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Isolation of the Fathead Minnow Nidovirus from Muskellunge Experiencing Lingering Mortality

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Abstract

In 2011, the Fathead Minnow nidovirus (FHMNV; Genus *Bafinivirus*, Family *Coronaviridae*, Order *Nidovirales*) was isolated from pond-raised juvenile Muskellunge *Esox masquinongy* suffering from lingering mortality at the

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Wild Rose Hatchery in Wild Rose, Wisconsin. Moribund Muskellunge exhibited tubular necrosis in the kidneys as well as multifocal coalescing necrotizing hepatitis. The FHMNV was also isolated from apparently healthy juvenile Muskellunge at the Wolf Lake State Fish Hatchery in Mattawan, Michigan. The identity of the two syncytia-forming viruses (designated MUS-WR and MUS-WL from Wild Rose Hatchery and Wolf Lake State Fish Hatchery, respectively) 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 naive juvenile Muskellunge through intraperitoneal injection with two viral concentrations (63 and 6.3×10^3 TCID₅₀/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 revealed differences between the MUS-WL FHMNV, MUS-WR FHMNV, and two other FHMNV originally isolated from moribund Fathead Minnows *Pimephales promelas* including the index FHMNV strain (GU002364). Based on a partial helicase gene sequence, a reverse transcriptase PCR assay was developed that is specific to FHMNV. These results give evidence that the risks posed to Muskellunge by FHMNV should be taken seriously.

The Muskellunge *Esox masquinongy* is one of the most important freshwater fishes in North America from the ecologic and economic points of view. 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 are raised to the fingerling or yearling stage in state fish hatcheries before stocking in major inland lakes and rivers. During the rearing phase in hatcheries, Muskellunge are fed first *Artemia* and then pelleted food, followed by live baitfish, predominantly Fathead Minnow *Pimephales promelas* (Wahl 1999). This widely used practice has raised major concerns regarding the pathogens that wild baitfish may introduce to captive Muskellunge fingerlings. This is particularly alarming since fish health inspection of baitfish that includes virus isolation is not a common practice (Goodwin et al. 2004).

In the past few years, a number of microbial pathogens and parasites have been identified in baitfish (Goodwin et al. 2010; Phelps et al. 2014). One such pathogen is the Fathead Minnow nidovirus (FHMNV), a bacilliform, positive-sense RNA virus that was isolated from moribund Fathead Minnows exhibiting external hemorrhages in a baitfish facility in Arkansas (Iwanowicz and Goodwin 2002). The FHMNV was originally believed to be a rhabdovirus (Iwanowicz and Goodwin 2002), but later genetic analysis identified FHMNV as a member of the Genus *Bafinivirus*, Family *Coronaviridae*, Order *Nidovirales* (Batts et al. 2012). The FHMNV genome (27,291 nucleotides, GenBank accession: GU002364) contains four major open-reading frames encoding polyprotein, spike glycoprotein, membrane, and nucleocapsid proteins (Batts et al. 2012). While FHMNV has been originally described as a pathogen of the Fathead Minnow, its pathogenicity to other fish species, especially piscivorous fish, has never been ascertained.

In 2011, an episode of mortality among cultured juvenile Muskellunge was reported from Wild Rose Hatchery, Wisconsin, (WRH) about 1 month after their diet was

switched from pellets to live Fathead Minnows. The FHMNV was isolated from these moribund Muskellunge. Shortly thereafter, FHMNV was also isolated from apparently healthy juvenile Muskellunge raised at Wolf Lake State Fish Hatchery, Michigan, (WLSFH). Herein, we report on the isolation of two Muskellunge FHMNV strains (designated MUS-WR and MUS-WL from Wild Rose Hatchery and Wolf Lake State Fish Hatchery, respectively), describe a new reverse transcriptase PCR (RT-PCR) assay for the specific detection of the FHMNV, and discuss the genetic relationship between Muskellunge isolates and the FHMNV index strain. Moreover, River's postulates were ascertained using the MUS-WL FHMNV strain in naive Muskellunge.

