge sampled from WLSFH appeared normal externally aside from a few individuals with mildly congested fins. Upon necropsy, these fish exhibited liver pallor and almost half of them (27/60) harbored larval nematodes.

Viral isolation and identification

Following inoculation on EPC cell line, 7/8 WRSFH tissue samples resulted in a syncytial formation in EPC within 7 days of inoculation (Figure 1-2). A syncytia-forming agent was also isolated from 3 out of 12 pools of WLSFH muskellunge tissues (5 fish/pool). The

syncytia-forming agent was confirmed to be FHMNV by an RT-PCR assay targeting the FHMNV spike glycoprotein gene, developed by Batts et al (2012).

Results of experimental infection

Juvenile muskellunge experimentally infected with MUS-WL showed no morbidity or mortality for the first two weeks following exposure to the virus. By the 16th day post infection (pi) fish started to die (Figure 1-3). By the end of the 30 day study period, muskellunge had reached 100% mortality at both low and high doses. Infected fish exhibited severe gill pallor, with several individuals also exhibiting petechial hemorrhages throughout the eyes, fins, and skin (Figure 1-4). Upon necropsy of dead and moribund fish, the majority of individuals tended to have enlarged spleens and gallbladders, as well as mottled livers.

FHMNV was reisolated from all infected muskellunge tissue samples. EPC cells showed syncytia formation as early as 14 days post infection. On the contrary, no CPE was observed in wells containing negative control muskellunge tissues. RNA extracted from wells exhibiting CPE amplified a 276 bp amplicon in an RT-PCR assay using FHMNV glycoprotein gene-specific primers (Batts et al 2012).

Histopathology

Histopathology performed on both spontaneously and experimentally infected muskellunge revealed a similar tissue alteration. WRSFH spontaneously infected muskellunge exhibited mild to severe acute, multifocal and locally extensive, often coalescing necrotizing hepatitis (Figure 1-1a, b); the kidney exhibited moderate acute multifocal tubular necrosis (Figure 1-1c); while the spleen appeared normal with a slightly increased mitotic rate.

In experimentally infected fish, similar necrotic changes in hematopoietic tissues were observed. For example, coalescing to diffuse necrosis and edema were seen within the splenic

stroma (Figure 1-5a), as well as severe widespread necrosis of red blood cells and lymphocytes (Figure 1-5a). Diffuse necrosis of the interstitium and edema were noted in the anterior kidney (Figure 1-5b). Likewise, multifocal renal tubular necrosis and nephritis, along with necrosis of the interstitium were observed in the posterior kidney (Figure 1-5c). In general, kidneys showed moderate widespread necrosis of hematopoietic cells (Figure 1-5b), as well as mild tubular epithelial degeneration (Figure 1-5c).

Discussion

Syncytia formation and positive RT-PCR amplification using FHMNV primers demonstrated that the isolated viruses from both muskellunge cases are indeed FHMNV. This is the first time that FHMNV has been isolated from muskellunge exhibiting clinical signs and experiencing mortalities. FHMNV is known to be a virus of cyprinids and its ability to spontaneously infecting cultured muskellunge causing lingering mortality was surprising. Upon experimental infection, both low and high doses of MUS-WL caused morbidity and mortality of all exposed fish, underscoring the high potentials of this virus in causing losses in muskellunge.

FHMNV was found to cause tissue damage in multiple organs (liver, kidneys and spleen), as well as significant necrosis of the hematopoietic tissues. These results indicate FHMNV causes systemic infections in muskellunge, however, more analysis are needed to better understand the transmission dynamics and pathogenicity of this viral infection in muskellunge. The order *Nidovirales* is comprised of a diverse group of viruses which infect a variety of different hosts and tissue types. Despite the alarming mortalities and risks associated with animal nidoviruses, further investigation is needed (Siddell & Snijder, 2008).

Nidoviruses are relatively uncommon in fish, with only two species currently described; the white bream virus (WBV) and fathead minnow nidovirus (FHMNV) (Schütze et al. 2006; Batts et al. 2012). The WBV was isolated from a European cyprinid species, the white bream (*Blicca bjoerkna L.*; order *Cypriniformes*) in 2001. This isolation occurred doing a routine monitoring program of microbial pathogens in wild fish populations (Schütze et al. 2006). Additional information on the host range and pathogenicity of this virus are currently unknown. Slightly more information is known about the FHMNV, which was isolated from fathead minnows during a major fish kill event on a baitfish farm in Arkansas during the year 2001. This virus was also found to be highly pathogenic to fathead minnows in a laboratory setting, and was discovered to cause lesions in the anterior kidney, liver and spleen (Iwanowicz & Goodwin, 2002).

In addition to causing mortalities in fish, nidoviruses have also been found to cause severe disease in humans (*Severe Acute Respiratory Syndrome (SARS)*) (Gu &Korteweg 2007), companion animals (*feline infectious peritonitis (FIP)*) (Diaz & Poma 2009), as well as in economically important livestock (*gill-associated virus (GAV) of penaid prawns*) (Spann et al 2003). These different viruses target a variety of tissue types. Some tissues affected include those of the respiratory tract (Gu & Korteweg 2007), central nervous system (Gu & Korteweg 2007, Diaz and Poma 2009), as well as lymphoid tissues and various immune cells (Spann et al 2013, Gu & Korteweg 2007).

River's postulates have been fulfilled for FHMNV infection in muskellunge, highlighting the capability of this virus to infect multiple hosts.. It is of utmost importance to determine the host range of this virus, particularly looking at the susceptibility of native cyprinids and piscivorous species being used in conservation rehabilitation programs. In addition, future work

needs to be completed in order to better understand the tropism, pathogenicity, and transmission of this newly emerged virus.

The fact that FHMNV, which until now was believed only to be a pathogen of the fathead minnow, is highly pathogenic to an apex predator in the Great Lakes which regularly contacts this baitfish is particularly alarming especially in hatchery conditions. Currently there are no regulations for baitfish farmers to certify their minnows as being free of FHMNV prior to sale. In addition, muskellunge share a variety of inland and major lakes with various minnow species, which could potentially expose them to FHMNV in their natural habitat. This finding highlights the need to increase research targeted towards the relationship between predator-prey interactions and disease transmission. More stringent bio control measures need to be put into place to prevent transmission and spread of FHMNV. Additionally, it would be wise to increase regulations and have stricter enforcement within the baitfish industry to prevent spread of the virus to new areas.

APPENDIX

Table 1-1. List of reverse transcriptase polymerase chain reaction (RT-PCR) primers used in this study.

Target taxon	Primer	Target gene	Length (bp)	*Position	MUS-WL	MUS-WR	FHMNV-181	WBV	HCovV	CavV	Reference
Bafinivirus	Forward: 5′-ATGAAGTCACACTATCWCAAC-3′ Reverse: 5′-GACATWGCAACAATRGCTC-3	helicase	517	18904-19420	+	+	+	+	-	-	**This study**
FHMNV	Forward: 5'-TTTTGTTGAATTTATAGCTCTT-3' Reverse: 5'-TGGCCATATCCTTAAGGG-3'	spike glycoprotein	278	24713–24990	+	+	+	-	-	-	Batts et al. 2012
FHMNV	Forward: 5′-CGAATTCGGCGTATCATAC-3′ Reverse: 5'-GTACAGTGTATTGATGTTGTGG-3'	helicase	1000	18543-19542	+	+	+	-	-	-	This study

(*) denotes the nucleotide position in the fathead minnow nidovirus (FHMNV) reference genome (GenBank accession: GU002364). (+) denotes an isolate tested positive using a particular RT-PCR primer set. (-) denotes an isolate tested negative using a particular RT-PCR primer set.

Figure 1-1: A normal EPC (epithelioma papulosum cyprini) cell monolayer that is uninfected (left). EPC cells infected with the fathead minnow nidovirus (FHMNV) isolated from muskellunge at WLSFH in EPC cells (right).

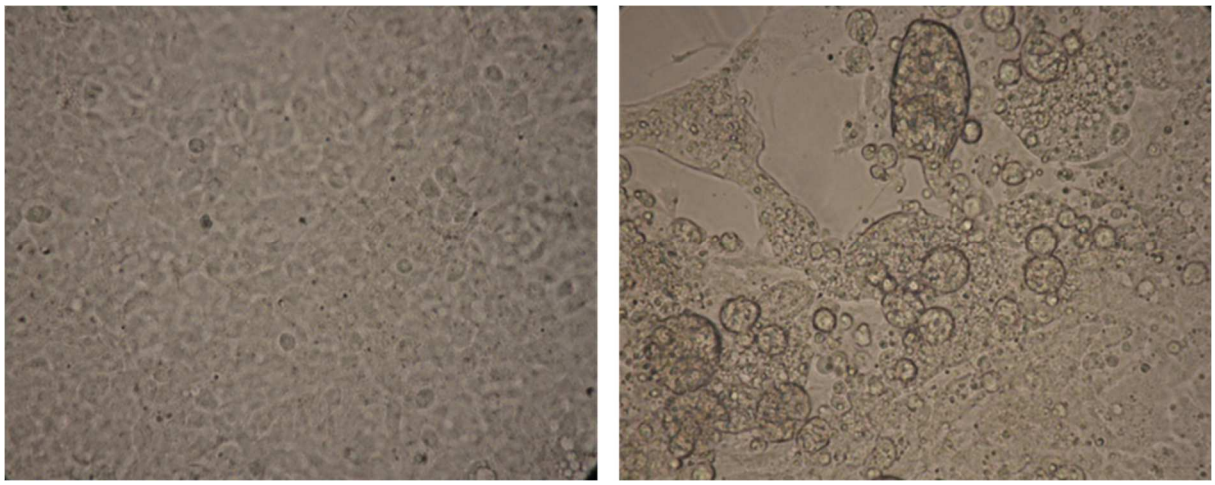


Figure 1-2: Phylogenetic tree (50% majority-rule consensus) generated using Bayesian Inference (MrBayes 3.1.2) of *Bafinivirus* (Order *Nidovirales*) based on concatenated helicase and spike glycoprotein gene sequences. Isolates obtained from muskelleunge (*Esox masquinongy*) (MUS) were obtained from fish reared at Wolf Lake State Fish Hatchery, Michigan, USA (WL) and Wild Rose State Fish Hatchery, Wisconsin, USA (WL). White bream virus (WBV) was used to root the tree. Numbers at the nodes are Bayesian posterior probabilities. FHMNV= Fathead minnow nidovirus.

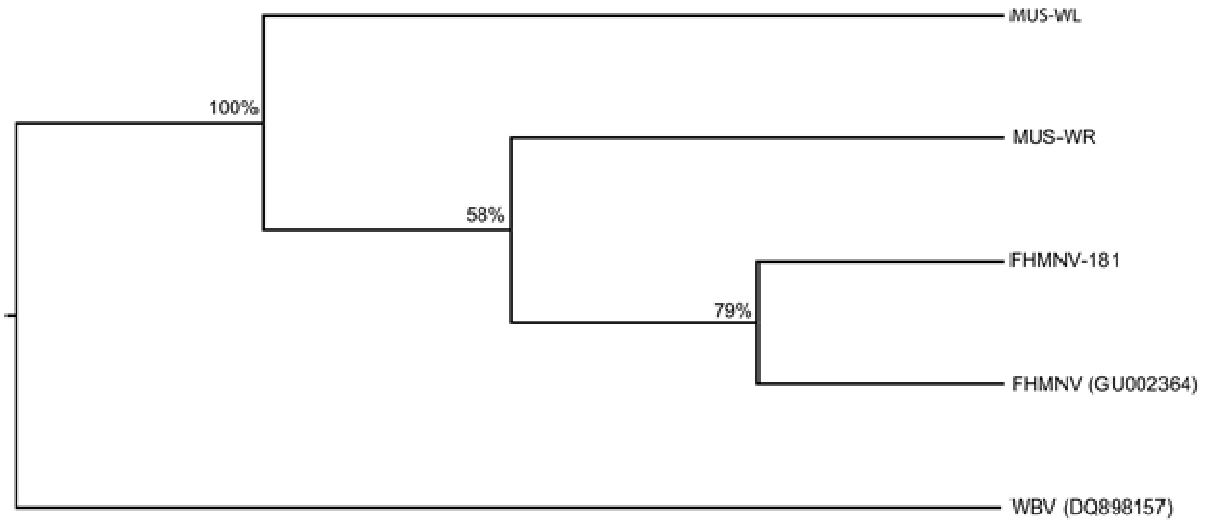


Figure 1-3. Mortality curve for muskellunge (n=10 per treatment) infected by IP-injection with 100µL of low (630 $TCID_{50}$/ml), high (63,000 $TCID_{50}$/ml) and control doses (sterile media) of fathead minnow nidovirus (FHMNV) over a 30 day study period.

