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CHARACTERIZATION AND RISK ASSESSMENT OF A NOVEL VIRUS ISOLATED FROM WHITE SUCKER FISH (*CATOSTOMUS COMMERSONII)* IN WISCONSIN

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CHARACTERIZATION AND RISK ASSESSMENT OF A NOVEL VIRUS

ISOLATED FROM WHITE SUCKER FISH

(*CATOSTOMUS COMERSONII*)

IN WISCONSIN

By Heather C. Hutchings

We recommend acceptance of this thesis in partial fulfillment of the candidate's requirements for the degree of Master of Science in Microbiology.

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ABSTRACT

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White suckers are important both ecologically and economically as baitfish. Wild white suckers and baitfish suckers are affected by viral diseases. In 2010, the La Crosse Fish Health Center (LFHC) isolated a novel virus from white suckers being sold as baitfish in Wisconsin. Since then, the LFHC has presumptively detected this virus in wild white suckers and in white suckers being sold as baitfish, but has not had a definitive assay. Previous research generated genomic information and that suggested the virus belonged to the family *Bunyaviridae*. The virus was named white sucker bunyavirus (WSBV). Further sequence analysis confirmed that this virus was a bunyavirus, but was distantly related to all known bunyaviruses. This sequence information was used to develop a diagnostic RT-PCR assay for WSBV. The assay was used to detect WSBV during a preliminary survey and experimental infection. In the survey, no fish from the twelve watersheds surveyed tested positive for WSBV. The assay has since been used to identify WSBV from 11 previous presumptive isolations collected by the LFHC. The experimental infection demonstrated WSBV was pathogenic to juvenile white suckers at high doses (10^7 PFU). Environmental agencies should now consider WSBV significant viral agent of white sucker fish.

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INTRODUCTION

The white sucker (*Catostomus commersonii)* is an abundant fish species in North America (11, 17). It is part of the sucker family (*Catostomidae*), in the subfamily *Catostominae,* which includes the genera *Catostomus, Moxostoma, Ictiobus* and numerous others (53). There are 17 species of suckers from the family *Catostomidae* that live in Wisconsin waters. Some of the better known species in the area include the big mouth buffalo (*Ictiobus cyprinellus*), black buffalo (*Ictiobus niger*), smallmouth buffalo (*Ictiobus bubalus*), golden redhorse (*Moxostoma erythrurum*), silver redhorse (*Moxostoma anisurum*) and shorthead redhorse (*Moxostoma macrolepidotum*) (4). Only two species of the genus *Catostomus* live in Wisconsin, the white sucker and the longnose sucker (*Catostomus catostomus*) (4).

White suckers prefer cool water and are found in swift moving streams, slow moving rivers and large bodies of water throughout North America (4). White suckers also prefer streams that have overhanging cover of trees and shrubs (12, 17, 30). Being a cool water fish, the preferred water temperature for the white sucker is between 15°C and 20°C (12, 30).

White suckers take several years to mature and reach their adult size and if they avoid being eaten these fish can grow and breed for up to 17 years (30). The average size for an adult fish is 12 to 20 inches and their coloring is black to olive on their back with a whitish belly (4). White suckers lack barbels and an adipose fin and they only have teeth in their throat (17). The young fish (<1 year) have prominent black spots on their sides

that disappear as they reach sexually maturity (4). It takes one to two years for male fish to reach sexual maturity while females take two to three years. The fish normally spawn in the spring in cool shallow streams, where females can each release between 20,000 and 50,000 eggs (30).

White suckers are abundant fish and are benthic feeders; they play a vital role in the aquatic food chain, feeding on algae, insects, mollusks, and small crustaceans. As omnivores, they may be able to ingest pollutants that affect both plant and animal life. Because of their vast distribution and adaptability to environmental conditions, white suckers are considered a biological monitor species and are being used in many studies as an indicator of environmental health (11, 26, 23, 39).

White suckers serve as forage for many large sport fish species (4, 11). In a study done by Bozek *et al.* (1999), species in the family *Catostomidae* were the most common prey for muskellunge (*Esox masquinongy*) during the spring. In hatcheries, juvenile white suckers are also frequently used as feed for large sport fish such as walleye (*Sander vitreus*) and northern pike (*Esox lucius*) (4), which are in turn commonly used to stock rivers and lakes for sport fish anglers (8).

Because white suckers are natural forage for large sport fish, they are often used as bait in Wisconsin and are the second highest selling baitfish species in the state behind fathead minnows (*Pimephales promelas*) (28, 35, 36). Wisconsin is the second-leading state in baitfish sales, which generate 4.65 million dollars annually (35). Baitfish revenue in Wisconsin comes mainly from the sale of bait to anglers. When the demand for bait is high, juvenile white suckers can sell for $2.50 per pound in bait shops (26). Baitfish can be farm raised or wild caught, but since white suckers rarely spawn in ponds, baitfish

dealers normally collect fry from the wild and then stock them (40,000-100,000 fry per acre) in aquaculture ponds to be raised for bait (26). Alternatively, eggs and milt can be collected from adult fish to artificially spawn fry in aquaculture settings (26). When anglers purchase bait from baitfish shops or dealers, this bait and/or water can, in turn, end up in contact with wild fish in many different bodies of water. This contact with wild fish and the waters they inhabit may lead to the spread of pathogens carried by the baitfish (33). Due to the economic importance of the baitfish industry, the Wisconsin Department of Agriculture, Trade and Consumer Protection (DATCP) and the Department of Natural Resources (DNR) are concerned about the effect of baitfish pathogens on the health of Wisconsin's fish populations and limiting the spread of diseases between farm-raised fish and wild fish (36).

Viruses That Affect White Sucker Fish

Fish viruses have been extensively studied in recent years because of the threats they pose to aquatic ecosystems and aquaculture farms. Viruses are spreading to new waterbodies via transport of fish and fish carcasses (41, 46). Baitfish are the most frequently transported live fish and are capable of spreading viruses to endemic fish populations (46). These viruses can become more virulent and expand their host range upon introduction to new waters, causing greater damage to fish health (42, 52). As a result, continued monitoring and classification of viruses in baitfish and wild fish is important.

Several viruses are known to infect white suckers, and signs of infection range from carrier state (asymptomatic) to wart-like lesions and hemorrhaging, to death. Viruses from four different families, the *Birnaviridae* and *Reoviridae*, and possibly

Rhabdoviridae (15) and *Retroviridae,* have been found in white suckers. The infectious pancreatic necrosis virus (IPNV), in the family *Birnaviridae,* has been isolated from wild white suckers and affects many other species of fish (50). The golden shiner virus (GSV), in the family *Reoviridae,* has been found in white suckers used as bait (33). Viral hemorrhagic septicemia virus (VHSV strain IVb), in the family *Rhabdoviridae,* has contributed to many large fish kills in the Midwest and is thought to be carried by many species of fish (15, 37, 56). Finally, retroviral-like particles have been found in lesions and growths on the lips of white suckers, suggesting a viral etiology possibly in the family *Retroviridae* (6). Although the first three viral families have not been shown to cause disease in white suckers, there is concern that infected white suckers could possibly spread the viruses to other species of fish. All of the above families of viruses have been found in different species of large predatory fish (15). Thus, it is possible that using white suckers as bait could lead to the spread of viruses from white suckers to predatory fish.

Infectious Pancreatic Necrosis Virus

Of the viruses that affect the white sucker, IPNV has the widest host range. The virus can infect over 30 families of fish and ten species of crustaceans (47, 57). IPNV is in the genus *Aquabirnavirus* and is a non-enveloped icosahedral virus that is 60 nm in diameter (2, 20, 57). Its genome consists of two segments of double-stranded RNA (dsRNA) named the A and B segments. The virus has ten different serotypes that are found all over the world; in North America groups A1, A6, A7, A8 and A9 are most prevalent and in Europe A2, A3, A4, A5 and B1 are most prevalent (55, 57).