METHODS

Clinical and Laboratory Examination and Virus Isolation and Identification

Fish and clinical examination.—Muskellunge fingerlings were reared intensively in raceways supplied by 10°C well water and were fed commercial pellets (Reed Mariculture, Campbell, California; Bio Oregon, Longview, Washington) at WLSFH. In late August 2011, these fish of approximately 11.5 cm TL were transferred to WRH, where 800 of them were immediately stocked into a 2,023-m², lined, outdoor pond, while another 1,000 Muskellunge were stocked into a 4,047-m², lined, outdoor pond. The ponds were supplied with 21°C well water that had been first degassed and oxygenated, then passed through trout-rearing raceways and treated with ultraviolet (UV) light before filling the ponds. These ponds were prestocked with 726 kg (1,600 lb) of Fathead Minnows (TL < 4 cm) that had been reared intensively on pellet feed at a commercial baitfish facility and, according to the Wisconsin Department of Agriculture, Trade and Consumer Protection requirements, were health certified, which included visual examination for disease signs and laboratory testing for the viral hemorrhagic

septicemia virus (*Novirhabdovirus*, *Rhabdoviridae*). Within 3 weeks of arrival at WRH, the Muskellunge became sick, and some suffered morbidity and mortality. Initial low-level mortality rates increased to 10–20 fish (0.5–1%) per day within 60 d. In early October, a sample of eight moribund Muskellunge with a mean TL of 15.5 cm (SD, 0.64) and mean weight of 14.5 g (SD, 2.0) from WRH was submitted for analysis to the Aquatic Animal Health Laboratory at Michigan State University (MSU-AAHL) to identify the cause of mortality. The fish exhibited pale gills and external petechial hemorrhages and were individually subjected to clinical and laboratory health assessments according to the guidelines outlined by the American Fisheries Society's Fish Health Section (AFS-FHS 2012, 2014). In late November of the same year, 60 Muskellunge with a mean TL of 19.5 cm (SD, 1.27) and mean weight of 28.9 g (SD, 6.84) from the originating stock at WLSFH were collected after being fed on commercial minnows for 7 weeks and were analyzed in pools of five fish at MSU-AAHL for the presence of pathogens. These fish had not been experiencing elevated mortality and were apparently healthy. The two hatcheries obtained their minnows from different baitfish collection facilities.

Fish were examined for the presence of lesions or external parasites. Skin and gill smears were made and examined microscopically for the presence of ectoparasites. Fish were grossly examined under aseptic conditions, and the abdominal cavity and internal organs were 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 Fisheries Society's Blue Book (AFS-FHS 2012, 2014). For virus testing, kidneys, spleen, and heart were aseptically collected in Whirl-Pak plastic bags.

Tissue processing for viral isolation.—Tissue samples were diluted with Earle's salt-based minimum essential medium (MEM; Invitrogen Life Technologies, Carlsbad, California), supplemented with 12 mM tris buffer (Sigma-Aldrich, St. Louis, Missouri), penicillin (100 IU/mL; Invitrogen), streptomycin (0.1 mg/mL; Invitrogen), and amphotericin B (2.5 µg/mL; Invitrogen) to produce a 1:4 dilution (w/v) of original tissues. Kidney, spleen, and heart samples were then homogenized using a Biomaster Stomacher (Wolf Laboratories, York, UK) at the high speed setting for 2 min. Following homogenization, samples were centrifuged at $2,000 \times g$ for 30 min, 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, Logan, Utah) and L-glutamine (29.2 mg/mL; Invitrogen). Infected plates were incubated at 15°C for up to 21 d and observed for the formation of cytopathic effects (CPE).