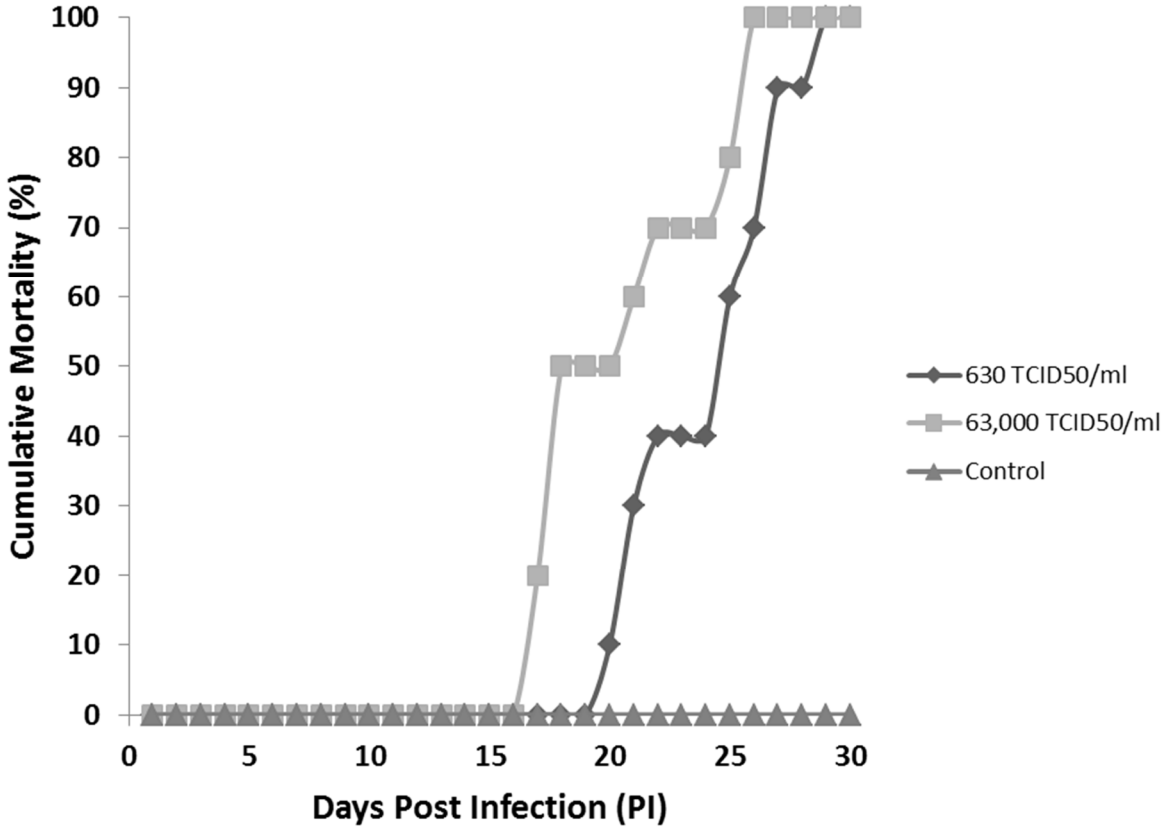


Figure 1-4. Clinical signs exhibited by muskellunge infected with fathead minnow nidovirus (FHMNV). Multifocal petechial hemorrhages on abdomen (left); Petechial ocular hemorrhage (right).

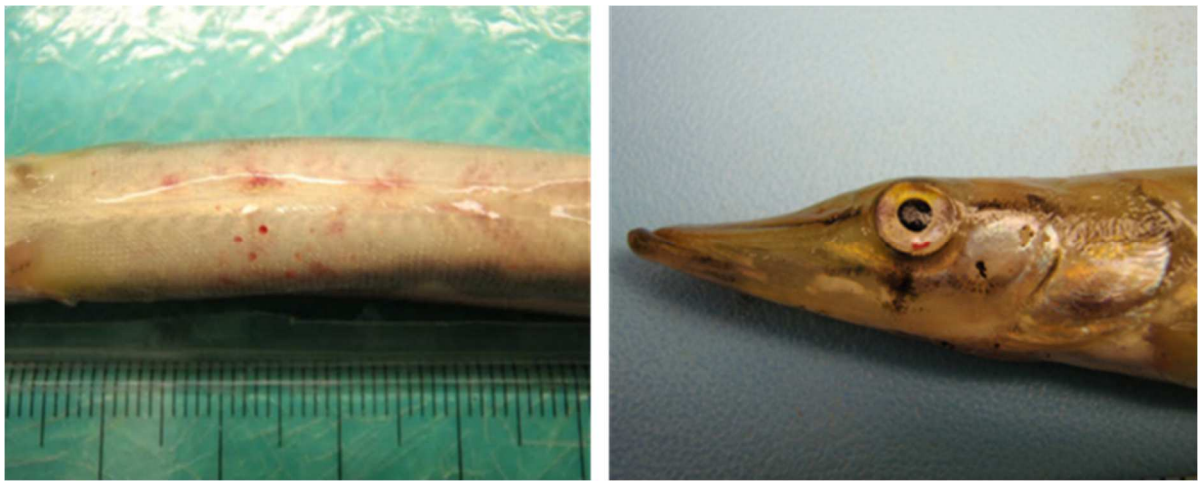


Figure 1-5: Hematoxylin and eosin (H & E) stained tissue sections from FHMNV-spontaneously infected WRSFH muskellunge. A) Liver showing multi-focal, locally extensive, coalescing necrotizing hepatitis (arrow denotes section of tissue shown in panel B). B) Magnified section of liver exhibiting coalescing necrotizing hepatitis. C) Kidney exhibiting moderate, acute multi-focal tubular necrosis (arrows).

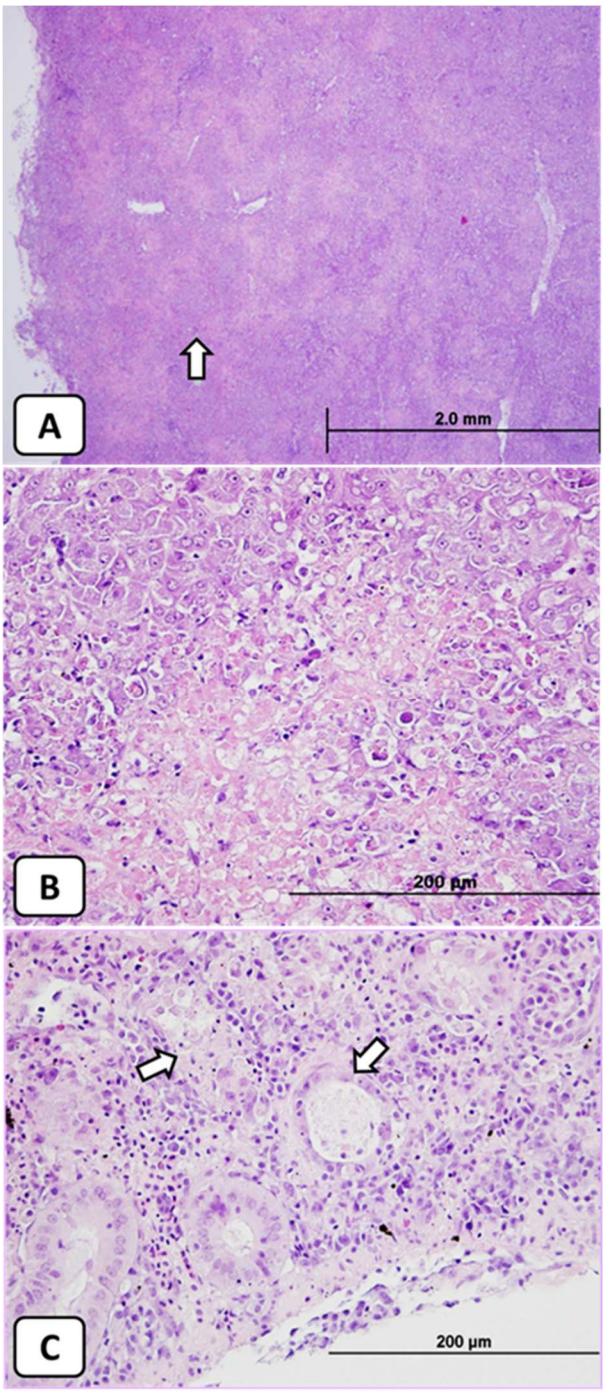
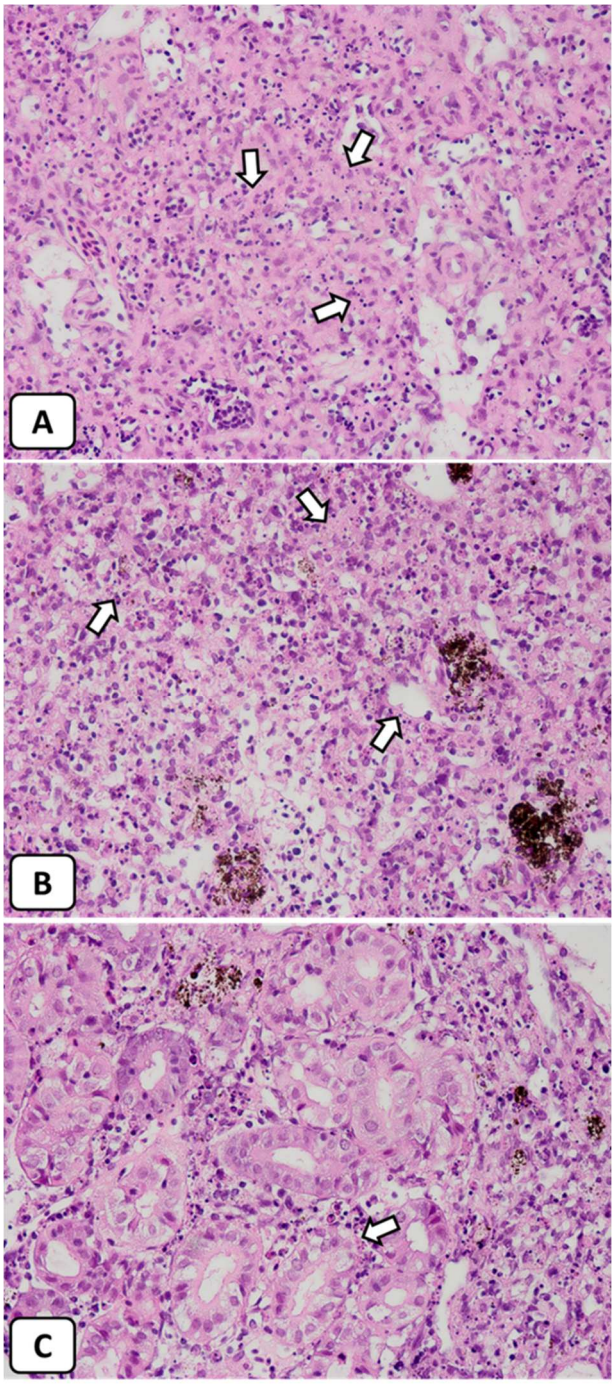


Figure 1-6. Hematoxylin and eosin (H & E) stained tissue sections from muskellunge experimentally challenged with fathead minnow nidovirus. Tissues were collected and fixed 22 days post-infection. A) Coalescing to diffuse necrosis (arrows) and edema within the stroma of the spleen (40x magnification); B) Diffuse necrosis of the interstitium (arrows) and edema within the anterior kidney (40x); C) Multifocal renal tubular necrosis and nephritis (arrow), along with necrosis of the interstitium in the posterior kidney (40x).



CHAPTER 2

Pathogenicity of the fathead minnow nidovirus (FHMNV) to muskellunge (*Esox masquinongy*)

Abstract

The recent isolation of a new fathead minnow nidovirus (FHMNV) strain from hatchery raised muskellunge (MUS-WL) has raised concerns regarding its risks to muskellunge rehabilitation efforts in North America. This study was designed to investigate the pathogenicity and disease course of this distinct FHMNV strain (MUS-WL). Experimental infection of naïve muskellunge by intraperitoneal injection (IP) using doses ranging from 6-6.3 x 10^3 $TCID_{50}$/ml of FHMNV revealed the median lethal dose (LD_{50}) to be 11.2 $TCID_{50}$/ml, underscoring the relative high pathogenicity of this virus to muskellunge. When muskellunge were experimentally infected by immersion or through the ingestion of infected live fathead minnows to mimic the probable natural routes of infection, mortalities reached >90%. Clinical signs and histopathology were consistent across all methods of experimental infection. Infected muskellunge exhibited external petechial hemorrhages in addition to severe gill pallor. Internally, infected muskellunge showed primarily pale livers that were occasionally hemorrhagic or congested in appearance. Histopathology revealed multifocal areas of hepatocellular necrosis, as well as widespread necrosis throughout the splenic and renal tissues. FHMNV induced systemic infection in muskellunge with the virus detected in the serum early on in the disease course then spread to multiple organs including gills, kidneys, brain and liver. Infected muskellunge shed the virus as early as 2 days postinfection. Data generated in this study demonstrate that FHMNV is infectious to muskellunge by multiple infection routes causing serious damage to vital organs. Spread of

this emerging coronavirus in naïve muskellunge stocks may have significant impacts on rehabilitation efforts of this important species in North America.

Introduction

Viruses of the family *coronaviridae* (order *Nidovirales*), are of particular concern due to their association with high mortality in humans (e.g., *Severe Acute Respiratory Syndrome*), companion animals (e.g., *feline infectious peritonitis*), as well as in economically important livestock (e.g., *infectious bronchitis of chickens*, *yellow head disease of penaeid prawns*) (Siddell & Snijder, 2008). So far, there have only been two described coronaviruses isolated from fish. Both of these viruses infect fish of the family *cyprinidae*, and have been assigned to a novel genus, *Bafinivirus* (Granzow et al 2001, Batts et al 2012). The first of these viruses is the white bream virus (WBV), which was isolated from white bream (*Blicca bjoerkna*) during a routine health examination of wild fish in Germany during the year 2001 (Granzow et al, 2001); the second is the fathead minnow nidovirus (FHMNV) which was isolated from fathead minnows during a mortality event on a baitfish farm in Arkansas (Iwanowicz & Goodwin 2002). Relatively little work has been done to determine the host range and pathogenicity of these viruses.