The virus was first isolated in the United States in 1957 from brook trout (*Salvelinus fontinalis*) from the Leetown National Fish Hatchery in West Virginia (20,

47). It was thought to mainly affect salmonid species but was later isolated from healthy white suckers in 1972 (50). IPNV is found in both freshwater and marine environments, but it causes the most economic damage in large fish hatcheries (27, 47). Effluents from fish hatcheries that have been contaminated by introduction of infected brood stock or carrier fish are thought to be the main source of the virus in wild fish (47, 57). In one survey of hatchery effluent (IPNV was isolated annually from hatchery stock), IPNV was found 19.3 km downstream of the hatchery in the water and in brook trout tissues (32). Young fry and fingerlings of salmonid species are the most susceptible to disease caused by the virus (20, 27). In contrast, older fish with the infection are mainly asymptomatic, but these fish can become lifelong carriers of the virus and spread it to other fish via horizontal transmission and to their offspring via vertical transmission (20, 47, 58).

Signs of IPNV infection in salmonids include behavioral changes such as corkscrew spinning and ataxia, and physical changes such as darkening of skin, paleness of gills, abdominal swelling and sometimes ventral hemorrhaging (47). These signs are due to viral replication and lysing of cells in these tissues. Signs of disease are normally observed in young fingerlings, but have been observed in salmon over six months of age (51). In older fish or fry that have survived the disease, a carrier state exists in which no signs are observed and the virus is shed intermittently into the environment (47). The conditions in which the fish are raised influence the susceptibility to infection by this virus. For example, overcrowding of tanks, which can lead to physical stress on the fish, plays a key role in the pathogenicity of the virus due to a reduced immune response by the fish (47, 51).

Golden Shiner Virus

The golden shiner virus was first isolated from a golden shiner minnow (*Notemigonus crysoleucas*) kill at a hatchery in Arkansas in 1977 (10). The virus is in the family *Reoviridae*, and the genus *Aquareovirus* group C. Like other Aquareoviruses, GSV infection of fish cell culture lines demonstrate the formation of large syncytia of infected cells (2, 16). These viruses are non-enveloped, 80 nm icosahedral virions, with genomes comprised of 11 segments of dsRNA (2, 16, 19).

The virus can replicate in temperatures as low as 15°C, but replicates best between 25°C and 30°C which correlates with late summer temperatures when fish mortality events are reported (9, 34, 49). Fish infected with GSV may display hemorrhaging around eyes, visceral fat and muscle (10, 34). These signs are often seen in aquaculture populations, such as in baitfish, due to the environmental and physiological stresses placed on these fish. Most infections with the virus in wild populations are subclinical (10, 19).

GSV has been found in several species of native fish including the white sucker, fathead minnow, creek chub (*Semotilus atromaculatus*) and emerald shiner (*Notropis atherinoides*) (33). The mortality of golden shiners infected with GSV is usually around 5% of the population in natural infections (24, 48). GSV can, however, cause mortality of 50% among golden shiners that are kept in crowded conditions with warmer temperatures (48). Mortality in other fish populations and species is unknown.

Viral Hemorrhagic Septicemia Virus

Viral hemorrhagic septicemia virus (VHSV) is in the genus *Novirhabdovirus* of the family *Rhabdoviridae* and has non-segmented, negative sense, single stranded RNA

genome (14, 18, 19, 37, 42). The virions are enveloped, bullet shaped, with a length of 170-180 nm and a width of 60-70 nm (19, 21).

VHSV can cause extreme economic losses in both freshwater and marine fish (13, 14, 19, 25, 28, 29, 37, 41, 52, 56). VHSV was first isolated from rainbow trout (*Oncorhynchus mykiss*) in a Denmark aquaculture farm in 1962 (18). Since that time it has caused multiple mortality events throughout Europe. It was first found in North America in 1988 when it was isolated from Coho salmon (*Oncorhychus kisutch*) from hatcheries in the Pacific Northwest (19, 37). VHSV was not isolated in the Great Lakes region until 2003 when it was detected in a muskellunge caught in Lake St. Clair, between Ontario, Canada and Michigan (28, 56). Large fish kills were observed in 2006 that affected freshwater drum (*Aplodinotus grunniens*), muskellunge, white bass (*Morone chrysops)*, round goby (*Neogobius melanostomus*), gizzard shad (*Dorosoma cepedianum*) and yellow perch (*Perca flavescens)* in the St. Lawrence River, Lake St. Clair and Lake Ontario (28, 56).

Five different genotypes of VHSV have been identified: I, II, III IV and IVb (19, 28, 56). Genotypes I-III are found in continental Europe and the British Isles (19, 56). Genotype IV can be found in North America, Japan and Korea, while IVb has only been found in the Great Lakes region (56). Due to the isolation and lack of divergence of the genotype IVb, it is thought to have occurred from one introduction event, possibly from contaminated ballast water being released into the lake system (56). It is now known to affect over 28 species of fish in the Great Lakes region (14, 46, 56). Muskellunge were found to be highly susceptible to IVb through experimental infections, but no such experimentation has been done with white suckers (25).

Fish become infected with VHSV, a virus that is stable in water, through the gills and contact with contaminated fomites and infected fish (37, 43, 46). Infection most frequently occurs at temperatures between 1°C and 12°C and the signs fish exhibit include hemorrhages at the base of fins, in skeletal muscle and internal organs (13, 19, 41, 43, 46, 56). Many species of fish that are virally infected display the same symptoms and not all of them are tested for VHSV. Because of this the host range of VHSV is thought to be very large and many host fish species remain to be determined including the white sucker (15).

Retrovirus-Associated Sucker Papilloma

Tumors on the lips of benthic feeding fish, such as the white sucker, have been commonly reported (38). The tumors are usually raised, firm, yellowish to whitish in color, round to oval shaped, with a size of around 0.5 cm in diameter (6, 19, 38). Other lesions, either firm papillomas or mucoid plaques, have also been observed on the eyes, body and fins of white sucker fish (6). Homogenates made from the lesions have shown elevated levels of reverse transcriptase activity which indicates presence of retroviral agents (6, 19, 22, 38, 45). The firm papilloma lesions have been found to contain 100 nm C-type retrovirus particles when examined under electron microscopy (6, 19, 38, 45). Papilloma growth is more common in older fish and fish living in polluted water but has been seen in fish that were maintained in well water that was pollutant free (38). Walleye also have papilloma-like lesions that have retroviruses associated with them. These viruses include walleye dermal sarcoma virus and walleye epidermal hyperplasia virus 1 and 2 (22, 31, 38). More research is needed to confirm the exact mechanism by which papilloma tumors develop in white suckers but a viral etiology is suspected (6, 19, 22, 38,

45). These tumors are usually benign in small numbers, but may deleteriously affect a fish's quality of life if present in high numbers.

Uncharacterized Virus of White Sucker Fish

In 2010, an unknown virus was found by the United States Fish and Wildlife Service, La Crosse Fish Health Center (LFHC) in white suckers obtained from baitfish dealers in Wisconsin. The fish were tested as part of a settlement after four Wisconsin baitfish dealers were convicted of illegally transporting live fish across state lines without the proper permits, a violation of the federal Lacey Act (33). Over 1,200 white suckers, some of which displayed hemorrhaging around eyes, fin bases, gills and skin, were tested for viruses using tissue homogenates from the kidneys and spleens. Homogenates from pools of five fish were placed on fish tissue culture cell lines and observed for cytopathic effects (CPE). CPE from the virus, later determined to be white sucker bunyavirus (WSBV), was observed on chinook salmon embryo (CHSE) cells and *Epithelioma papulosum cyprini* (EPC, isolated from carp epidermal herpes virus-induced lesions) cells at 15°C and 20°C, respectively (33). The fish homogenates that displayed CPE in this study were also tested using diagnostic PCR for GSV, VHSV, Spring Viremia of Carp Virus, IPNV, Largemouth Bass Virus, Fathead Minnow Nidovirus, and Bluegill Picornavirus (33). Failure to amplify a known virus meant that a novel virus was likely present. The isolate was then sent to Dr. Hoffman's lab at the University of Wisconsin-La Crosse for further analysis that was started by Marisa Barbknecht, M.S., who amplified the genome by random RT-PCR and created a library of clones. Sequencing and BLAST (Basic Local Alignment Search Tool) analysis (via the NCBI website) of the clones

revealed that the isolate was likely a member of the family *Bunyaviridae.* Collectively, the sequence of about 8,000 nt of the genome were determined.