Cell culture and virus propagation.—Stocks of EPC cell line were grown and maintained in 150-cm² tissue culture flasks (Corning, Corning, New York) at 25°C using MEM supplemented with L-glutamine (29.2 mg/mL), penicillin (100 IU/mL), streptomycin (0.1 mg/mL), 10% fetal bovine serum, and sodium bicarbonate (7.5% w/v; Sigma). Virus stocks were produced in EPC cells and harvested when CPE predominated the cell sheet, then the supernatant was aliquoted and stored at –80°C until used. To determine the virus concentration, the median tissue culture infectious dose (TCID₅₀) assay was performed on EPC cells and calculated as described by Reed and Muench (1938).

Virus identification and development of FHMNV-specific RT-PCR.—Samples inoculated onto EPC cells that exhibited CPE were subjected to further identification using RT-PCR. Total RNA was extracted from fish tissue using the Qiagen RNeasy mini kit (Qiagen, Valencia, California). Viral RNA was extracted from infected EPC cells using a QIAamp viral RNA kit (Qiagen) following the manufacturer's protocol. Total complementary DNA (cDNA) was synthesized by the Reverse Transcription System (Promega, Madison, Wisconsin) using a random hexamer primer, and then used as a template for PCR amplification.

Several RT-PCR assay protocols were used in this study to determine the identity of the isolated viruses. Since isolated viruses produced giant cell formation (syncytia) in the EPC cell line, our efforts were directed to aquareoviruses and nidoviruses of nonsalmonids known to cause this kind of CPE. To test for aquareoviruses, the RT-PCR assay developed by Seng et al. (2004) for the rapid and accurate detection of a variety of aquareovirus strains isolated from different host species and origin was employed in this study. For the identification of FHMNV, two primer sets were used. The first is an RT-PCR assay that specifically targets a region of the spike glycoprotein gene of FHMNV developed by Batts et al. (2012). Additionally, since the helicase genes of nidoviruses contain a number of conserved domains (Lehmann et al. 2015), an RT-PCR was developed and used to specifically target a region of the helicase gene of the FHMNV and allow for greater phylogenetic characterization of FHMNV isolates. The RT-PCR assays for the detection of FHMNV were performed using the primers listed in Table 1. Each 25 µL of the PCR mixture consisted of 12.5 µL of 2× Green GoTaq Master Mix (Promega), 1.0 µM of each primer, and 2.5 µL of template cDNA. The RT-PCR conditions for all assays were as follows: initial 94.0°C for 4 min, followed by 35 cycles of 94.0°C for 30 s, 50.0°C for 30 s, and 72.0°C for 1 min, and a final extension of 72.0°C for 7 min.

The cDNA for all assays was synthesized by the Reverse Transcription System (Promega) using random primers according to the manufacturer's instruction. The PCR mixture consisted of 12.5 µL of 2× Green GoTaq Master Mix (Promega), 1.0 µM of each primer, and 2.5 µL of template cDNA. A negative control containing no DNA was included in

TABLE 1. List of RT-PCR primers used in this study. Isolates recovered from Muskellunge (MUS) were obtained from fish reared at Wolf Lake State Fish Hatchery, Michigan, and Wild Rose Hatchery, Wisconsin, and are designated MUS-WL and MUS-WR, respectively. Position denotes the nucleotide position in the Fathead Minnow nidovirus (FHMNV) reference genome (GenBank accession: GU002364). A plus (+) symbol denotes an isolate tested positive using a particular RT-PCR primer set; a minus (–) symbol denotes an isolate tested negative using a particular RT-PCR primer set. FHMNV = Fathead Minnow nidovirus, WBV = White Bream virus, HCoV = Human coronavirus, CavV = Cavally virus.

Target taxon	Primer	Target gene	Length (bp)	Position	MUS-WL	MUS-WR	FHMNV-181	WBV	HCovV	CavV	Reference
FHMNV	Forward: 5'-TTTTGTTGA ATTATAGCTCTT-3' Reverse: 5'-TGGCCATAT CCTTAAGGG-3'	Spike glycoprotein	278	24713–24990	+	+	+	–	–	–	Batts et al. (2012)
FHMNV	Forward: 5'-CGAATTCGG CGTATCATAC-3' Reverse: 5'-GTACAGTGTA TTGATGTTGTGG-3'	Helicase	1,000	18543–19542	+	+	+	–	–	–	This study

each set of PCR mixtures. Amplified RT-PCR products were visualized with agarose gel electrophoresis and bidirectionally sequenced. Sanger chromatograms produced for amplified sequences were analyzed with BioEdit version 7.2.5 (1997–2013, Tom Hall) to confirm base calling. The amplified sequences for each assay were deposited in GenBank (accession numbers pending).