In 2011, FHMNV was isolated from hatchery raised muskellunge in Wisconsin that were suffering from a mortality episode; and was shortly followed by isolation of the same virus from apparently healthy juvenile muskellunge raised in a hatchery in Michigan (Chapter 2). This was surprising since FHMNV was believed to only infect cyprinids. Upon experimental infection via the intraperitoneal route, FHMNV was found to be pathogenic to muskellunge fulfilling River's postulates (Chapter 2). Phylogenetic analysis revealed that one of the muskellunge FHMNV isolates, designated MUS-WL, was distinctly different from other FHMNV strains (Chapter 2).

Muskellunge are an extremely ecologically and economically important sportfish species that are resident of the Laurentian Great Lakes region. They are highly desirable due to their large size, and support a significant sport fishery particularly in Lake St. Clair and the Detroit River. To keep up with the demand for this species, the State of Michigan has created muskellunge rehabilitation programs whereby gametes are collected from wild adults, and raised in state fish hatcheries to a certain size before being stocked in water bodies throughout the state. In the 1950's there were only 15 lakes and streams that supported the muskellunge fishery in Michigan, however, supplemental hatchery production of muskellunge has increased this number significantly (Dexter 2004). During their holding time in state fish hatcheries, muskellunge are fed fathead minnows which typically come from out of state. Currently, the only reportable fish diseases in Michigan for minnows include heterosporis and viral hemorrhagic septicemia virus (VHSV) (MDNR 2007). Baitfish are not required to be certified free of FHMNV prior to sale. It is for this reason that there are increasing concerns among fishery managers about the emergence of FHMNV in hatchery raised muskellunge. To this end, this study was designed to better understand the pathogenicity of the MUS-WL strain of FHMNV and to determine its disease course in muskellunge.

Methods

Cell culture and virus propagation

Virus stocks of MUS-WL FHMNV strain were produced in *Epithelioma papulosum cyprini* (EPC; Fijan et al. 1983), aliquoted, and kept at -80° C until used. To determine the virus concentration, a median tissue culture infective dose $TCID_{50}$ assay was performed using EPC cells as described by Reed & Muench (1938). Titrated virus stocks were aliquoted in cryogenic vials (Corning Inc., Corning, NY, USA) and kept at -80° C until used.

Cell lines were grown and maintained in 150 cm^2 tissue culture flasks (Corning) at 25° C using growth formulation of Earle's salt-based minimal essential medium (MEM) (Invitrogen Life Technologies, Carlsbad, CA, USA) supplemented with 29.2 mg ml^{-1} L-glutamine (Invitrogen), penicillin (100 IU ml^{-1}) and streptomycin (0.1 mg ml^{-1}) (Invitrogen), 10% fetal bovine serum (Hyclone, South Logan, UT, USA), and sodium bicarbonate (7.5% w/v; Sigma Chemical Co, St. Louis, MO, USA).

Experimental infection by IP-injection

Fish and maintenance

Certified disease free muskellunge (approximately 6 months post hatch) were obtained from Chautauqua fish hatchery located in Chautauqua, NY. Prior to use in experimental infection, five fish were euthanized using an overdose of tricaine methanesulfonate (MS-222; Argent Chemical Laboratories, Redmond, WA, USA) (25 mg ml^{-1}) and tested for the presence of pathogens including FHMNV. Briefly, kidneys and spleen were aseptically removed and homogenized using a Biomaster Stomacher (Wolf Laboratories, York, UK) at the high speed setting for 2 minutes. Homogenates were diluted with Earle's salt-based MEM, supplemented with 12mM Tris buffer (Sigma Chemical Co, St. Louis, MO, USA), penicillin (100 IU ml^{-1}) (Invitrogen), and amphotericin B (250 μg ml^{-1}, Invitrogen) to produce a 1:4 dilution of original tissues. Samples were centrifuged at 5000 rpm for 30 minutes, and the supernatant from each individual sample was inoculated into triplicate wells of a 96-well plate containing EPC cells grown with MEM (5% fetal bovine serum). Infected plates were incubated at 15° C for 7 days, and observed for the formation of cytopathic effects (CPE). Infected cells were removed from the plates and placed in a -80° C freezer, then thawed after approximately 20 minutes. The samples were then centrifuged, and the supernatant was inoculated for a second time onto EPC

cell lines. After 7 days, the wells were assessed for the presence of FHMNV by the Loop Mediated Isothermal Amplification (LAMP) assay (Zhang et al 2014). The LAMP reaction was carried out in a 20µl reaction mixture containing 1.6 µM each of FIP and BIP primers; 0.8 µM each of LF and LB primers; 0.2 µM each of F3 and B3 primers; 1×isothermalamplification buffer (20 mM Tris–HCl, 10 mM (NH4)2SO4, 50 mMKCl, 2 mM MgSO4, 0.1% Tween 20, pH 8.8); 6 mM MgCl2;1 M betaine; 1.4 mM each deoxynucleoside triphosphate (dNTP);0.2 mM MnCl2; 20 µM Calcein; 2U AMV Reverse Transcriptase(Invitrogen); 8U Bst DNA polymerase (New England Biolabs, Beverly,MA, USA); and 1µl of template RNA. The mixture was incubated for 60 min in a Mastercycler gradient PCR machine (Eppendorf, Hauppauge, NY, USA) at 63℃ (Zhang et al 2014).

Experimental fish were allowed to acclimate to laboratory conditions for 3 weeks, during which time the temperature was progressively lowered to 11 +/- 1℃ before the experiment was initiated. All fish were initially held in approximately 420L fiberglass tanks in a continuous flow-through system supplied with oxygenated and chilled well water at the Michigan State University-Research Containment Facility, East Lansing, MI. Fish were fed certified disease free fathead minnows.

Experimental infection

Juvenile, naïve muskellunge were challenged with MUS-WL FHMNV using a standard intraperitoneal (IP) injection protocol to ensure all fish were simultaneously infected with four different doses of the virus ranging from 6.3 - 6.3 x 10^3 $TCID_{50}$/ml. Five tanks total were used; the first tank being a control tank which received only sterile media and then four tanks (one for each infective dose). 100 µl of each dose level was administered to 10 fish shortly after being anesthetized with MS-222 (100-150 ppm). Fish were then recovered from anesthesia in a 72 L

polyethylene tank (Aquatic Eco-Systems, Apopoka, FL, USA) in a continuous flow-through system. All fish were monitored daily for an observation period of 60 d postinfection (p.i.). The inflowing water was supplied by an underground well and chilled to 11 +/- 1° C throughout the duration of the experiment. The outflow was united into a common tract entering an ultraviolet sterilization unit (Aquafine, Mississauga, ON, Canada). Tanks were monitored every 8 to 12 h daily for moribund or dead fish.

Virus recovery

Kidney, spleen, heart and liver were collected from dead/moribund fish. Samples from fish tissues from each dose were tested for the presence of virus by Loop Mediated Isothermal Amplification (LAMP) as well as by cell culture as described previously.

Histopathology

Lesions that were grossly observed from dead and severely moribund (euthanized with an overdose of MS-222; 25 mg ml^{-1}) fish as well as kidney/spleen/heart/liver tissues were collected from all fish. The samples were fixed in 10% formalin and embedded in paraffin prior to sectioning. Tissue sections (5 μm) were stained with hematoxylin and eosin (H&E) as detailed in Prophet et al. (1992). Tissue alterations were determined by microscopical examinations. Professor Scott Fitzgerald, a board-certified veterinary pathologist at the Michigan State University Diagnostic Center for Population and Animal Health (DCPAH), was consulted when needed.

Experimental infection by immersion

Fish and maintenance

Certified disease free juvenile muskellunge were obtained from Wolf Lake State Fish Hatchery located in Kalamazoo, MI. Experimental fish were allowed to acclimate to laboratory

conditions for 3 weeks, during which time the temperature was progressively lowered to 11 +/- 1℃ before the experiment was initiated. All fish were initially held in approximately 420 L fiberglass tanks in a continuous flow-through system supplied with oxygenated and facility-chilled well water (Michigan State University-Research Containment Facility, East Lansing, MI). Fish were fed using certified disease free rosy red minnows.

Experimental infection

Juvenile, naïve muskellunge were experimentally infected with a low (4.64×10^2 $TCID_{50}$/ml) and high dose (5.88×10^3 $TCID_{50}$/ml) by an immersion protocol previously described by Kim & Faisal (2012). During the infection process, 10 individual muskellunge per treatment were immersed in 6L of diluted virus within an aerated 5 gallon glass aquaria for one hour. Following the treatment period, fish were removed from their respective aquaria and rinsed with chilled freshwater before being transferred to their round 72 L experimental tanks. Virus recovery and histopathology were performed as described previously.

Ingestion of infected fathead minnows

Experimental infection

10 fathead minnows were infected with 6.4×10^3 $TCID_{50}$/ml and were fed 5 days post infection to 10 muskellunge that had been fasting for 5 days. Prior to feeding the tank flow was turned up, and following one hour minnows that were not eaten were removed to prevent viral shedding into the water. This was repeated once per week for four additional feedings, after which uninfected minnows were fed once weekly. Tanks were monitored every 8-12 hours for dead or moribund fish.

Viral Shedding and disease course

For this experiment, 30 fish total were infected with a single dose of 3.73 x 10^2 $TCID_{50}$/ml by immersion as described previously, and six of these fish were housed in five separate 72L tanks. Thirty control fish were immersed in an equivalent dilution of sterile media, and were housed in three separate 72L tanks in a designated control area. One fish from each infected and control tank (five infected fish, three control fish) were sampled and euthanized with an overdose of MS-222 every two days for a study period of twelve days (6 total sampling times). Prior to euthanasia, fish were tested for viral shedding as described by Kim & Faisal (2012). To determine the viral concentration shed by infected muskellunge, water samples collected during viral shedding trials were tested using a TCID50 protocol as described by Reed & Meunch (1938). Blood, kidney, spleen, heart, liver and brain were collected from each fish following euthanasia and stored at -80℃ until completion of the 15 day study period. After being allowed to clot at 4℃, blood samples were spun at 2000 xg for 10 minutes at 4℃. Serum was aliquoted and stored with the other tissue samples at -80℃.

Results

Experimental infection by IP-injection

By the end of the 60 day study period, the highest dose of 6.3 x 10^3 $TCID_{50}$/ml had experienced 88.9% mortality, the doses of 6.3 x 10^2 $TCID_{50}$/ml reached 100% mortality, and the 6.3 x 10^1 $TCID_{50}$/ml reached 71.4% mortality. The lowest dose of 6 $TCID_{50}$/ml dose and the control dose did not experience any mortalities (Figure 2-1). Based on cumulative mortality data, the intraperitoneal median lethal dose (IP-LD_{50}) for FHMNV infection in muskellunge was calculated to be 11.2 $TCID_{50}$/ml. The majority of infected fish exhibited severe gill pallor (Figure 2-2). Some individual fish also exhibited petechial hemorrhaging in the eyes, fins and

musculature (Figure 2-2). Internally, many fish were found to have pale livers (Figure 2-2) and enlarged spleens.

Experimental infection by immersion

Mortalities began around 8 days post infection, and steeply increased with the high dose reaching 100% mortality by 15 days post infection (Figure 2-3). By day 24 of the 30 day study period, the low dose group (4.64×10^2 $TCID_{50}$/ml) had also reached 100% mortality (Figure 2-3). Control individuals experienced no mortalities. When infected fish tissues were inoculated on EPC cell lines, 7/10 individuals from the low dose group and 4/10 individuals from the high dose group exhibited CPE following two 14 day passages. All infected fish tissues from the high dose group and 9/10 infected fish tissues from the low dose group were positive for FHMNV by qLAMP.

Muskellunge infected with FHMNV by immersion exhibited multifocal hepatocellular necrosis (Figure 2-4a). In addition, these fish also exhibited severe widespread necrosis of the splenic tissues (Figure 2-4c) and of the interstitial tissue of the anterior kidney (Figure 2-4b). No abnormalities were observed in brain or heart tissues. All changes in the gills appeared to be artifact in origin.

Experimental infection by ingestion of infected fathead minnows

Muskellunge that were experimentally infected with FHMNV through feeding of infected fathead minnows reached 90% mortality after five consecutive feedings (50 days post initial feeding) (Figure 2-5). Following inoculation on EPC cell lines, 8/10 infected fish tissues exhibited CPE after two 14 day passages. FHMNV was detected from 9/10 infected fish tissue samples by qLAMP.

Viral Shedding and disease course

Muskellunge infected with FHMNV by immersion using a dose of 3.73 x 10^2 $TCID_{50}$/ml began to shed virus as early as two days following infection at levels of 2.43 x 10^2 $TCID_{50}$/ml (Figure 2-6). Viral shedding reached 1 x 10^4 $TCID_{50}$/ml at eight days postinfection, after which it dropped down to 5.67 x 10^2 $TCID_{50}$/ml at 10 days and 1 x 10^3 $TCID_{50}$/ml at twelve days post infection (Figure 2-6).

FHMNV started to be reisolated from the serum of one of five infected fish two days postinfection (p.i.) (Table 2-1). By day 4 p.i., the virus was detected in the serum and brain of 1/5 infected fish (Table 2-1). On day 6 p.i., the virus appeared in the kidneys of 2/5 fish, serum of 3/5 fish, and gill of 1/5 fish (Table 2-1). By day 8 p.i. the virus was only detected from gill tissue in 3/5 fish (Table 2-1). By day 12 p.i. the virus had spread to multiple organs of infected fish (Table 2-1). No virus was detected from any control individuals.