The *Bunyaviridae* family consists of negative-sense, segmented RNA viruses that are mainly arthropod-borne (with the exception of *Hantavirus*) and infect birds, plants and mammals. There are five genera in this family, and they include: *Orthobunyavirus, Hantavirus, Nairovirus, Phlebovirus* and *Tospovirus.* The first four genera are associated with human and animal diseases causing hemorrhagic fevers and flu-like sickness. The final genus includes arthropod transmitted viruses that cause necrosis of plant tissues. A key feature of all bunyaviruses is that they contain three genomic segments, which are named the L (large), M (medium) and S (small). The L segment codes for an RNA-dependent RNA polymerase (RDRP) while the M segment codes for a polyprotein that is cleaved into envelope glycoprotein 1, envelope glycoprotein 2 and a non-structural protein. The S segment codes for the nucleocapsid protein and in some viruses a non-structural protein. There are no vaccines to prevent the spread of viruses in this family and control of arthropod vectors is the main preventative measure. Although these viruses have a wide host range, there are no documented fish viruses in this family (44).

RESEARCH OBJECTIVES

Because WSBV could be one of the first fish viruses in the *Bunyaviridae* family, it merited further exploration due to the uniqueness of this virus and possible economic and ecological consequences this virus poses to populations of fish in Wisconsin. Therefore, the main goals of this study were to characterize WSBV and perform a risk assessment using both a wild fish survey in Wisconsin and a controlled experimental infection of white suckers with WSBV. To confirm the presence of WSBV, a diagnostic RT-PCR was developed. This assay was then used to identify isolations from the wild fish survey and to confirm infection of fish during the experimental infection. Three research objectives were used to complete this study.

1. Develop a RT-PCR based diagnostic assay based on partial sequence.
2. Survey for the virus in white sucker fish living in lakes and streams in Wisconsin.
3. Experimentally infect white suckers to characterize any pathology associated with WSBV.

METHODS

Cells and Virus

EPC cells were cultured in Eagle's Minimum Essential Medium (MEM) supplemented with 10% fetal bovine serum (FBS). The cells were incubated at 20°C. The WSBV isolate that was characterized and used for RT-PCR assay development was isolated from white suckers collected from baitfish dealers in Wisconsin in 2010 (LFHC isolate 195).

Microscopic Observation of Viral Infection

Observation of WSBV viral infection via inverted microscopy at 100X magnification was used, which is the same procedure used in a diagnostic laboratory. EPC cells (95% confluent) growing in 6-well tissue culture plates were inoculated with either 500 µl of clarified medium from WSBV infected EPC cells or 500 µl phosphate buffered saline (PBS, as a negative control) via replacement of the growth medium with inoculum. Cells were incubated at 25°C for one hour to promote viral attachment. Following attachment, three ml MEM (10% FBS) was added. The cells were incubated at 20°C and monitored daily for CPE.

Concentration of Virus

To propagate WSBV, EPC cells in 175 cm^2 flasks at 100% confluency were used. To infect the cells, 500 µl clarified medium from infected EPC cells was used and growth media was replaced with MEM without FBS. Cells were incubated at 20°C. When cells were 90-100% detached from flask (often observed 3-5 dpi), the medium was collected

and clarified via slow speed centrifugation (1,600 X *g*) for 15 min at 4°C. This clarified medium was then concentrated in one of two ways, depending on downstream use of virus.

For diagnostic assay development for the virus, sucrose gradient centrifugation was used. Virus in clarified medium was concentrated by layering the medium over a five ml 20% (w/v) sucrose cushion in multiple 30 ml ultracentrifuge tubes and centrifugation (131,000 X *g*) at 4°C for three hours. After centrifugation, the supernatant was removed and virus particles were resuspended in 100 µl PBS and frozen at -80°C.

For the experimental infection with white sucker juveniles, PEG-6000 precipitation was used. The media from infected EPC cells was clarified by centrifugation (14,000 X *g*) at 4°C for 20 min. The supernatant was then transferred to an ice bath. Sodium chloride was added to the supernatant to make a 2.3% (w/v) salt solution. PEG-6000 was added to this solution to make a 7.0% PEG-6000 solution. This solution was then rocked at 4°C for 24 hours to allow for precipitation of virus particles. After the 24 hour period the solution was centrifuged (14,000 X *g*) at 4°C for 20 min to pellet virus. The pellet was washed with one ml of 10 mM Tris-HCL, 2 mM EDTA, 150 mM NaCl (TES) buffer. The washed pellet was resuspended in two ml TES pH 7.2 and stored at -80°C until use. Both concentrated stocks of virus were titered via plaque assay.

Plaque Assay

EPC cells in 6-well tissue culture plates were grown to 90-100% confluency. Virus was serially diluted in PBS and added to cells. The plate was incubated at 25°C for one hour. The diluent was then removed from the wells and 2.5 ml of a 0.75% methylcellulose/ 0.5X MEM (10% FBS) overlay was added to the wells. Once plaques

were visualized via inverted microscopy (3-7 dpi), the assay was stopped with 500 µl of 1.0 % crystal violet in 20% ethanol. The stain was added directly atop the overlay and allowed to stain for 24 hours. The overlay was removed and the cells were destained with PBS and plaque forming units per ml were determined (PFU/ml).

Genome Analysis

Sequences obtained by Marisa Barbknecht M.S. (University of Wisconsin-La Crosse) and Nicholas Phelps Ph.D. (University of Minnesota College of Veterinary Medicine) were analyzed using San Diego Super Computer Center (SDSC) Biology Workbench 3.2 (http://workbench.sdsc.edu). Sixframe analysis was performed to identify the longest open reading frame (ORF) from each sequence. The predicted protein sequences from the long ORFs were compared with entries in the GenBank Viral database using protein Basic Local Alignment Search Tool (BLAST).

WSBV Diagnostic One Step RT-PCR Development

SDSC Biology Workbench 3.2 was used to design two primer pairs to amplify regions in the M segment of the WSBV genome (Table 1). The T_m was designed to be around 55°C for both pairs to make specificity comparisons possible.

Table 1. WSBV diagnostic RT-PCR primer pair candidates and expected product sizes.

Primer Pair	Forward Primer	Reverse Primer	Product Size (bp)
#1	5'CATGCATCTACGGAATGTGG	5'CCTGTGCCCAGTAGAGAAGC	222
#2	5'AACAGGGGAGAGTAGGAGCA	5'GCTGTGTTCCCAATCCTCAT	282

To determine the specificity of the potential diagnostic primer pairs to the WSBV genome, RT-PCR reactions were compared to find the pair with the least amount of background. RNA was extracted from the sucrose concentrated virus with the Qiagen

MinElute Virus Spin kit. The Qiagen One-step RT-PCR kit was used with five µl of WSBV RNA, using the reagents per manufacturer's recommendations. The cycle parameters were 50°C for 30 min (reverse transcription), 95°C for 15 min, 30 cycles at 94°C for 30s, 55°C for 30s, 72°C for one min, and a final extension at 72°C for 10 min.