To determine the specificity of the developed FHMNV-helicase gene assay, the following viruses were included in the analysis: White Bream virus (WBV; *Bafinivirus*, *Coronaviridae*) grown in EPC cell line, human coronavirus (HCoV-229E, *Alphacoronavirus*, *Coronaviridae*) grown in HuH-7 cell line, Cavally virus (CavV, *Alphamesonivirus*, *Mesoniviridae*) grown in C6/36 cell line, and FHMNV-181 isolated from Fathead Minnow and grown in EPC cell line. These viruses were selected because WBV is the most closely related virus to FHMNV and the only other known virus within the genus *Bafinivirus* (Batts et al. 2012). The other selected viruses represent more distantly related nidoviruses (Zirkel et al. 2011). Corey Puzach at the La Crosse Fish Health Laboratory, U.S. Fish and Wildlife Service, Onalaska, Wisconsin, kindly provided the FHMNV-181 (originally isolated from Fathead Minnow) and John Ziebuhr from the Institute for Medical Virology at Justus Liebig University Giessen, Giessen, Germany, kindly provided the RNA for HCoV-229E, CavV, and WBV. Total RNA from FHMNV strains 181, MUS-WL, and MUS-WR was isolated as described above.

Sequence and phylogenetic analyses.—All sequences mentioned above (both helicase and spike glycoprotein genes) were included in the data set for phylogenetic analysis. The nucleotide sequences of each gene were aligned independently with ClustalW as implemented in MEGA 5.0 (Tamura et al. 2011) using default settings and then concatenated. The length of the final alignment with gaps after trimming primer sequences was 1,278 bp and corresponded to nucleotide positions 18,551–19,533 (983 bp) and 17,776–18,767 (992 bp) for FHMNV (GU002364) and WBV (DQ898157) helicase genes, respectively, and nucleotide positions 24,714–24,989 (276 bp), 24,045–24,326 (282bp), for FHMNV (GU002364) and WBV (DQ898157) spike glycoproteins, respectively.

Prior to phylogenetic analysis, the program jModelTest (Darriba et al. 2012) was used to select the best-fitting substitution model according to the corrected Akaike information criterion (AIC_c) (Hurvich and Tsai 1993); the model with the lowest AIC_c value was identified as the best model in terms of fit and parsimony (Burnham and Anderson 2002). A total of 24 candidate models, including models with equal or unequal base frequencies, with or without a proportion of invariable sites (+I), and with or without rate variation among sites (+G) were tested. The best-fit model of nucleotide substitution was using the general time reversible (GTR) model (Rodriguez et al. 1990). Tree topologies were inferred with a Bayesian

approach using MRBAYES version 3.2.4 (Ronquist and Huelsenbeck 2003) with the selected model of nucleotide substitution. Bayesian analysis included four Markov chain–Monte Carlos (MCMC) for 4,000,000 generations, and trees were sampled every 100th generation. After discarding the first 25% of samples as burn-in samples, the remaining data were used to generate a 50% majority-rule consensus tree. The WBV (DQ898157) was used to root the tree.