Discussion

Novel genotypes of coronaviruses continue to emerge, presumably due to natural recombination (Zhang et al 2014). For example, Zhang et al. (2014) who studied the different genotypes of the human coronavirus-0C43 (HCoV-0C43) over the course of 7 years, noticed the emergence of novel genotypes. Further, Lau et al. (2011) reported that different HCoV-0C43 genotypes vary in their pathogenicity. Similar variation in pathogenicity has also been observed among different genotypes of the mouse hepatitis virus (MHV). Compton et al. (1993) linked differences in the spike (S) proteins to variable tropisms. In addition to MHV genotypes varying in pathogenicity, (Compton et al 1993). It is for these reasons that we focused our experimental challenges on the MUS-WL strain of FHMNV, since it was determined to be distinctly different from previous FHMNV strains and was isolated from muskellunge (Chapter 2).

MUS-WL strain of FHMNV displayed a relatively low LD_{50} in juvenile muskellunge by IP injection (11.2 $TCID_{50}$/ml). This value was slightly higher than the IP-LD_{50} for viral hemorrhagic septicemia (VHSV) (genotype IVb) in muskellunge which has been calculated to be as low as 3.2 $TCID_{50}$/ml (Faisal & Kim 2010). VHSV has caused significant losses in Great Lakes muskellunge, so the fact that FHMNV has a similar pathogenicity to this virus is particularly alarming. Coronaviruses in general have a variety of LD50 values; these values can be drastically different across strains of the same virus. For example, MHV strain A59 has an LD_{50} of 5714 $TCID_{50}$/ml, while MHV strain 2 LD50 is 285 $TCID_{50}$/ml, while ML-7, ML-8, ML-10, and LA-7 of MHV are all nonlethal (>71,500 $TCID_{50}$/ml) (Sarma et al 2001). It is possible that different strains of FHMNV may vary in their pathogenicity as well; however more research would need to be completed in order to confirm this.

The World Organisation for Animal Health (OIE) requires fish species to exhibit morbidity/mortality following experimental infection by immersion in order to consider a species susceptible to viral infection (OIE 2012). The ability of FHMNV to overcome the muskellunge natural barriers, as shown in the immersion and ingestion experimental infection studies, demonstrates that the FHMNV inherent pathogenicity to muskellunge and underscores the high susceptibility of muskellunge to this virus. This finding has a far reaching impact as far as managing this disease in muskellunge rehabilitation programs considering the high dependency of these efforts on baitfish. To date, muskellunge is the only non-cyprinid species from which FHMNV was found associated with mortalities (Chapter 2). The other two reported cyprinid hosts for FHMNV are creek chub (*Semotilus atromaculatus*) (McCann 2012) and the fathead minnow (Iwanowicz & Goodwin 2002) in addition to muskellunge. Infection with muskellunge

associated with high morbidity and mortality underscores the need to screen more fish species for their susceptibility to this emerging coronavirus.

Clinical signs and histopathological changes observed in FHMNV infected muskellunge were consistent across the different methods of infection. The majority of individuals exhibited severe gill pallor, splenomegaly, as well as pale and occasionally congested livers. Several individuals also showed mild, multi-focal petechial hemorrhages in the eyes, fins and musculature. Histopathologically, multi-focal and widespread necrosis was observed in the kidneys, spleen and liver. Necrosis of the renal, splenic and hepatic tissues was consistent with histopathology results from previous IP-injection studies of both muskellunge (Chapter 2) and fathead minnow (Iwanowicz & Goodwin 2002). Such necrotic changes in the hematopoietic tissues along with hemorrhages could be the cause of the gill pallor observed in infected muskellunge. In the same context, we observed an increased prevalence of fungal infection in infected but not in uninfected muskellunge (data not shown) which may have resulted from immunosuppression due to the widespread necrosis in hematopoietic tissues.

Results of this study demonstrated that FHMNV is being constantly shed into the surrounding water, therefore, infected fish probably constitute a main route for the virus spread. Similar results were also reported for another viral infection of muskellunge, the viral hemorrhagic septicemia virus (Kim & Faisal 2012). The detection of FHMNV in the serum of infected individuals as early as 2 days p.i underscores the systemic nature of the virus and its ability to spread in most tissue as well as overcoming the brain barrier. Detection of FHMNV in the kidneys and liver is consistent with what has been seen histopathologically in both IP-injection and immersion experimental challenges. The fact that FHMNV was also detected in gill, serum, and brain tissues was interesting, as other animal coronaviruses are able to spread

through the blood vessels, central nervous system, and respiratory system following infection (Lavi et al, 1999).

The lack of regulation in the baitfish industry is particularly alarming now that we know this virus is highly pathogenic to muskellunge by IP-injection, immersion as well as through ingestion. FHMNV could potentially have devastating impacts on both wild and captive muskellunge, and consequently to the sport fishing industry which contributes billions of US dollars to the economy each year (NOAA Great Lakes Environmental Research Laboratory). This virus has been demonstrated to cause significant morbidity and mortality in hatcheries, the infectious etiology has been proven, and it has the potential to negatively affect wild fish populations (muskellunge and native cyprinids). Based on these characteristics, it is proposed that FHMNV should be considered a reportable fish pathogen, particularly for the Great Lakes basin (Phillips et al 2014). FHMNV has been associated with mortality events in additional states as well, and has the potential to cause damage on a national level where esocids and native cyprinids are intermingled.

In conclusion, FHMNV is highly pathogenic to muskellunge. Infected muskellunge actively shed FHMNV as early as 2 days p.i., and may become infected with the virus both through the water and by ingestion of infected baitfish. If FHMNV were to enter a hatchery setting via infected baitfish, the virus could continue to spread through viral shedding of infected muskellunge as well as through ingestion. Muskellunge are also known to cannibalize one another (Meade et al 1983), which could potentially be another route of transmission. In order to protect muskellunge in the Great Lakes ecosystem, more stringent health certification requirements of baitfish is necessary, for FHMNV in particular.

APPENDIX

Table 2-1: FHMNV was first detected in the serum (1/5 fish) at 2 days p.i. On day 4 p.i., the virus began to appear in the brain tissues in addition to serum (1/5 fish). By day 6 p.i. the virus disappeared from the brain, but was found in kidney (2/5 fish) and gill (1/5 fish) tissues in addition to serum (2/5 fish). By day 8 p.i., FHMNV was only detected in gill tissues (3/5 fish). By day 12 p.i. the virus was detected in the gill (2/5 fish), brain (1/5 fish) and liver (1/5 fish) of infected fish.

Days Post Infection (PI)	**Sample**	**FHMNV copies/mg of tissue**
2	Serum (5)	265.8
4	Brain (2)	16
4	Serum (2)	9.2
6	Kidney (1)	630
6	Serum (1)	1920
6	Kidney (2)	14.7
6	Serum (2)	8
6	Gill (3)	1.5
6	Serum (5)	1557.5
8	Gill (1)	321.1
8	Gill (4)	69.1
8	Gill (5)	197.5
12	Gill (1)	2.9
12	Liver (2)	35.1
12	Brain (3)	117.5
12	Gill (4)	1.2

Figure 2-1: Percent cumulative mortality for muskellunge infected with fathead minnow nidovirus (FHMNV) intraperitoneally with four different doses varying from 6-6.3 x 10^3 $TCID_{50}$/ml over a 60 day study period.

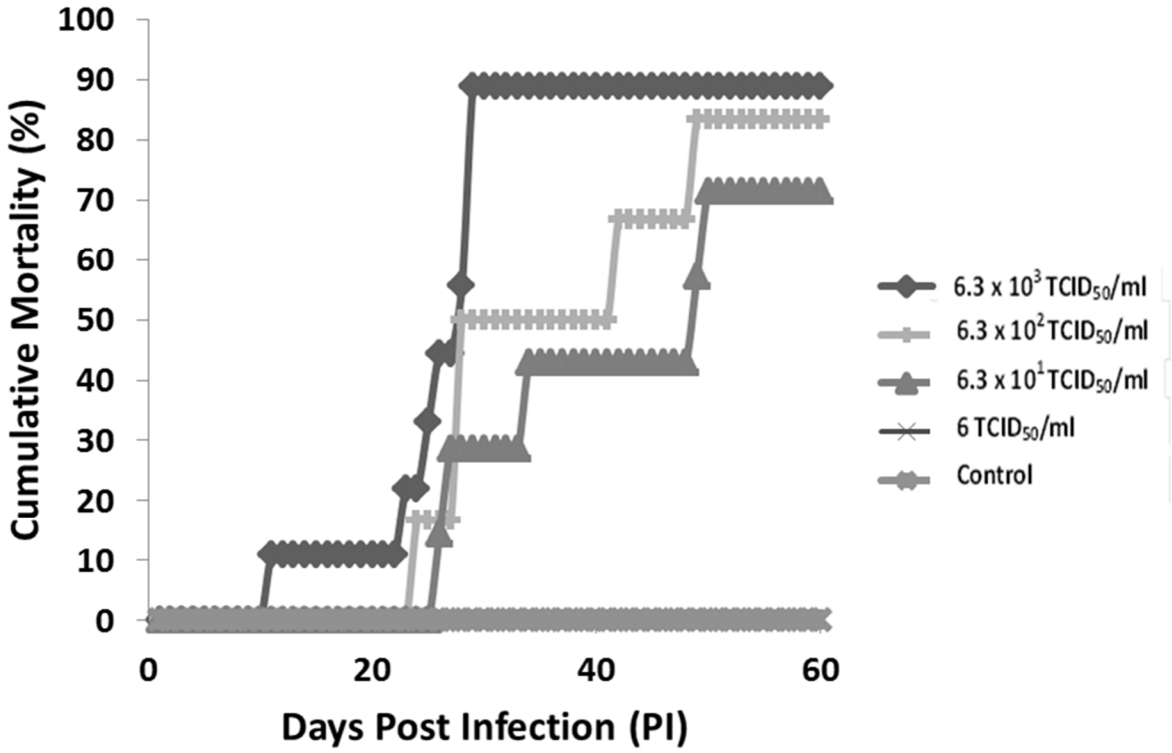


Figure 2-2: Muskellunge infected with FHMNV by IP-injection exhibited multifocal echymotic hemorrhage on caudal peduncle (top left), severe gill pallor (top right), moderate ocular hemorrhage (bottom left), and severe liver pallor (bottom right).

Figure 2-3: Percent cumulative mortality for muskellunge infected with the fathead minnow nidovirus at two doses over 30 days by immersion.

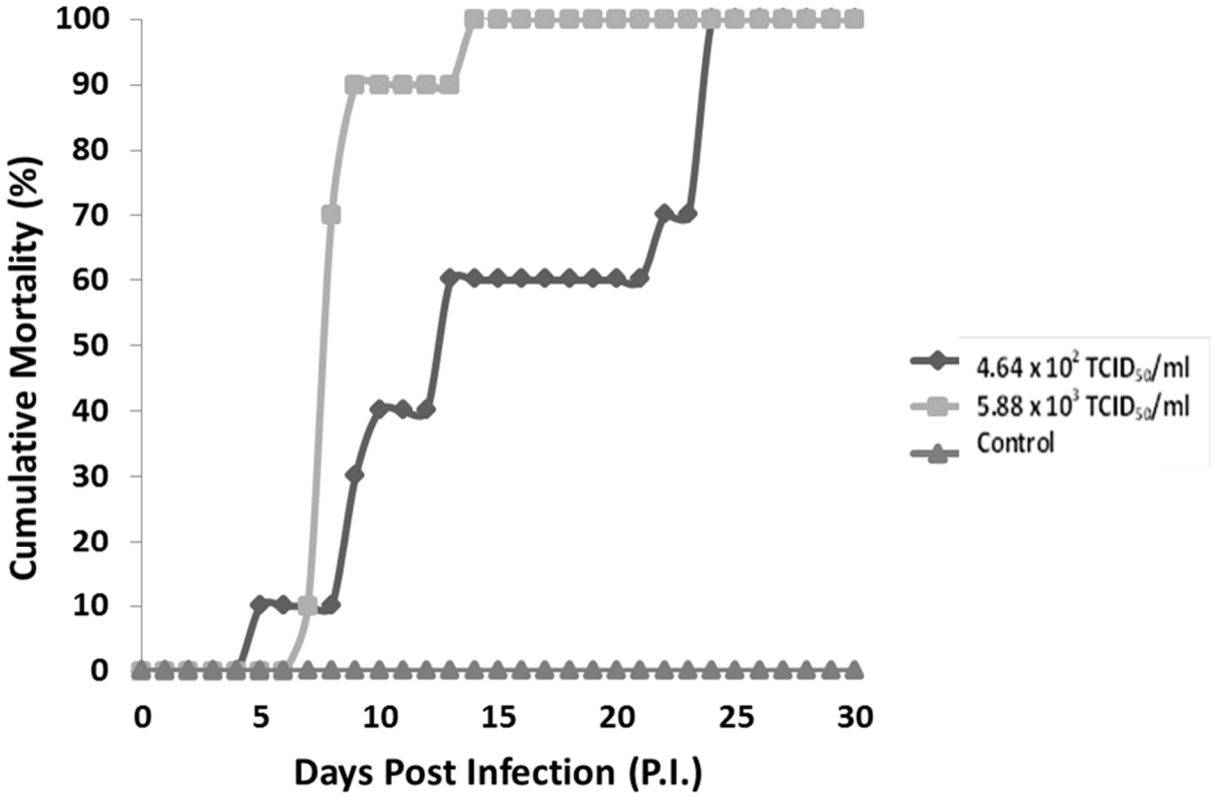


Figure 2-4: Hematoxylin and eosin (H & E) stained tissue sections from muskellunge infected with FHMNV by immersion. A) Muskellunge liver exhibiting multifocal hepatocellular necrosis (40X magnification). B) Severe widespread necrosis of the interstitial tissue throughout the anterior kidney of infected muskellunge (40X). C) Moderate widespread necrosis of splenic tissues (40X).