To assess PCR specificity of the primers to other viruses, the primers were tested against RNA extracts from other fish viruses and uninfected cells. Clarified media from VHSV, IPNV and SVCV positive tissue culture cell supernatants were obtained from the LFHC. Nucleic acids were extracted from 200 µl of media from each of the samples, and 200 µl of medium from uninfected EPC cells using the MinElute Virus Spin Kit (Qiagen) using manufacturer specifications. This kit extracts all nucleic acids from a given sample. Amplification of the different nucleic acids were tested using the conditions described above with both primer pairs. The products were electrophoresed on a 1% agarose gel and visualized via ethidium bromide staining.

The assay was used to detect the presence of WSBV during the preliminary survey and experimental infection. Media from tissue culture samples inoculated with kidney and spleen homogenates from white suckers were analyzed. Nucleic acids were extracted from 200 µl of clarified media using the MinElute Virus Spin Kit (Qiagen). The one-step RT-PCR reaction was performed on each extract, including uninoculated media extracts as a negative control. Products were transferred to a 1% agarose gel, electrophoresed and visualized via ethidium bromide staining.

RNA Detection Limit of RT-PCR

The detection limit for the one-step RT-PCR assay was tested using both primer pairs against RNA extracted from sucrose concentrated WSBV. The titer of virus in the

supernatant was determined via plaque assay to be 2.8 x 10^7 PFU/ml. The RNA was diluted 1:100 in PBS and then in a series of two-fold dilutions. Five µl of each dilution and the undiluted RNA was added to the RT-PCR reagents as described above. The products were electrophoresed on a 1% agarose gel and visualized with ethidium bromide staining. The virus concentration in the greatest dilution that still produced a band was determined to be the detection limit.

Preliminary Wisconsin Survey of WSBV

Between May and October 2014, adult and juvenile white suckers were collected from 12 different watersheds in Wisconsin using electroshocking and seine nets (Figure 1). Thirty fish from each watershed were collected, monitored for signs of viral infection, and euthanized with a lethal concentration of tricaine methane sulfonate (MS-222).

Figure 1. Watersheds sampled in 2014 wild white sucker survey for WSBV.

Approximately 0.2 g of kidney and spleen tissue was removed from each of the fish with forceps. Five-fish pools of tissue were diluted 1:10 with three ml Hank's balanced salt solution (HBSS), homogenized in a stomacher, and clarified via slow speed centrifugation (1,400 X *g*) for 15 min at 4°C. Clarified tissue homogenates (1.5 ml) were diluted 1:1 in HBSS and incubated overnight at 4°C. Duplicate wells of EPC cells in 24-well plates were inoculated with 0.1 ml of dilute homogenate from each pool and

incubated for one hour at room temperature on a plate rocker to facilitate attachment. After attachment, 0.5 ml of complete bicarbonate media (1X MEM, 10% FBS, 0.5% $NaCO_3$) was added to each well. The EPC cells were then incubated at 20°C for 28 days and monitored twice a week for CPE. Negative controls consisted of mock inoculated cells.

If CPE was observed, media from these wells were clarified via slow-speed centrifugation and nucleic acids were extracted from 200 µl supernatant using the MinElute Virus Spin Kit (Qiagen). The remaining supernatant was stored at -20°C. If no CPE was observed within 14 days post-inoculation, a blind passage was performed in order to rule out low virus titer/slow-growing virus. Growth medium was pooled from 6 wells lacking CPE and centrifuged at low speed (1,400 X *g*) for 15 min at 4°C. Cells in 24-well plates were inoculated as previously described and incubated for another 14 days, monitoring twice a week. All of the samples (including CPE negative) were tested via the diagnostic RT-PCR assay described previously (Figure 2).

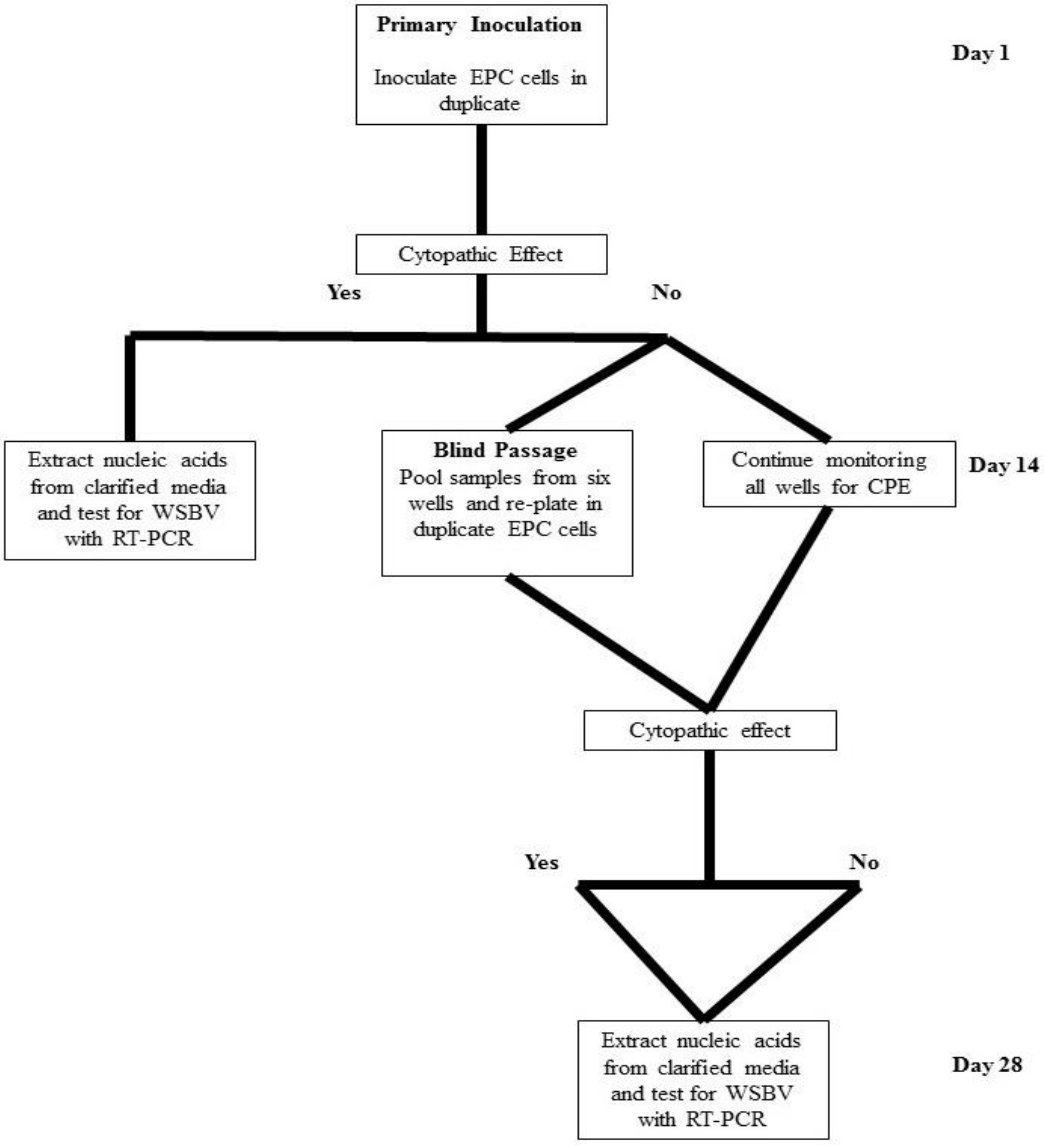

Figure 2. White sucker tissue processing and testing for WSBV in wild white sucker survey and juvenile white sucker experimental infection.