Challenging Naive Muskellunge with the FHMNV Wolf Lake Isolate

Fish and maintenance.—Disease-free, raceway-raised, juvenile Muskellunge (approximately 6 months posthatch; mean TL, 13.99 cm [SD, 0.81]; mean weight, 8.92 g [SD, 1.84]) were obtained from the Chautauqua Hatchery, Mayville, New York, where they were intensely reared at a water temperature of approximately 17°C. The Muskellunge were certified as disease-free based on the guidelines of the American Fisheries Society's Fish Health Section (AFS–FHS 2012, 2014). Prior to use in experimental infection, a random subsample of five fish were euthanized using an overdose (25 mg/mL) of tricaine methanesulfonate (MS-222; Western Chemical, Ferndale, Washington) and tested for the presence of pathogens including the FHMNV, as described above. Muskellunge were fed certified, disease-free, Fathead Minnows (mean TL, 5.92 cm [SD, 0.93]; mean weight, 2.65 g [SD, 1.08]) by allowing the two species to cohabitate. The minnows were certified to be free of the viral hemorrhagic septicemia virus, infectious pancreatic necrosis virus, spring viremia of carp virus, *Aeromonas salmonicida*, *Yersinia ruckeri*, and *Heterosporis* sp. Thirty experimental fish were allowed to acclimate to laboratory conditions for 3 weeks, during which time the temperature was lowered to $11 \pm 1^\circ\text{C}$ from 16°C by gradually increasing the ratio of chilled water added to the tanks. 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, Michigan, in accordance with the Institutional Animal Care and Use Committee (IACUC) guidelines (AUF approval: 09/10-140-00).

Experimental infection.—To fulfill River's postulates, naive Muskellunge were challenged with the MUS-WL FHMNV strain using the intraperitoneal (i.p.) injection route to ensure all fish were infected with a known dose of the virus. Three groups of fish ($n = 10$ each) were used in this experiment. The first group received 100 μL of 630 TCID₅₀/mL (low dose group); the second group received 100 μL of 6.3×10^4 TCID₅₀/mL (high dose group); the third group received 100 μL of sterile MEM (control group). All fish were monitored daily for an observation period of 30 d postinfection (PI). The tank outflow was united into a common tract entering an UV-light sterilization unit (Aquafine, Valencia, California) prior to discharge. Tanks were monitored every 8 to 12 h for the collection of moribund or dead fish.

Virus reisolation.—Kidneys, spleen, and heart were collected from each dead fish immediately following their death. Samples were also collected from moribund fish exhibiting lack of normal swimming behavior (e.g., loss of buoyancy regulation, floating, spinning, lethargy, absence of escape reflex) along with severe petechial, focal, and generalized external hemorrhaging and marked exophthalmia. Tissue samples were divided to test for the presence of virus by both the FHMNV-specific RT-PCR developed by Batts et al. (2012) and by cell culture as described previously.

Histopathological analyses.—To determine tissue alterations associated with FHMNV infections, portions of the liver, spleen, and kidney of naturally infected Muskellunge at WRH, as well as those from experimentally infected fish, were collected (moribund and dead fish). Samples were preserved in 10% buffered formalin, dehydrated in a graded series of alcohols, embedded in paraffin, cut into 5- μ m-thick serial sections, and stained with Mayer's hematoxylin and eosin (H&E) as detailed in Prophet et al. (1992).

RESULTS

Clinical and Laboratory Examination

Moribund Muskellunge from WRSFH exhibited gill pallor and multifocal petechial hemorrhages on the skin and eyes. Internally, Muskellunge livers appeared mottled, and both livers and posterior kidneys exhibited a firm consistency. One of the fish harbored larval nematodes (*Contracaecum* sp.). Motile aeromonads were isolated from seven out of eight fish. Muskellunge sampled from WLSFH appeared normal externally, aside from a few individuals (12%) with mildly congested fins. Upon necropsy, these fish exhibited liver pallor, and almost half (27 of 60) harbored larval nematodes (*Contracaecum* sp.).