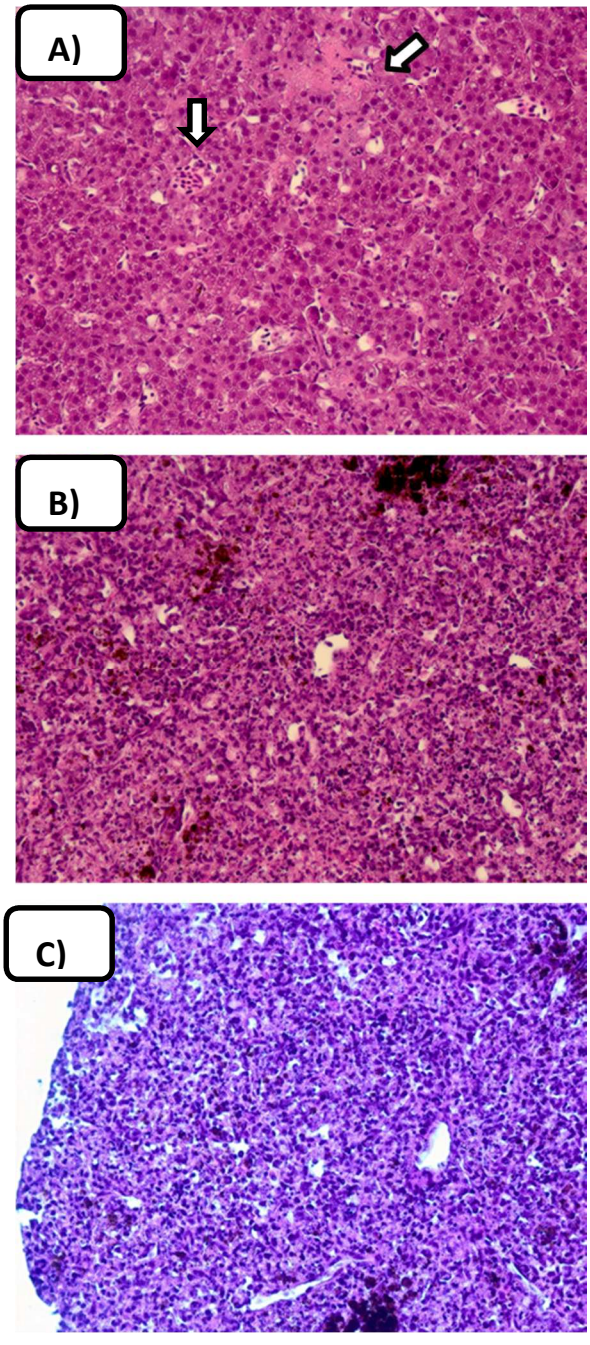


Figure 2-5: Mortality curve for muskellunge that were fed fathead minnows infected with a dose of 6.4 x 10^3 $TCID_{50}$/ml weekly for five weeks. None of the control fish died throughout the experiment.

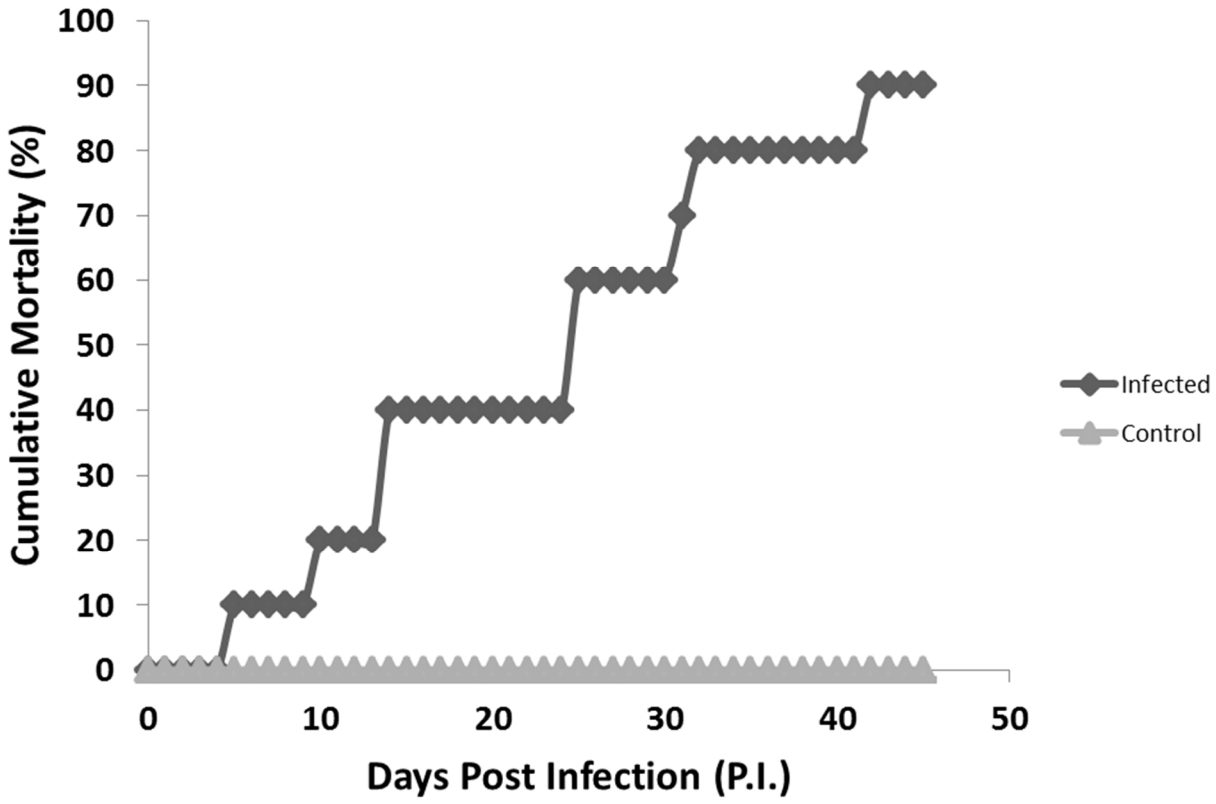
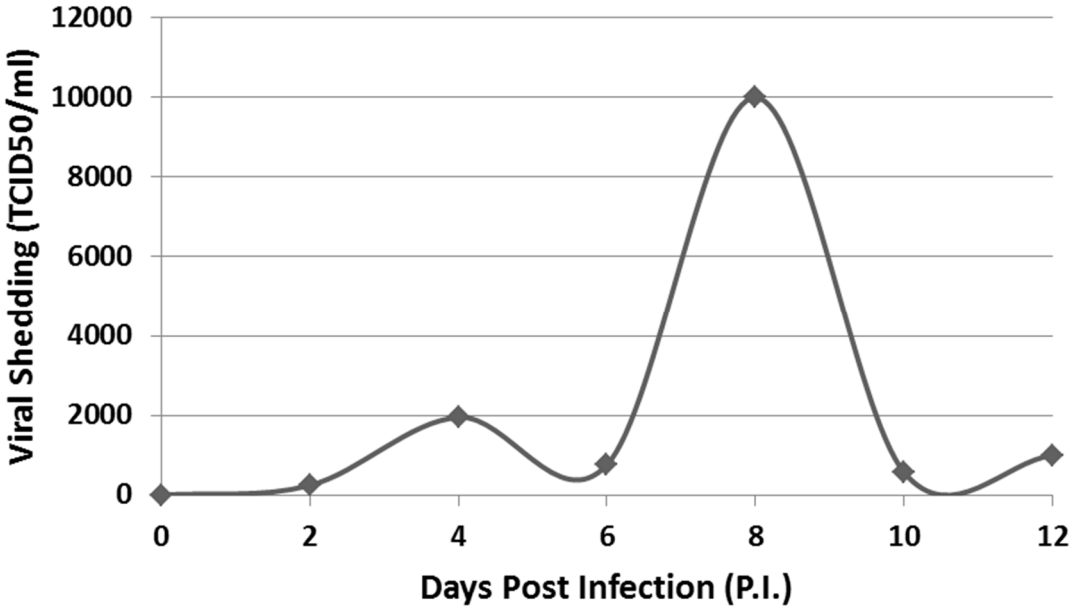


Figure 2-6: Curve showing the amount of viral shedding in muskellunge experimentally infected by immersion with FHMNV. The immersion dose was 3.73 x 10^2 $TCID_{50}$/ml. Fish were tested for viral shedding every two days for a period of twelve days (6 total sampling events).



CHAPTER 3

Fathead Minnow Nidovirus (FHMNV) infects two other native cyprinids; the spotfin shiner (*Cyprinella spiloptera*) and the golden shiner (*Notemigonus crysoleucas*)

Abstract

Since the initial isolation of the fathead minnow nidovirus (FHMNV), concerns have been raised regarding the risks it may pose to other fish species. In this study, seven fish species, resident to the Laurentian Great Lakes were challenged intraperitoneally (IP) with Two doses of the MUS-WL strain of FHMNV; 6.3×10^2 and 6.3×10^4 $TCID_{50}$/ml in a controlled laboratory setting. IP-injected spotfin shiner and golden shiner suffered morbidity and mortality during the 40 day observation period, while other species tested including creek chub (*Semotilus atromaculatus*), rainbow trout (*Oncorhynchus mykiss*), largemouth bass (*Micropterus salmoides*) and walleye (*Sander vitreus*) showed no clinical signs or endured mortality. FHMNV was re-isolated on EPC cell line from the tissues of infected spotfin shiners and golden shiners which harbored high number of virus RNA copies as measured by the quantitative loop mediated isothermal amplification (qLAMP). FHMNV infected spotfin shiner and golden shiner exhibited petechial hemorrhages throughout the eyes, fins and skin, exopthalmia, edematous kidneys, and liver pallor which mimicked those exhibited by fathead minnow infected with the same virus concentrations. Histopathologic analysis revealed multifocal areas of necrosis in the kidneys, spleen and liver of infected fish. Spotfin shiner and golden shiner were then infected with two doses of FHMNV (3.2×10^3 and 8×10^3 $TCID_{50}$/ml) by immersion to mimic more natural modes of infection. Spotfin shiner experienced 60% mortality at both doses, while golden shiners

did not experience mortality neither developed any clinical signs have been developed following a 40 day observation period. Overall, piscivorous fish tested in this study do not seem to be at risk for infection, while cyprinids appear to vary in their susceptibility to MUS-WL FHMNV

Introduction

In 1997, a novel coronavirus of the genus *Bafinivirus* (order *Nidovirales*), the fathead minnow nidovirus (FHMNV), was isolated from a mortality event of fathead minnows of both wild-type and rosy-red phenotypes on a baitfish farm in Arkansas, USA (Iwanowicz & Goodwin 2002, Batts et al., 2012). The disease caused by FHMNV was systemic in nature with moribund fish exhibiting external petechial hemorrhages. River's postulates were fulfilled for this virus since fathead minnows experimentally infected via the intraperitoneal (IP) route suffered from high levels of mortality and hemorrhagic diathesis similar to that observed in spontaneously infected minnows (Iwanowicz & Goodwin 2002).

FHMNV has also been isolated from non-cyprinids. The studies outlined in Chapters 2&3 demonstrated that muskellunge (*Esox masquinongy*) is susceptible to FHMNV infection which is associated with high morbidity and mortality. River's postulates have already been fulfilled for the MUS-WL strain of FHMNV in muskellunge by IP-injection; immersion, and ingestion. Muskellunge experimentally infected with FHMNV experienced 100% mortality, and exhibited clinical signs including external petechial hemorrhages, severe gill pallor, as well as liver pallor (chapter 2). Histopathological investigation revealed that FHMNV seems to primarily target the hematopoietic tissues in the kidneys, spleen and liver causing widespread necrosis. This isolation of FHMNV from hatchery muskellunge was alarming as it may threaten the baitfish-dependent muskellunge fishery rehabilitation programs practiced by multiple North American fishery agencies.

Since the initial isolation of FHMNV in Arkansas, it has been isolated from fathead minnows reared in Wisconsin, Minnesota and Illinois (Batts et al 2012). In addition, it has also been detected from another cyprinid; the creek chub (*Semotilus atromaculatus*) (McCann 2012), as well as from hatchery raised muskellunge in Michigan and Wisconsin in 2011 (chapter 2). Despite its pronounced morbidity and mortality, little work has been done to assess the pathogenicity and host range of this virus. This is particularly alarming as FHMNV may pose significant risks to native cyprinids which play a central role in the stability of the foodweb and to the future of the baitfish industry.

In the USA, the baitfish industry was estimated to generate annual revenues of US $170 million shipping about 10 billion fish per year, with fathead minnows and golden shiners being the most popular species used (Goodwin et al 2011). There has been increasing concern over pathogens in baitfish, as we have begun to detect them more frequently. Interestingly, recent studies have determined fathead minnows are statistically more likely (P=0.021) to be infected with a virus compared to other popular baitfish species used (golden shiners and white suckers) (McCann, 2012). Despite this increased detection of pathogens, the baitfish industry is mostly unregulated when it comes to disease screening requirements (Goodwin et al, 2011). FHMNV in particular is currently not listed as an OIE reportable disease, and therefore baitfish are not required to be screened for this virus prior to being transferred to various states and water bodies. To this end, this study was designed to elucidate the potential pathogenicity of FHMNV to representative fish species resident in the Great Lakes basin that may be at risk for FHMNV infection either through the baitfish industry or rehabilitation programs.