Experimental Challenge of Juvenile White Suckers with WSBV

The experimental infection was performed on juvenile white suckers obtained from a baitfish dealer in Minnesota. These fish were hatched from eggs and milt collected from adult fish from Lake Ida, Minnesota. The fish are grown in a pond supplied with well water on the farm. Sixty juvenile fish (<1 year) were purchased and tested for any

presence of virus via tissue culture and the presence of WSBV, SVCV, VHSV and IPNV using diagnostic PCR assays techniques as described previously. Once the fish were determined free of these viruses via PCR another 80 fish were purchased for the infection.

Four fiberglass tanks were obtained from the UMESC, disinfected, and filled with 24 gallons of chlorinated tap water at the LFHC. Each tank was equipped with an alcohol thermometer and a 30-gallon aquarium filter. Tanks were allowed to sit for five days before the fish were added. In each tank, 30 ml of API© nitrifying bacteria were added to the filters and aquarium salt was added to a 0.15% concentration. The salt aided in helping the fish excrete excess ammonia and the bacteria aided in processing excess ammonia from the water.

The fish were added (20/tank) and tanks were numbered, with a wooden board separating tanks #1 and #2 from tanks #3 and #4. Plastic netting was placed over the tanks to prevent fish from jumping out of the tanks (Figure 3).

Figure 3. Experimental infection tanks at the La Crosse Fish Health Center.

Inoculation and Monitoring of Fish

WSBV (isolate 195) from the LFHC was used in the infection, and was propagated in EPC cells and concentrated via PEG-6000 precipitation. The virus was titered via plaque assay and determined to be 1.0×10^8 PFU/ml. All the fish from tanks #1 and #2 were netted and placed in a solution of MS-222 (~100 mg/kg of body weight) until the fish were sedated enough to handle. Restraining the fish was done by holding the head and tail area exposing the abdomen for the injection. These fish were injected one millimeter into the body cavity with a 25 gauge needle containing 0.1 ml of 1.0×10^7 PFU of WSBV in TES buffer. The fish were placed in a recovery tank until they recovered from anesthesia and were able to be returned to their respective tanks.

Fish from the control tank #3 were sedated in the same way and injected with 0.1 ml TES buffer to rule out the possibility of adverse effects from injection stress or the buffer. The fish in a second control tank #4 remained unhandled and served as a control for water conditions/transportation stress.

The tanks were monitored and the temperature recorded daily for a period of three weeks. The fish were fed pellet feed twice over the infection period. Signs of infection (erratic swimming, hemorrhaging and malaise) and mortalities were documented from each tank and the fish were processed for viral analysis.

Tissue Collection and Processing

Fish that died during the infection were individually processed the same day by collecting 0.2 g kidney and spleen from each fish. If the fish died on a weekend they were stored at 4°C on ice until they could be processed. The tissue was diluted/centrifuged and incubated as described for the survey. To compare physical differences between control

fish to the infected fish, four control fish were collected (two from tank #3 and two from tank #4) and euthanized during the second week of the infection. Kidney and spleen tissue was collected from these individual control fish and processed. At the end of the three weeks, the remaining fish were euthanized with a lethal dose of MS-222 and processed. The remaining control fish tissues were pooled into five-fish pools while the infected fish were processed individually.

Tissue Culture

Tissue samples that had been processed and diluted were plated on 80% confluent EPC cells in 24-well tissue culture plates and incubated at 20°C. These plates were monitored twice a week for CPE. Once CPE was noted (3-7 dpi) nucleic acids were extracted using the Viral RNA Mini Kit (Qiagen). The control fish samples were allowed to incubate for 28 days before nucleic acids were extracted using the same kit (Figure 2).

Diagnostic Assay

RNA extracted from all the samples was tested using the One-Step RT-PCR kit (Qiagen) diagnostic assay as described previously. Negative control nucleic acids came from uninfected EPC cell media. Positive controls came nucleic acids extracted from sucrose concentrated WSBV. Products were electrophoresed on a 1% agarose gel and visualized via ethidium bromide staining.

RESULTS

Molecular Characterization of WSBV

To molecularly characterize WSBV, the RNA for the partial L protein encoding segment was used because the L segment encodes the RNA-dependent RNA polymerase (RDRP) which is highly conserved in this family of viruses (Figure 4). Protein BLAST searches performed with partial WSBV L protein segment (1873 aa) aligned with members of the family *Bunyaviridae*, with the closest matches occurring with members of the *Orthobunyavirus* genus. WSBV was the closest to five viruses in this genus: Khurdun virus, Tete virus, Shuni virus, Pacui virus and Douglas virus.

The SDS Biology Workbench software Align program was used to conduct global pairwise alignments of WSBV with these five viruses, and another partially sequenced virus isolated from largemouth bass (*Micropterus salmoides*) that appears to be a bunyavirus and is tentatively named largemouth bass bunyavirus (LMBBV). Percent similarities between each of these viruses with WSBV were calculated using the Align program. WSBV has higher similarity with LMBBV and lower similarity with the other orthobunyaviruses (Table 2).

ClustalW was used to do a multiple sequence alignment with WSBV, four of the known orthobunyavirus L segments and the LMBBV L segment (Figure 5). There are some conserved residues between all seven viruses most notably the VHSDDN motif. This motif contains the SDD tripeptide sequence which is a catalytic domain for nucleotide polymerization common between all segmented negative sense RNA viruses

(44). These conserved residues are in the middle of the L segment and occur between residues 900 and 1145 in all of the viral sequences.

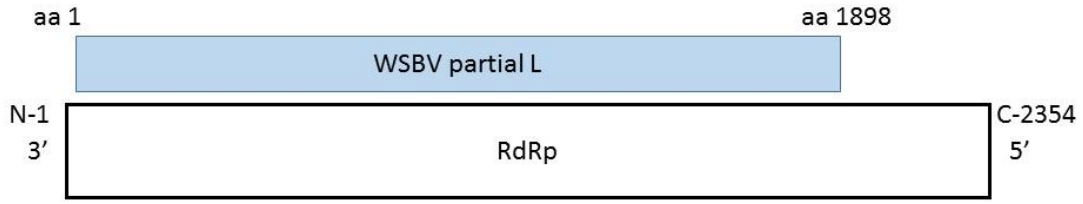

Figure 4. Schematic of WSBV RDRP coding segment overlap with a typical orthobunyavirus RDRP segment. Negative-sense orientation shown with amino acid numbering of sequences.

Table 2. Orthobunyavirus GenBank viral L protein sequence pairwise alignments with partial WSBV sequence encoding the L protein.

GenBank Accession	Virus	Amino Acid length	Percent similarity
AHL27166.1	Khurdun virus	2164	24.3
AJT55735.1	Tete virus	2281	23.7
CCH15003.1	Shuni virus	2253	22.3
AIN55741.1	Pacui virus	2253	24.0
CCG93471.1	Douglas virus	2253	23.2
NONE*	LMBBV	2225	43.8