Viral Isolation and Identification

Following inoculation of EPC cell line, seven out of eight WRSFH fish tissue samples resulted in syncytia formation within 7 d of inoculation (Figure 1). A syncytia was also observed in 3 out of 12 pools of tissues collected from WLSFH Muskellunge (five fish per pool). To identify the syncytia-forming agent, a series of assays was performed. All isolates tested negative for aquaviruses using the primer set developed by Seng et al. (2004). In contrast, the isolates tested positive using both the FHMNV-specific primer targeting the FHMNV spike glycoprotein gene of Batts et al. (2012), which yielded a 278-bp amplicon, and the FHMNV-specific primers targeting the FHMNV helicase gene developed in this study, which yielded amplicons measuring 1,000 bp. The specificity of the RT-PCR assays using the newly developed primer set targeting the helicase gene was confirmed as it amplified MUS-WL isolates, MUS-WR isolates, and FHMNV-181, but not the other coronaviruses (WBV, HCoV-229E, and CavV). The same

result was observed using the RT-PCR of Batts et al. (2012). These findings demonstrated that the viruses isolated from both Muskellunge cases are FHMNV.

Sequence and phylogenetic analyses of Muskellunge FHMNV isolates.—Comparison of amplified gene sequences of the two Muskellunge FHMNV isolates (MUS-WL and MUS-WR) showed that the spike glycoprotein gene sequences were identical (100%) and the helicase gene sequences were nearly identical (99.7%). When the amplified sequences of MUS-WL and MUS-WR were compared with those isolated from Fathead Minnows (FHMNV-181 and GU002364), similarities of 99.6% were observed for the spike glycoprotein gene, and similarities ranging from 99.4% (MUS-WL compared with FHMNV-181) to 100% (MUS-WL compared with the FHMNV index strain GU002364) were observed for the helicase gene sequences. Pairwise analysis of nucleotide substitutions in the spike glycoprotein gene sequences did not result in different amino acid sequences among all isolates. However, multiple differences in amino acid sequences were observed for helicase gene sequences (Table 2).

Due to the high level of sequence similarity observed for both the helicase and spike glycoprotein genes, an additional phylogenetic analysis based on concatenated helicase-spike glycoprotein gene sequences was performed. As depicted in Figure 2, the two Fathead Minnow sequences clustered together, while the MUS-WR sequence appeared as a paraphyletic taxon, albeit with a low level of node support (58%). Interestingly, the posterior probabilities of branching points based on Bayesian inference indicated that the MUS-WL is different from the other FHMNV isolates, as evidenced by the high degree of node support (100%).

Results of Challenging Muskellunge with FHMNV

Prior to infection, an analysis of a subsample of experimental fish proved they were free from viruses (on EPC cell line), bacteria, or parasites. Juvenile Muskellunge experimentally infected with MUS-WL showed no morbidity or mortality for the first 2 weeks PI. By day 16 PI fish started to die (Figure 3). By the end of the 30-d study period, all Muskellunge died at both low and high doses. All infected fish exhibited severe gill pallor, with several individuals (15–35%) also exhibiting petechial hemorrhages throughout the fins, eyes, and skin (Figure 4). Upon necropsy of dead and moribund fish, several individuals tended to have enlarged spleens (20%) and gall-bladders (30%), as well as liver pallor (60%).

The FHMNV was reisolated from all experimentally infected Muskellunge tissue samples. All EPC cell sheets were dominated by syncytia as early as 14 d PI. No CPE was observed in wells containing negative control Muskellunge tissues. Wells exhibiting CPE tested positive for FHMNV using the RT-PCR assay of Batts et al. (2012).

Histopathology.—Histopathology was performed on Muskellunge from the WRSFH diagnostic case and the

FIGURE 1. (A) Noninfected EPC cell monolayer. (B) EPC cells infected with the Fathead Minnow nidovirus MUS-WL strain; notice syncytia formation in (B). Isolates recovered from Muskellunge (MUS) were obtained from fish reared at Wolf Lake State Fish Hatchery, Michigan, and designated MUS-WL.

experimental infections. Muskellunge revealed similar tissue changes. Naturally infected Muskellunge from WRH exhibited mild to severe, acute, multifocal and locally extensive, often coalescing necrotizing hepatitis (Figure 5a, b); the kidney exhibited moderate, acute, multifocal, tubular necrosis (Figure 5c); while the spleen appeared normal with a slightly increased mitotic rate.