Methods

Fish and maintenance

A total of 7 fish species were used in this study. **C**ertified disease free fathead minnows were obtained from the Animal and Plant Health Inspection Services, US Department of Agriculture (Ames, IA), certified disease-free spotfin shiner, golden shiner and largemouth bass from the the LaCrosse Fish Health Center, US Fish and Wildlife Service (Onalaska, WI, and rainbow trout from Wolf Lake State Fish Hatchery (Mattawan, MI). Certified disease-free walleye (gametes collected from St. Mary's River broodstock) were obtained from the Sault Tribe facility in Chippewa County, MI. In addition, creek chub that were wild caught using hook and line from a stream in Kalamazoo, MI were also included in this study. Prior to use in experimental infection, five fish from each species were euthanized using an overdose of tricaine methanesulfonate (MS-222; Argent Chemical Laboratories) (25 mg ml^{-1}) and tested for the presence of pathogens including the FHMNV as described below.

Experimental fish were allowed to acclimate to laboratory conditions for 3 weeks, during which time the temperature was progressively lowered to $11 \pm 1^{\circ}$ C before the experiment was initiated. All fish were initially housed in separate 420 L fiberglass tanks in a continuous flow-through system supplied with oxygenated and facility-chilled well water with outflow uniting into a common tract entering an ultraviolet sterilization unit (Aquafine Corporation, Valencia, CA, USA). Experimental fish were held at the Michigan State University-Research Containment Facility, East Lansing, MI in accordance with the International Animal Care and Use Committee (IACUC) guidelines (Approval #: RC102183).

Cell culture and virus propagation

Virus stocks of the muskellunge nidovirus (MUS-WL) isolate (Chapter 2) were produced in *Epithelioma papulosum cyprini* (EPC; Fijan et al. 1983), aliquoted, and kept at -80° C until used. To determine the virus concentration, a $TCID_{50}$ assay was performed on EPC cell lines as described by Reed & Muench (1938). Titrated virus stocks were aliquoted in cryogenic vials (Corning, Corning, NY, USA) and kept at -80° C until used.

Cell lines were grown and maintained in 150cm^2 tissue culture flasks (Corning) at 25° C using growth formulation of Earle's salt-based minimal essential medium (MEM) (Invitrogen Life Technologies, Carlsbad, CA, USA) supplemented with 29.2mg ml^{-1} L-glutamine (Invitrogen), penicillin (100 IU ml^{-1}) and streptomycin (0.1 mg ml^{-1}) (Invitrogen), 10% fetal bovine serum (Hyclone, South Logan, UT, USA), and sodium bicarbonate (7.5% w/v; Sigma-Aldrich, St. Louis, MO, USA).

Experimental infection of fathead minnow to determine the range of virus concentration to be used for susceptibility screening

Certified disease free fathead minnows were infected with FHMNV using a standard intraperitoneal (IP) injection protocol to ensure all fish were simultaneously infected with six different doses of the virus ranging from 6-6.3 x 10^4 $TCID_{50}$/ml. Six tanks total were used; the first tank being a negative control tank which received only sterile media and then five tanks (one for each infective dose; 6, 6.3 x 10^1, 6.3 x 10^2, 6.3 x 10^3 and 6.3 x 10^4 $TCID_{50}$/ml respectively). 100 µl of each dose level was administered to each of 10 fish shortly after being anesthetized with MS-222 (100-150 ppm). Fish were then recovered from anesthesia in a 72 L polyethylene tank (Aquatic Eco-Systems) in a continuous flow-through system. All fish were monitored every 8-12 hours daily for an observation period of 60 d post-infection (p.i.) Kidneys,

spleen, heart and liver were collected from dead/moribund fish and processed for viral testing as described below.

Experimental infection by IP-injection for susceptibility screening

Each species were challenged with FHMNV using a standard intraperitoneal (IP) injection protocol to ensure all fish were infected with a known dose of the virus. Three groups of fish per species (n=10) were used in this experiment, the first group received 100 µl of 630 $TCID_{50}$ /ml (low dose group), while the second group received the same volume of 63,000 $TCID_{50}$/ml (high dose group). The third group received 100 µl of sterile media (negative control group). All fish were monitored daily for an observation period of 30 days post infection (p.i.) Tanks were monitored every 8 to 12 h daily for moribund or dead fish. Kidneys and spleen were collected and processed for viral testing as described below. If infected fish did not experience any mortality by the end of the study period, fish tissues were collected in pools (5 fish/pool) and processed for viral testing in the same way.

Experimental infection of spotfin shiner and golden shiner by immersion

Based on the initial screening, spotfin shiner and golden shiner were selected for testing the pathogenicity of FHMNV by immersion. Certified disease free spotfin shiners and golden shiners were experimentally infected with a low (3160 $TCID_{50}$/ml) and high dose (8000 $TCID_{50}$/ml) by an immersion protocol previously described by Kim & Faisal (2012). During the infection process, 10 individual fish from each species per treatment were immersed in 6 L of diluted virus within an aerated 5 gallon glass aquaria for one hour. Following the treatment period, fish were removed from their respective aquaria and rinsed with chilled freshwater before being transferred to their round 72L experimental tanks.

Tissue processing for viral isolation

Tissue samples were diluted with Earle's salt-based minimal essential medium (MEM), supplemented with 12 mM Tris buffer (Sigma Chemical Co, St. Louis, MO, USA), penicillin (100 IU ml^{-1}) (), and amphotericin B (250 µg ml^{-1}, Invitrogen) to produce a 1:4 dilution of original tissues w/v. Samples were then homogenized using a Biomaster Stomacher (Wolf Laboratories, York, UK) at the high speed setting for two minutes. Following homogenization, samples were centrifuged at 5000 rpm for 30 minutes, and the supernatant from each individual sample was inoculated into triplicate wells of a 96-well plate containing EPC cells grown with MEM supplemented with 5% fetal bovine serum (Hyclone). Infected plates were incubated at 15° C for up to 21 days, and observed for the formation of cytopathic effects (CPE).

RNA Extractions and quantitative loop-mediated isothermal amplification (qLAMP)

Total RNA was extracted from 100µl of the supernatant from processed fish tissue using the Qiagen RNeasy mini kit (Qiagen, Inc., Valencia, CA, USA). The number of viral copies were quantified using a quantitative loop mediated isothermal amplification (qLAMP) assay specific to FHMNV that was developed by Zhang et al (2014). The reaction was carried out in a 25µL reaction mixture containing 2.5µL 10X isothermal amplification buffer, 1.5µL of $MgCl_2$ (100mM), 5µL of betaine (5M), 1.75µL of deoxynucleoside triphosphate (dNTP) (20mM), 2µL each of FIP and BIP primers (20mM), 1µL of LF and LB primers (20mM), 0.5µL of F3 and B3 primers (10mM), 2.25µL of RNA free water, 1µL of *Bst* DNA polymerase (New England Biolabs, Beverly, MA, USA), 0.5µL of MLV Reverse Transcriptase (Invitrogen), 1µL Calcein, and 0.5µL $MnCl_2$.

Histopathology

Lesions that were grossly observed from dead and severely moribund (euthanized with an overdose of MS-222; 25 mg ml^{-1}) fish as well as kidneys, spleen, heart and liver tissues were sampled for histopathology. External and internal lesions were fixed in 10% buffered formalin and embedded in paraffin prior to sectioning. Tissue sections (5 μm) were stained with hematoxylin and eosin (H&E) as detailed in Prophet et al. (1992). Tissue alterations were determined by microscopical examinations. Professor Scott Fitzgerald, a board-certified veterinary pathologist at the Michigan State University for Population and Animal Health (DCPAH), was consulted when needed.

Results

Experimental infection of fathead minnow to determine the range of FHMNV concentration to be used for scanning the susceptibility of other fish species

FHMNV infected minnows began experiencing mortalities around 10 days post infection (Figure 3-1). By the end of the 60 day study period, the highest dose of 6.3 x 10^4 $TCID_{50}$/ml had reached 100% mortality, the dose of 6.3 x 10^3 $TCID_{50}$/ml had reached 80% mortality, the dose of 6.3 x 10^2 $TCID_{50}$/ml had reached 90% mortality, and the dose of 6.3 x 10^1 $TCID_{50}$/ml had reached 50% mortality. The lowest dose of 6 $TCID_{50}$/ml and the control group both experienced no mortalities (Figure 3-1). Based on this experiment, doses of 6.3 x 10^2 and 6.3 x 10^4 $TCID_{50}$/ml were chosen for consequent FHMNV susceptibility scanning of additional species. Based on the mortality curves obtained for each virus concentration, the median lethal dose (LD_{50}) was calculated to be 6.3 x 10^1 $TCID_{50}$/ml.

Experimental infection by IP-injection

Fathead minnow

Fathead minnow was included in this experiment to act as a positive control of susceptibility and tissue alterations in response to FHMNV infection. Most mortality began to occur at approximately 10 days post infection (Figure 3-5a). Moribund fish exhibited behaviors of erratic swimming, lethargy, and heavy gilling. Externally, dead/moribund fish showed gill pallor, expothalmia, and severe petechial and echymotic hemorrhages throughout the eyes, fins and skin (Figure 3-2). Internal examination of infected fish revealed enlarged spleens and edematous kidneys. Several individuals also exhibited pale livers with multifocal petechial hemorrhages throughout. All tissue samples from infected fish were positive for FHMNV by cell culture and qLAMP. No virus was detected from any control fish either by cell culture or qLAMP.

Spotfin Shiner

FHMNV infected spotfin shiners showed similar clinical signs to that of infected fathead minnows, including petechial hemorrhages throughout the eyes, fins and skin (Figure 3-3) as well as splenic enlargement. Liver pallor was also observed in several individuals. Mortalities began at 9 days post infection, sharply increasing and reaching 90% in the low dose by 22 days PI and 100% mortality in the high dose by 23 days p.i. (Figure 3-5b). All infected fish were positive for FHMNV by qLAMP, with virus copies reaching up to 5×10^6 copies/mg of tissue in the low dose group and 9×10^5 copies/mg of tissue in the high dose group. CPE was observed for 30% of the samples infected on EPC cell lines following two 14 day passages.

Golden Shiner

FHMNV was also found to be pathogenic to golden shiner when infected by IP-injection; however mortality was not as high as in the case of spotfin shiner. Infected golden shiners from the high dose began experiencing mortalities at 13 days p.i., and by 36 days p.i. the low dose had reached 60% mortality while the high dose had reached 80% mortality (Figure 3-5c). Clinical signs were consistent with what was observed in spotfin shiner, and included petechial and echymotic hemorrhages throughout the eyes, skin and fins as well as liver pallor (Figure 3-4). All individuals were positive for FHMNV by qLAMP, with virus copies reaching up to 5×10^5 copies/mg of tissue in the low dose group and 3×10^6 copies/mg of tissue in the high dose group. CPE was observed for 50% of the samples infected on EPC cell lines following two 14 day passages. No virus was detected from control fish.

Other species tested

Creek chub, walleye, rainbow trout and largemouth bass did not exhibit any mortalities following experimental infection. All tissues including those from control fish tested negative for FHMNV both by cell culture and qLAMP methods.

Experimental infection of spotfin shiner and golden shiner by immersion

Infected spotfin shiner experienced mortalities beginning from day 11 p.i. (Figure 3-6). By the end of the 40 day study period, both low and high immersion dose groups had reached 60% mortality (Figure 3-6). FHMNV was detected from all infected individuals except for one fish from the low dose group by qLAMP, with copy numbers detected at levels up to 1×10^4 copies/mg of tissue for both doses. CPE was observed for 60% of the spotfin shiner tissue samples infected on EPC cells following two 14 day passages.

Golden shiners infected with FHMNV by immersion did not show any clinical signs of infection, and did not exhibit any mortality. One pool of golden shiner tissues from the high dose group tested positive for FHMNV by qLAMP, however, all other pools tested negative for the presence of the virus. All golden shiner tissue pools were negative for FHMNV by cell culture.

Histopathology of FHMNV infected fish

Interestingly, spotfin shiner, golden shiner, and fathead minnow experimentally infected with FHMNV exhibited similar histopathological alterations in the form of multifocal areas of necrosis in the hematopoietic tissues (Figure 3-7a, 3-7d). Posterior kidney of FHMNV infected fathead minnow showed multi focal tubular necrosis and cell disorganization (Figure 3-7a). Additionally, multifocal areas of necrosis were observed in the spleen of infected fathead minnows (Figure 3-7b), spotfin shiners (Figure 3-8b) and golden shiners (Figure 3-8d). In spotfin shiner, diffuse necrosis of the interstitial tissue in the anterior kidney was seen (Figure 3-7d), as well as multifocal hepatocellular necrosis in the liver (Figure 3-8a, 3-7c). Golden shiner exhibited renal tubule necrosis accompanied by widespread necrosis of the interstitial tissue (Figure 3-8c).

Discussion

Results obtained from this study demonstrate that representative Great Lakes native fish species vary in their susceptibility to the MUS-WL isolate of FHMNV. The spotfin shiner seems to be susceptible to this virus since morbidity and mortality occurred following experimental infection, not only by intraperitoneal injection but also by immersion. While IP-injection studies yield some evidence for susceptibility, immersion challenges are necessary to deem a species 'susceptible' per OIE requirements (OIE chapter 2.3.9). By this standard golden shiner showed some evidence for susceptibility, since they exhibited both morbidity and mortality following

experimental infection by intraperitoneal injection but not by immersion. All other species screened including creek chub, rainbow trout, largemouth bass, and walleye were considered not susceptible as they did not experience any morbidity or mortality by intraperitoneal injection, at least, at the two virus concentrations used in this study.