*LMBBV is yet to be fully classified and only partial sequences are known

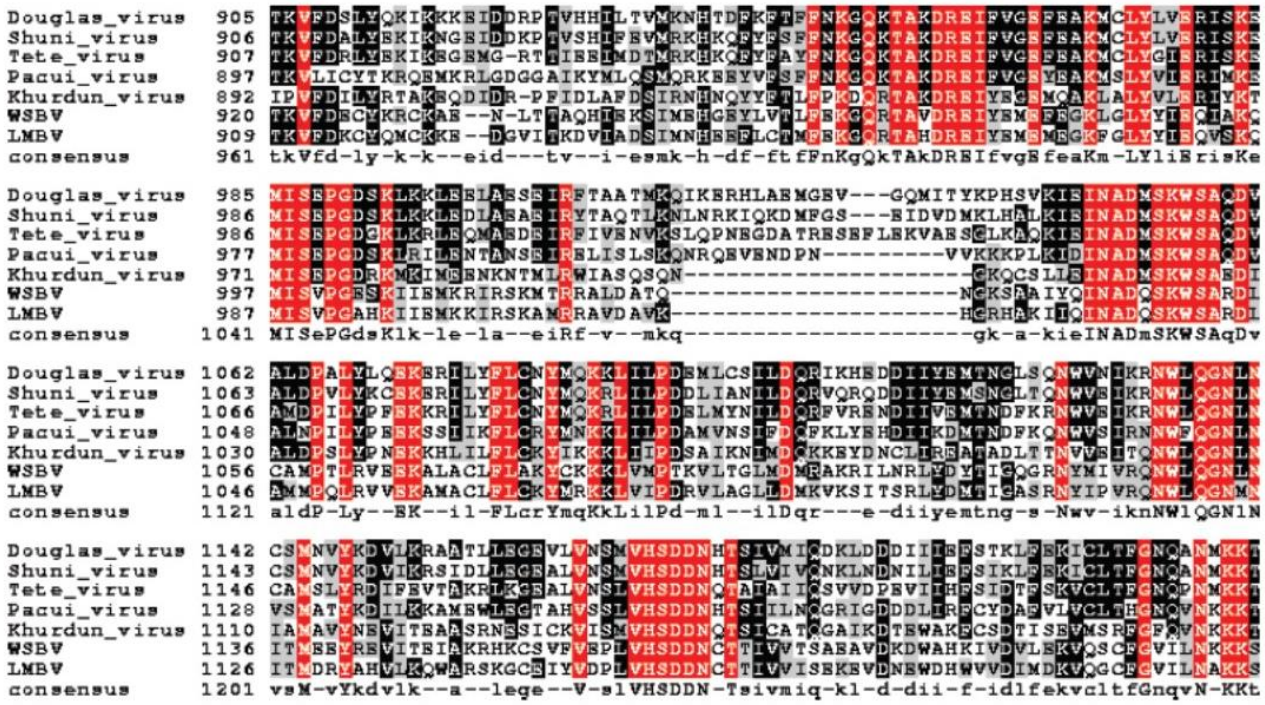

Figure 5. Middle portion of ClustalW protein alignment of WSBV partial L segment with five known orthobunyaviruses and a partial LMBBV L segment. Red background with white letters = completely conserved, black background with white letters = identical residues, light gray background with black letters = similar residues.

Microscopic Observation of WSBV- Induced CPE

To understand the CPE caused by WSBV and its development, low-power inverted phase contrast microscopy was used to follow a five-day WSBV infection in EPC cells. Infected and uninfected cells were observed two, three, and four days post infection (Figure 6). A MOI of 1.0 was used to infect the cells. By day two, virally damaged cells were apparent and formed bright clusters of crenated cells. Over time, as the virus spread, the cells detached in large numbers from the flask and by day five 90-100% of the cells were detached (data not shown).

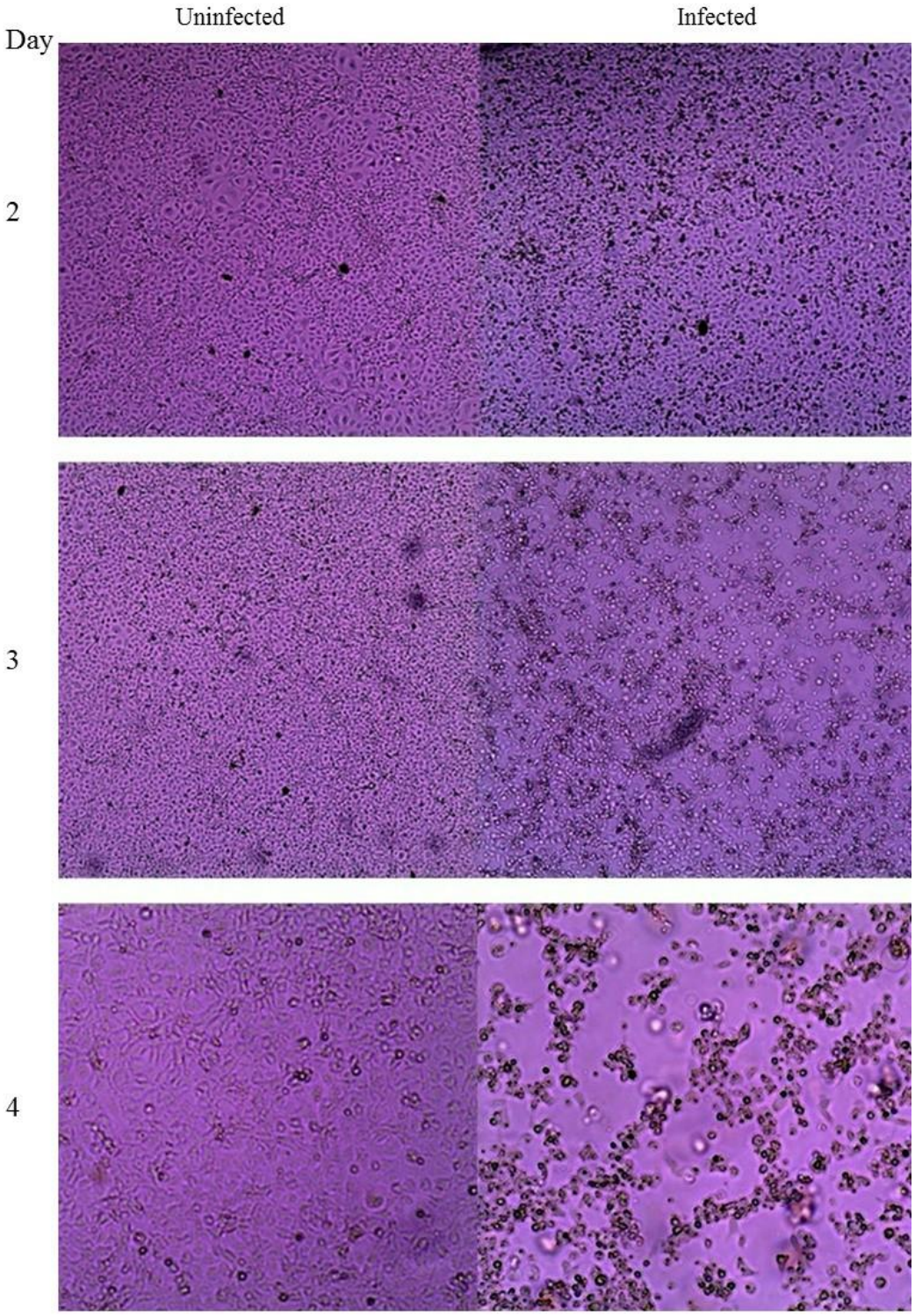

Figure 6. Development of cytopathic effects in EPC cells infected with WSBV (MOI 1.0). Cells were observed two, three, and four dpi with an inverted phase microscope. Days two and three are at 100X magnification and day four at 200X magnification.

Diagnostic One-Step RT-PCR Development

A diagnostic PCR assay for WSBV was needed to detect virus during the survey and experimental infection. This assay could also be used by fish health diagnostic labs for future detection of WSBV. Sequence information from WSBV was used to design two primer pairs to develop a one-step RT-PCR diagnostic assay (Table 1). The primer pairs amplified regions in the M segment of the WSBV genome most likely in glycoprotein coding regions (Figure 7). Primer pair #1 had the least amount of background (Figure 8). Primer pair #2 was discarded because it amplified nucleic acids from uninfected cell culture media producing amplicon the same size as the WSBV amplicon (data not shown). To make sure primer pair #1 would not amplify any sequences from other viral genomes, RT-PCR with primer pair #1 was performed with nucleic acid extracts from VHSV, SVCV, IPNV, WSBV infected cell media and uninfected EPC cell culture medium (Figure 9). The detection limit of viral RNA using primer pair #1 was found to be 7.0 x 10^5 PFU (Figure 10).