In experimentally infected fish, similar necrotic changes in hematopoietic tissues were observed. Coalescing to diffuse necrosis and edema within the stroma of the spleen was observed (Figure 6a), as was diffuse necrosis of the interstitium and edema in the anterior kidney (Figure 6b). Likewise, multifocal, renal, tubular necrosis and nephritis, along with necrosis of the interstitium, were noted in the posterior kidney (Figure 6c).

DISCUSSION

Syncytia formation, positive RT-PCR amplification using FHMNV-specific primers, and phylogenetic analysis demonstrated that the isolated viruses from both Muskellunge cases are indeed strains of FHMNV. In the case of WRSFH fish, this is the first time that FHMNV has been isolated from Muskellunge exhibiting clinical signs and experiencing mortality. Upon experimental infection, both low and high doses of the MUS-WL FHMNV strain caused morbidity, 100% mortality, and histopathological changes in tissues of all exposed fish, thereby fulfilling River's postulate that FHMNV is pathogenic to Muskellunge. This finding underscores the importance of determining the host range of this emerging virus and the potential risk it may pose to the Muskellunge conservation and enhancement programs in multiple U.S. states and Canada.

TABLE 2. Pairwise comparisons of nucleotide and amino acid sequences for helicase and spike glycoprotein genes among Fathead Minnow nidovirus isolates obtained from fish reared at Wolf Lake State Fish Hatchery, Michigan, and Wild Rose Hatchery, Wisconsin, and designated MUS-WL and MUS-WR, respectively. GU002364 refers to the FHMNV (Fathead Minnow nidovirus) index strain.

Isolate	Nucleotide			Amino acid		
	MUS-WL	MUS-WR	FHMNV-181	MUS-WL	MUS-WR	FHMNV-181
Helicase (983 nucleotide sites, 327 amino acid sites)						
MUS-WL						
MUS-WR	3			1		
FHMNV-181	6	3		2	1	
GU002364	3	0	3	1	0	1
Spike glycoprotein (276 nucleotide sites, 92 amino acid sites)						
MUS-WL						
MUS-WR	0			0		
FHMNV-181	1	1		0	0	
GU002364	1	1	0	0	0	0

FIGURE 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 recovered from Muskellunge (MUS) were obtained from fish reared at Wolf Lake State Fish Hatchery, Michigan, (WLSFH) and Wild Rose Hatchery, Wisconsin, (WRH) and are designated MUS-WL and MUS-WR, respectively. White Bream virus (WBV) was used to root the tree. Numbers at the nodes are Bayesian posterior probabilities. FHMNV= Fathead Minnow nidovirus.

FIGURE 3. Mortality curve over a 30-d period for Muskellunge ($n = 10$ per treatment) infected by i.p. injection with low (63 TCID₅₀/fish), high (6.3×10^3 TCID₅₀/fish), and control (sterile media only) doses of Fathead Minnow nidovirus.

FIGURE 4. Clinical signs exhibited by Muskellunge intraperitoneally infected with Fathead Minnow nidovirus MUS-WL strain. (A) Multifocal petechial hemorrhages on abdomen. (B) Petechial ocular hemorrhage.

That the MUS-WL FHMNV strain was not associated with morbidity or mortality at the time of collection can be attributed to one of multiple factors such as the fish were early in the incubation phase or that older fish may be less susceptible to infection with this virus. Upon experimental infection, the virus was isolated from internal organs suggesting, as was demonstrated in the case of Fathead Minnows, the infection is also systemic in Muskellunge. Since experimental infection in this study was performed by i.p. injection, it is currently unknown how the virus is transmitted under natural conditions (e.g., cohabitation with or ingestion of infected fish). Regardless of the route of infection (natural or by injection), it is clear that FHMNV can cause significant necrotic changes in hematopoietic tissues in multiple organs such as liver, kidneys, and spleen, a matter that may affect the ability of resident white blood cells to mount potent immune responses (Bromage et al. 2004; Ma et al. 2013). The studies of Iwanowicz and Goodwin (2002) described similar lesions in the liver, kidneys, and spleen of experimentally infected Fathead Minnows. There are currently no data available on cells or tissues targeted by the other bafiniviruses; the WBV was isolated from the White Bream *Blicca bjoerkna* (Order Cypriniformes) in 2001 during a wild fish disease survey and its pathogenicity has never been ascertained (Schütze et al. 2006). Other animal nidoviruses target a variety of tissue types such as the respiratory tract (Gu and Korteweg 2007) and central nervous system (Gu and Korteweg 2007; Diaz and Poma 2009), as well as lymphoid tissues and various immune cells (Spann et al. 2003; Gu and Korteweg 2007).