Both fathead minnow and spotfin shiner infected with MUS-WL FHMNV exhibited similar clinical signs and histopathological alteration in hematopoietic tissues indicating that the pathogenic mechanism employed by FHMNV are identical for both species. These findings were also consistent with what has been observed in both experimentally and spontaneously FHMNV infected muskellunge (Chapters 2&3). While golden shiner experimentally infected with the MUS-WL FHMNV by intraperitoneal injection exhibited identical clinical signs and histopathology, infection by immersion failed indicating that FHMNV is unable to overcome the golden shiner's intact body natural barriers.

Creek chub used in this study seem to be resistant to infection. This can be explained in a number ways. First, we were only able to obtain wild caught creek chub, therefore, there is a possibility these fish had already been exposed to FHMNV in their natural habitat prior to their capture. If that is the case, they would have already built up an immune response to the virus. Second, creek chub may be not susceptible to the muskellunge-adapted FHMNV. Last, the infectious dose used in this study were not enough to establish an infection. FHMNV was previously detected in one lot of creek chub during a routine disease screening of baitfish dealers in WI (McCann 2012), which suggests the potential for susceptibility. FHMNV does not appear to cause risks to piscivorous species tested (largemouth bass, walleye, and rainbow trout). All of these species tested negative for any traces of FHMNV both by qLAMP and cell culture. One potential reason for this is that these piscivorous species have an innate resistance to FHMNV.

There are a variety of different molecules necessary for nidoviruses to attach to host cells (Siddell & Snijder 2008), so it is entirely possible host cells of these species do not have the necessary receptors for FHMNV attachment. While walleye, rainbow trout and largemouth bass seem to be safe from FHMNV infection, this pathogen is still a significant problem for muskellunge which support a large sport fishing industry in the state of Michigan (chapters 2&3).

FHMNV's ability to infect multiple indigenous cyprinids is particularly alarming for several reasons. These species are very ecologically important as a food source for larger piscivorous species. Furthermore, fathead minnow, golden shiner and spotfin shiner are critical components of the baitfish industry which is of high economic importance. An FHMNV disease outbreak could cause serious monetary losses, due to its high pathogenicity to various cyprinids. Most of the fish used in this industry are cultured on baitfish farms, however, approximately 20% are still caught from the wild (Goodwin et al, 2011). Oftentimes, baitfish are shipped across state lines and then distributed either by retail or wholesale networks (Goodwin et al, 2011). This mixing and spreading of various baitfish species in combination with lack of disease screening requirements create ideal conditions for a virus to spread. In addition, many sportfishermen are unaware that of dumping their bait buckets at their various fishing destinations (Litvak & Mandrak, 2011) may contribute to the spread of a pathogenic virus such as the FHMNV.

The Great Lakes region in particular is a major importer of baitfish species to be used both for recreational fishing and as a forage source in rehabilitation programs for native piscivorous species. Currently, baitfish are not required to be certified free of FHMNV prior to their introduction to the Great Lakes watershed (MDNR). The results of this study warrant more intensive disease screening requirements for baitfish, as well as increased education and

regulation regarding baitfish use. An outbreak of FHMNV in the Great Lakes could have devastating impacts on native cyprinid populations, as well as on wild and hatchery raised muskellunge. FHMNV poses serious risks to Great Lakes native cyprinids, the baitfish industry, as well as muskellunge rehabilitation programs. Research needs to be completed to determine if and to what extent FHMNV has spread throughout the Great Lakes basin in order to develop effective managerial decisions

APPENDIX

Figure 3-1: Mortality curve for fathead minnow (n=10 per treatment) infected by IP-injection with 6 doses ranging from 6-6.3 x 10^4 $TCID_{50}$/ml of fathead minnow nidovirus (FHMNV) over a 60 day study period. The IP-LD_{50} for FHMNV infection in fathead minnow was calculated to be 6.3 x 10^1 $TCID_{50}$/ml.

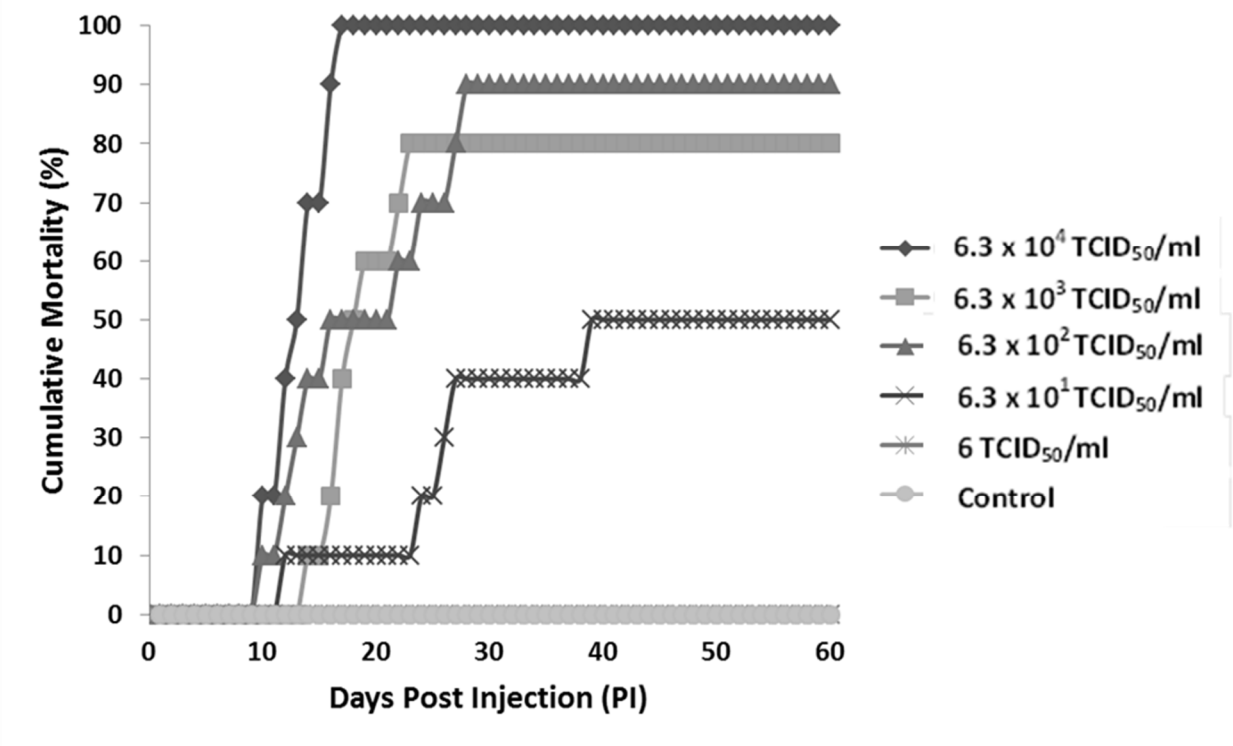


Figure 3-2: FHMNV infected fathead minnow exhibiting severe diffused hemorrhage throughout right pectoral fin (left) and lesion 1-5mm in diameter on right flank containing moderate diffused hemorrhage (right).

Figure 3-3: FHMNV infected spotfin shiner exhibiting moderate echymotic hemorrhage on the isthmus (left) and severe multifocal echymotic hemorrhage along the spine just caudal to the dorsal fin (right).

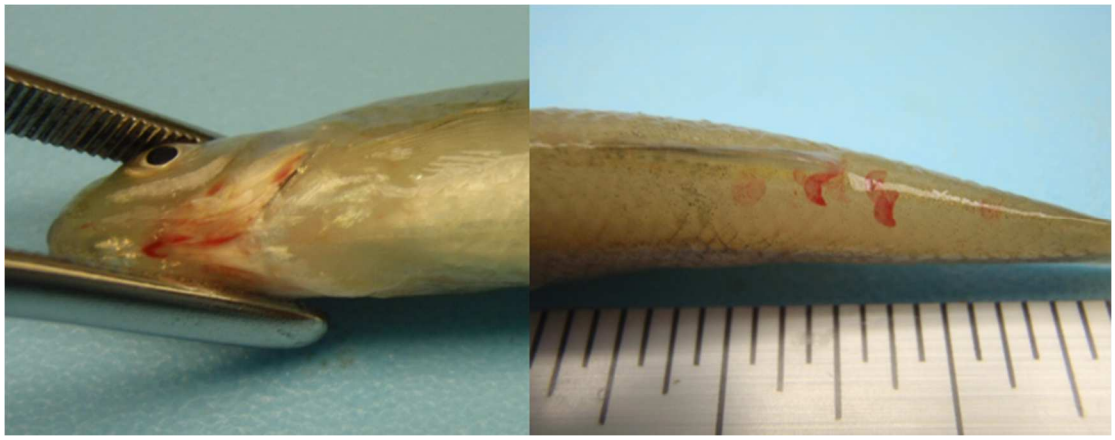


Figure 3-4: FHMNV infected golden shiner exhibiting severe diffuse hemorrhage just posterior to the brain (left) and severe multifocal petechial hemorrhages along the spine just below the dorsal fin on the left side (right).

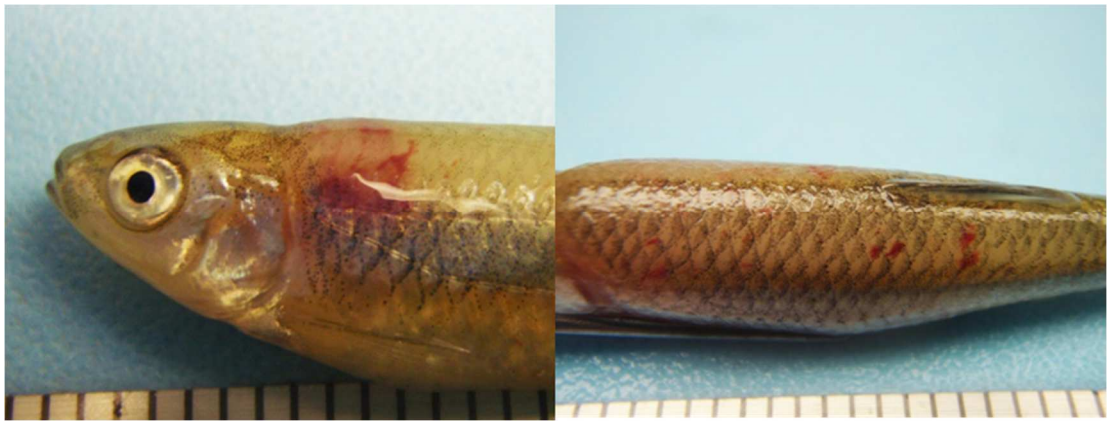


Figure 3-5: Mortality curves for experimentally challenged fish species that were susceptible to FHMNV infection by IP-injection. Each species was infected with 100µl of a low (630 $TCID_{50}$/ml), high (6.3 x 10^4 $TCID_{50}$/ml) and control dose (sterile media). The n=10 for each treatment group. A) Fathead Minnow. B) Spotfin Shiner. C) Golden Shiner.

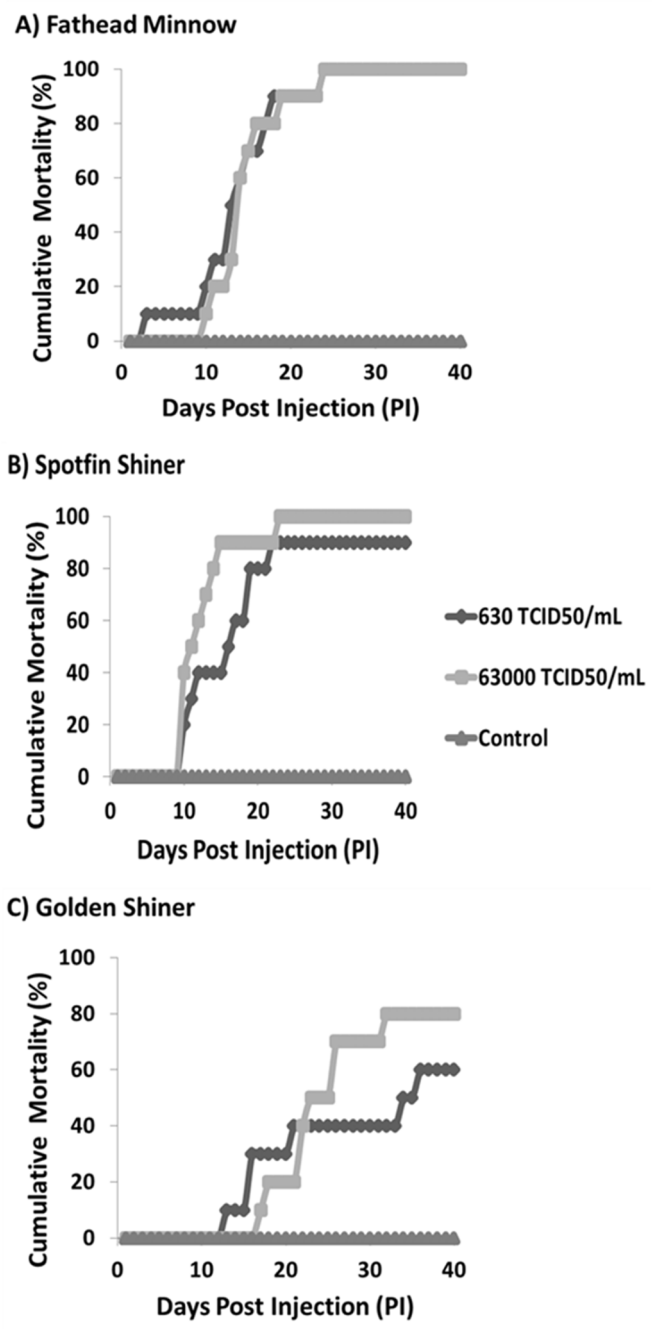


Figure 3-6: Cumulative percent mortality for spotfin shiner infected with low (3160 $TCID_{50}$/ml), high (8000 $TCID_{50}$/ml), and control (sterile media) doses of FHMNV by immersion. Infected fish from the high dose group began experiencing mortalities at 11 days PI, and by 27 days PI both groups of infected fish had reached 60% mortality.