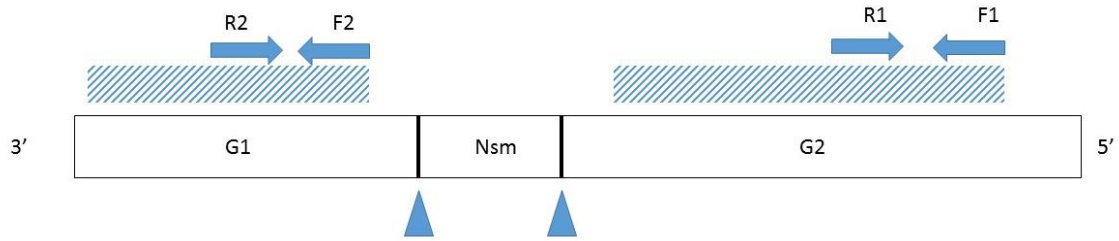

Figure 7. Schematic of the M segment of the WSBV negative-sense genome (6.9 kb). Blue dashed areas indicate the area of sequence used to develop diagnostic RT-PCR primer pairs (2449 nt). Blue arrows indicate where the primers are located in the sequence. Blue triangles indicate host protease cleavage sites. G1 stands for envelope glycoprotein one, G2 stands for envelope glycoprotein two and Nsm stands for a non-structural protein.

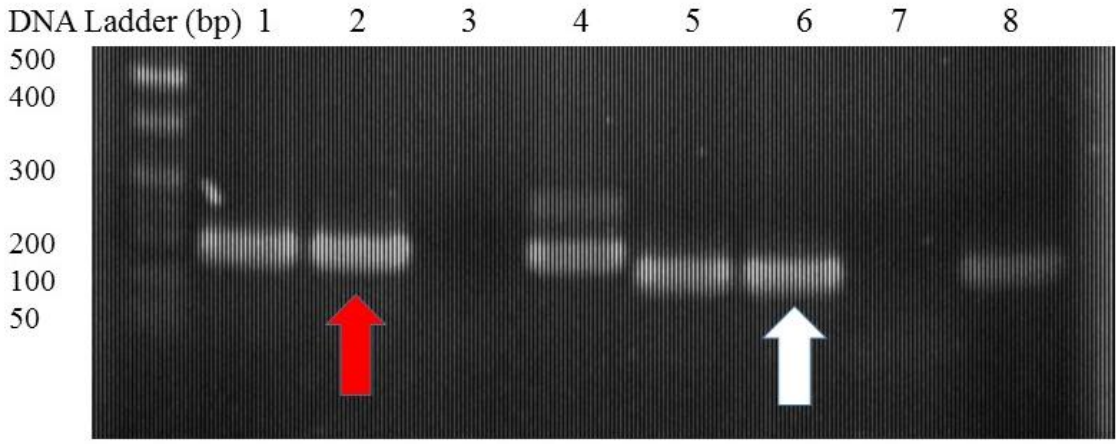

Figure 8. Product sizes from primer set #1 (white arrow) and primer set #2 (red arrow). Samples were run in duplicate with positive controls (plasmid DNA) in lane 4 and 8 and negative controls (no RNA) in lane 3 and 7.

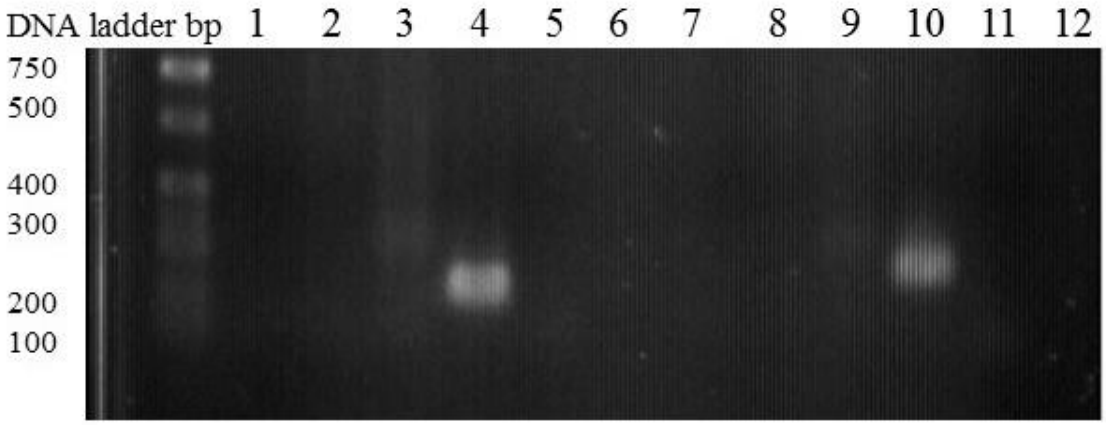

Figure 9. WSBV primer pairs specificity testing against other RNA viruses. Lanes 1-5 used primer pair #1 against VHSV, SVCV, IPNV, WSBV and no RNA negative control respectively. Lanes 7-11 used primer pair #2 against VHSV, SVCV, IPNV WSBV and no RNA negative control, respectively.

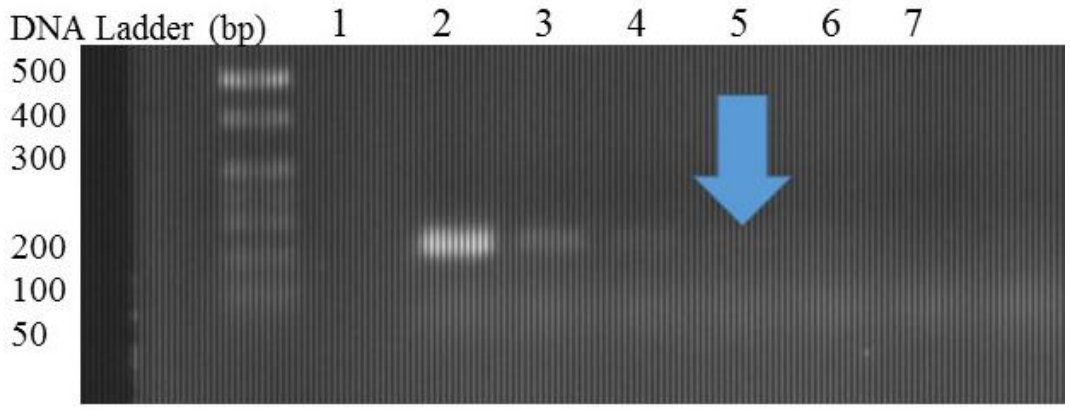

Figure 10. Detection limit of WSBV RNA with primer pair #1. Lane one is negative control with RNA from uninfected media. Lane two is undiluted WSBV RNA, lane three 100 fold dilution of WSBV RNA and two-fold dilutions of WSBV RNA lanes 4-7. Blue arrow indicates detection limit.

Preliminary Survey for WSBV in Wisconsin White Suckers

A preliminary survey of wild white suckers was performed to characterize the prevalence and geographic range of WSBV. Six lakes in Northern Wisconsin and six watersheds in Southeastern Wisconsin were surveyed (Figure 1). The fish from Northern Wisconsin were actively spawning at the time and were caught using nets. The fish from Jersey Valley Lake were also actively spawning and caught using electroshock. The remaining watersheds were sampled using electroshock methods. Cultures from kidney and spleen tissue samples showed no CPE and were negative by RT-PCR (Table 3).

Table 3. Survey of wild white sucker populations in Wisconsin for the presence of WSBV.