While sequence analysis of FHMNV helicase and spike glycoprotein gene sequences showed a high level of identity (99.4% and 100%, respectively) for all isolates, phylogenetic analysis revealed a few differences among FHMNV strains. The finding that Bayesian inference indicated that the node

support of the MUS-WL taxon was 100% suggests that MUS-WL is different from the other FHMNV strains of this study. Additionally, the finding that MUS-WR appeared as a paraphyletic taxon to the isolates obtained from Fathead Minnows (FHMNV-181 and GU002364) with a low level of node support indicates MUS-WR is more closely related to the FHMNV isolates obtained from Fathead Minnows than to the MUS-WL strain. Altogether, the data set suggests the presence of sequence variations among FHMNV isolates.

Molecular epidemiology studies have demonstrated that a novel genotype due to natural recombination can emerge for some coronaviruses. For example, phylogenetic analysis studies performed on 29 isolates of the human coronavirus OC43-OC43 (HCoV-OC43) demonstrated the presence of at least three distinct clusters of HCoV-OC43, most likely arising from recombination (Lau et al. 2011). By analogy, it is currently unknown whether the MUS-WL strain has mutated from another strain of FHMNV or vice versa. It is also unknown whether MUS-WL or MUS-WR FHMNV strains are associated with Muskellunge only; however, it is unlikely that the infection in WRH Muskellunge originated from WLSFH since both FHMNV strains have some nucleotide and amino acid differences from one another.

Molecular diagnostic assays that are able to identify viruses are valuable tools for cost-effective surveillance studies as well as for preliminary detection during outbreaks. Others have developed primer sets that target a seemingly conserved region of the spike glycoprotein to specifically detect FHMNV (Batts et al. 2012). In the current study, we developed an additional one-step RT-PCR that allows for the specific detection of FHMNV. The primers target a large portion of the helicase gene, a gene that plays a critical role in nidovirus replication, transcription, and virion morphogenesis (van Dinten et al. 2000; Seybert et al. 2005). Results indicated the assay performed as intended. We therefore propose that the

FIGURE 5. Tissue sections stained with H&E from a diagnostic case involving a natural outbreak of Fathead Minnow nidovirus in Wild Rose Hatchery Muskellunge. (A) Liver showing multifocal, 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, multifocal, tubular necrosis (arrows).

FIGURE 6. Tissue sections stained with H&E from Muskellunge experimentally challenged with Fathead Minnow nidovirus via the intraperitoneal route. Tissues from both doses were collected and fixed 22 d PI. (A) Coalescing to diffuse necrosis (arrows) and edema within the stroma of the spleen (400× magnification). (B) Diffuse necrosis of the interstitium (arrows) and edema within the anterior kidney (400×). (C) Multifocal, renal tubular necrosis and nephritis (arrow), along with necrosis of the interstitium in the posterior kidney (400×).

MNV-specific RT-PCR is an additional useful assay that can be used to detect FHMNV.

That FHMNV is pathogenic to an apex predator in the Great Lakes is particularly alarming, especially in hatchery conditions where baitfish are used as a source of forage for the Muskellunge. Currently, there are no regulations for baitfish farmers to certify their minnows as free of FHMNV prior to sale. This finding highlights the need to increase research targeted toward the relationship between predator–prey interactions and disease transmission. More stringent biocontrol measures need to be put into place to prevent transmission and spread of FHMNV.

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