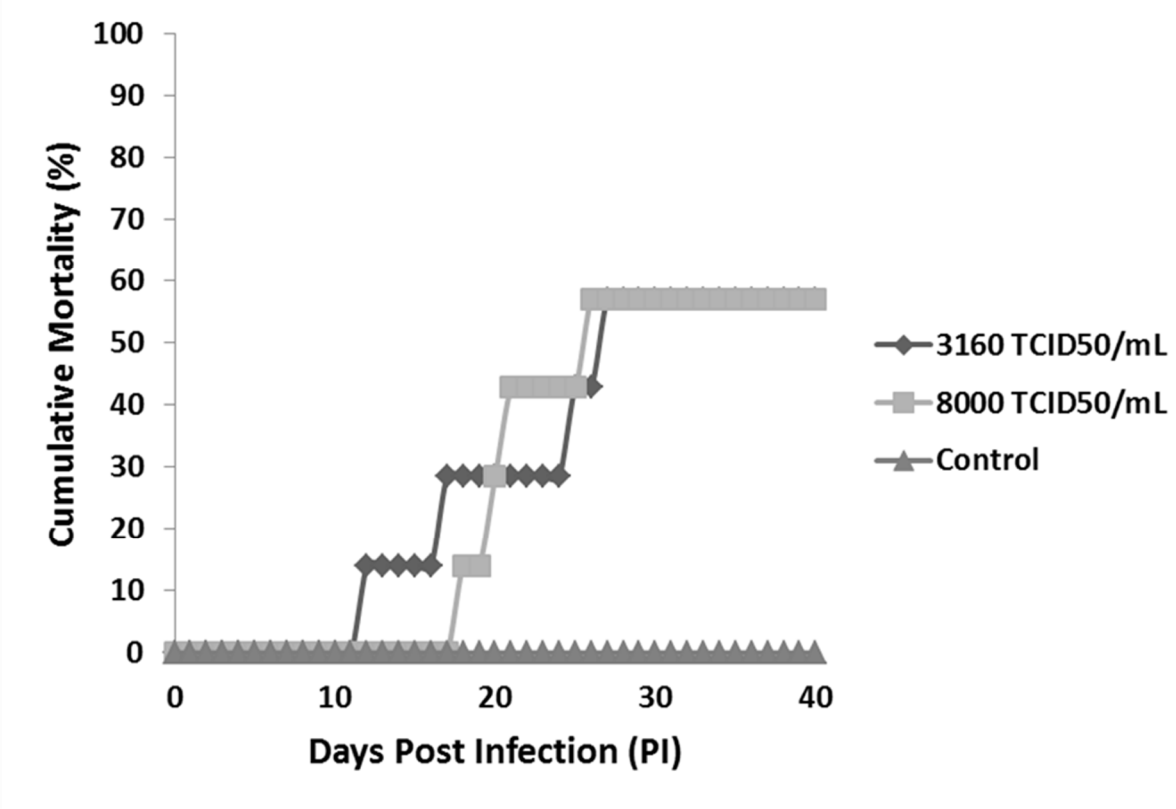


Figure 3-7: Hematoxylin and eosin (H & E) stained tissue sections from fathead minnows infected with FHMNV by IP-injection, and spotfin shiner experimentally infected with FHMNV by immersion. A) Posterior kidney of fathead minnow showing multi focal tubular necrosis and cell disorganization. B) Spleen of fathead minnow showing multi-focal areas of necrosis. C) Liver of spotfin shiner exhibiting multifocal hepatocellular necrosis. D) Diffuse necrosis of the interstitial tissue in the anterior kidney in spotfin shiner, evidenced by widespread nuclear fragmentation. 40X

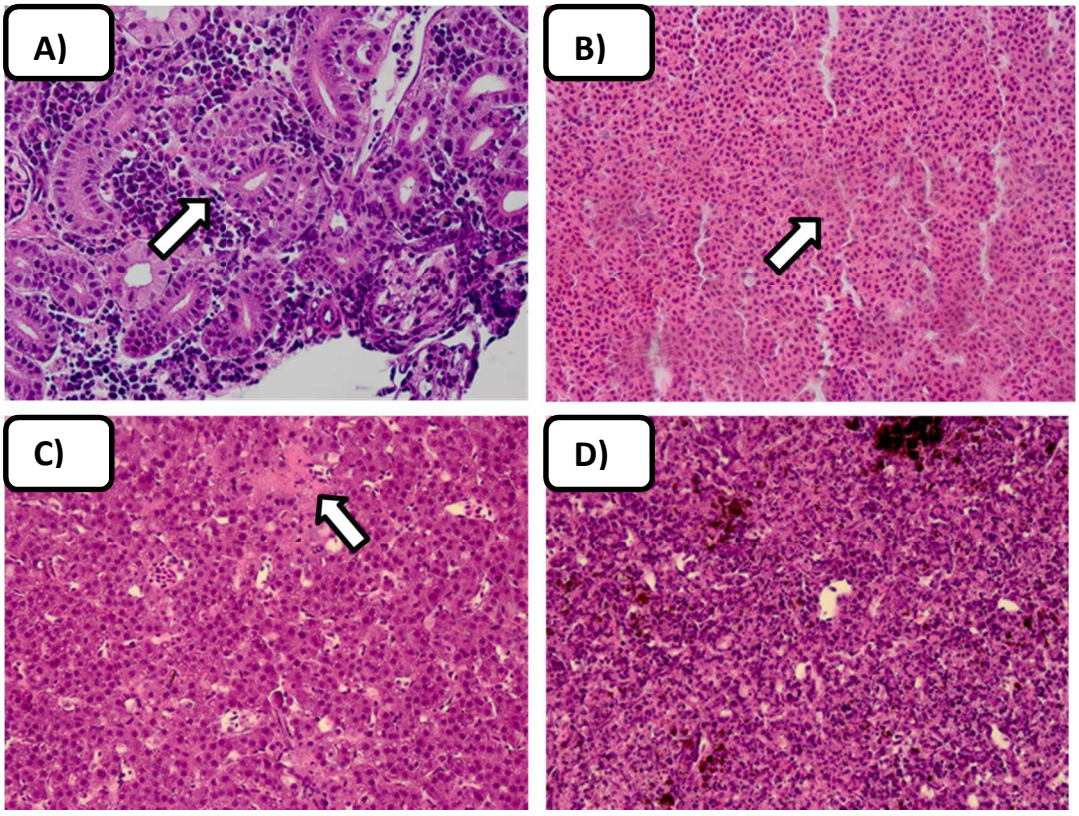
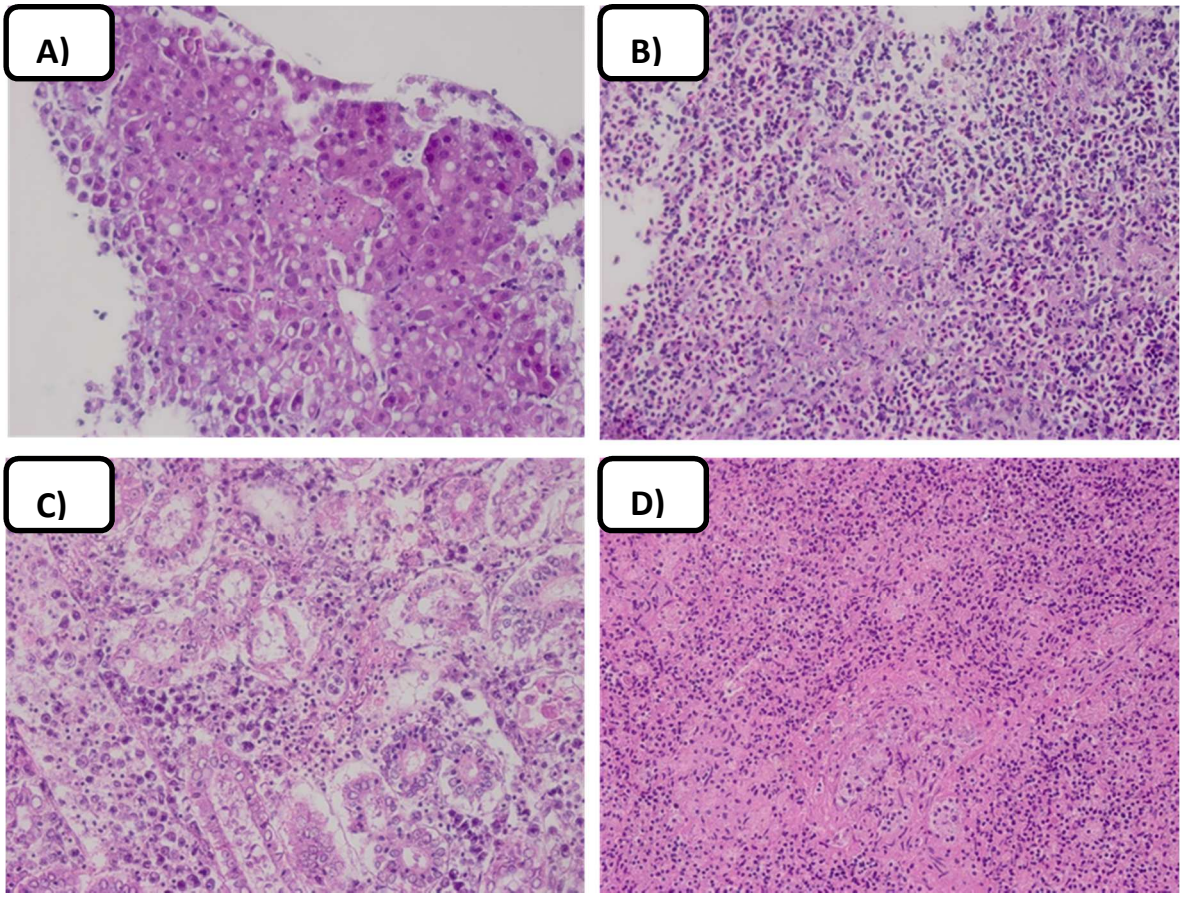


Figure 3-8: Hematoxylin and eosin (H & E) stained tissue sections from spotfin shiners and golden shiners infected with FHMNV by IP-injection A) liver of spotfin shiner exhibiting multifocal hepatocellular necrosis. B) Spleen of spotfin shiner exhibiting multifocal areas of necrosis. C) Posterior kidney of golden shiner exhibiting widespread renal tubule degeneration and necrosis of the interstitial tissue. D) Spleen of golden shiner exhibiting areas of multifocal necrosis. 40X



CONCLUSIONS

The results of these studies have shed light on the host range of the fathead minnow nidovirus (FHMNV), as well as its pathogenicity to muskellunge. In addition, data of this study demonstrates that this emerging coronavirus poses considerable risks to native cyprinids (particularly the fathead minnow and spotfin shiner) and potentially the golden shiner. It is also clear that infected baitfish and lack of disease certification requirements could have devastating impacts in regards to muskellunge rehabilitation efforts, especially due to these efforts dependence on baitfish as a food source; however, there are still many other questions which must be answered in order to assist fishery managers with the prevention and control of this dangerous pathogen.

While FHMNV has been isolated from multiple Midwestern states, including from muskellunge hatcheries in both Michigan and Wisconsin, there have been little if any studies to ascertain the geographic range of this pathogen. In order to protect the Great Lakes watershed and its resident fish populations, fish health surveys especially of baitfish dealers throughout the state of Michigan should be completed to assess the prevalence of FHMNV. In addition, stricter health certification regulations must be put into place in order to prevent the importation of FHMNV infected baitfish.

Additional viral shedding studies should also be completed with muskellunge and FHMNV in order to determine how long this species are capable of shedding the virus into the surrounding water. The viral shedding study completed for this thesis found that infected fish are capable of shedding virus up to 12 days post infection, and up to levels of 10,000 $TCID_{50}$/ml; however, the point at which infected fish cease to shed FHMNV was never determined. Even if fish appear to be healthy, they could likely be shedding high levels of FHMNV that could serve

as a source of infection for naïve fish either within a hatchery setting or in the wild. If FHMNV were to by chance enter a muskellunge hatchery, this data would be particularly useful in determining when these infected fish may be safe to stock in various rivers and lakes throughout the state.

Moreover, it is essential to better understand the immune response of recovered fish to FHMNV infection. While necrosis of the hematopoietic tissues and frequent infections of *Saprolegnia* to FHMNV experimentally infected fish provided some evidence for immunosuppression, the mechanism of this immunosuppression needs to be unraveled. Additionally, there is a need for the development of diagnostic assays such as an enzyme-linked immunosorbent assay (ELISA) for the detection of FHMNV antibodies which can be particularly advantageous in determining if this virus has spread throughout the Great Lakes basin. Serological assays would allow the indirect detection of FMNV through the presence of specific antibodies, whose presence is long lasting. While loop-mediated isothermal amplification (LAMP) is highly sensitive and able to detect low levels of the virus in infected fish tissues, this assay would be ineffective in determining if fish have been exposed to the virus and cleared the infection. An ELISA assay would not only be beneficial for detecting exposure to the virus, but would also allow for non-lethal sampling of these fish because antibodies are detectable in the serum. Non-lethal sampling is preferred, especially in the case of muskellunge which are such an ecologically and economically valuable piscivorous species.

In conclusion, while these results have given us far more information regarding the host range and pathogenicity of the newly emerging FHMNV, there is still much that must be learned in order to effectively protect native Great Lakes fish species and manage this disease.

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