Waterbody (Wisconsin County)	Month Fish Collected	Cytopathic Effects	RT-PCR Result
Jersey Valley (Vernon)	May	No	Negative
Rat Lake (Forest)	May	No	Negative
Big Muskellunge Lake (Vilas)	May	No	Negative
Trout Lake (Vilas)	May	No	Negative
Lac Vieux Desert (Vilas)	May	No	Negative
Lake Tomahawk (Oneida)	May	No	Negative
North Twin Lake (Vilas)	May	No	Negative
Mormon Coulee Creek (La Crosse)	August	No	Negative
Little La Crosse River (Monroe)	August	No	Negative
Timber Coulee Creek (Vernon)	August	No	Negative
Halfway Creek (La Crosse)	October	No	Negative
Beaver Creek (Trempealeau)	October	No	Negative

Experimental Challenge of Juvenile White Suckers with WSBV

The experimental challenge of juvenile suckers with WSBV was conducted to discover if the virus could be pathogenic in this species. An experimental infection with a WSBV isolate is the best way to characterize the signs of disease in a controlled setting. The fish used in this study were taken from a population that previously tested negative for WSBV, IPNV, VHSV and SVCV via PCR assay. Tissue culture results did, however, show CPE in four of the 12 lots from this original sampling (data not shown). The CPE did not look like any other known viral CPE and, since the PCR results were negative for the known viruses, the viral agent is considered unknown. A second group of fish from the same source was used for the experimental infection despite a possible unknown viral

agent in these fish because no other source of uninfected white suckers could be tracked back to a single waterbody.

The experimental challenge ran from February 17th 2014 until March 9th 2014 at the LFHC. Four separate 25-gallon tanks that housed 20 fish each were used which simulated crowded conditions seen in bait shops. The fish were not individually identified. The tanks were each separately aerated and filtered to make sure the water did not mix between any of the tanks. Eighty fish were used in the experimental challenge. Forty of these fish were infected with WSBV, 20 fish were injected with TES buffer only, and 20 fish remained unhandled after being placed in the tanks. The 20 fish that remained untouched were used to control for water conditions/transportation stress. When fish from this tank started to die, it was assumed that the water conditions had deteriorated or the fish were dying from starvation/stress. The water was not changed in the tanks but, water was added back to the tanks twice throughout the infection because of evaporation. The fish were monitored once a day during the infection, usually mid-morning, and dead fish were taken out of the tank to be processed.

Fish infected with WSBV started to die one week post infection and continued to die over the next three weeks (39 total). Fish from the control tanks (six total) started to die during the last (third) week of the experiment, possibly due to declining water quality or lack of feeding, despite being provided food (Figure 11). None of the control fish that died had food in their stomachs but other control fish had fed. During the second week, two healthy fish from each of the control tanks were taken and dissected to compare internal and external tissue morphology to the infected fish (Table 4).

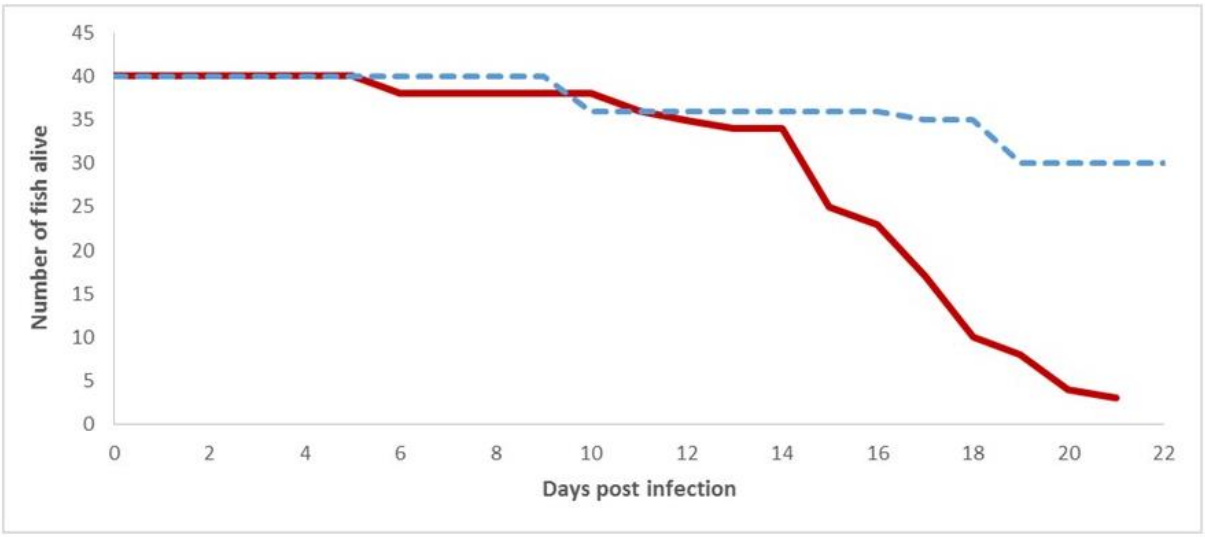

Figure 11. WSBV experimental infection death of fish over the 21 day infection period. WSBV infected fish red solid line (n=40) and all control fish blue dashed line (n=40). Control fish between days eight and ten were collected from control tanks and euthanized.

Of the experimentally infected fish, nine fish demonstrated erratic swimming throughout the infection, no erratic swimming was noted in the control fish (Table 4). This erratic swimming included spiraling, twitching and floating. Tremors were seen in two of the infected fish when they were taken out of the tank for dissection on March 6th. All of the experimentally infected fish demonstrated lethargy when compared to the control fish. This behavior was noted when the lights were turned off and back on. The control fish reacted by clustering and swimming quickly to get out of the light while the infected fish had little to no reaction.

Table 4. Spring 2015 WSBV experimental infection mortality and pathology of juvenile white suckers.

WSBV infected mortalities (n=40)	Days post infection**	Erratic swimming	Viral pathology
0	2	Yes	None
2	5	Yes	None
0	6	Yes	None
0	7	Yes	None
0	8	Yes	None
3*	10	No	None
1	11	No	Yellowing
1	12	Yes	None
0	13	Yes	None
9	14	Yes	Hemorrhages + yellowing
2	15	No	Yellowing
6	16	No	Yellowing
7	17	No	None
2	18	Yes	Hemorrhages
4	19	No	None
1	20	No	Hemorrhages
2	21	No	Hemorrhages
Mock infected mortalities (n=20)			
0***	10	No	None
1	18	No	None
4	20	No	None
Untouched control fish mortalities (n=20)			
0***	10	No	None
1	20	No	None

*One healthy fish was collected this day

**Forty fish were infected on day zero

***Two healthy fish were collected from each group

External examination of the infected fish revealed hemorrhaging from mouth, fins, and anal vent in five of the infected fish that had died (Figure 12). The infected fish overall became very pale, losing most of their juvenile spots which was noted in the tanks

and upon necropsy (Figure 13). It was difficult to judge if the lack of color after 12 hours was due to hemorrhaging in the deceased fish or due to necrosis.

Figure 12. WSBV infected white sucker (above) and uninfected white sucker (below). Infected fish shows hemorrhaging from various parts of the body. Pictures were taken 1 hour post mortem.

Figure 13. External appearance of WSBV infected white sucker (above) and an uninfected white sucker (below). Fish had been deceased for 1 hour before picture was taken. The infected fish exhibits pale appearance with lack of juvenile spots. Uninfected fish retained juvenile spots.

Necropsies were performed on all fish and internal signs of disease were compared between the infected fish and uninfected fish. Signs of internal organ damage were noted in 38/40 of the infected fish, mainly gallbladder and spleen damage. Spleens and gallbladders in the infected fish became very pale and mushy compared to the uninfected fish (data not shown). The livers of the infected fish were also very pale. These organs of the infected fish became hard to distinguish due to their deterioration, while the spleens, livers and gallbladders of the uninfected fish remained intact and easily identifiable. The damaged gallbladders of the infected fish left a yellow stain visible on the sides of the fish (Figure 14). The study was ended on the 9th of March and all the remaining fish were euthanized including one infected fish from tank #1, 14 buffer-injected fish from tank #3 and 17 fish from tank #4.

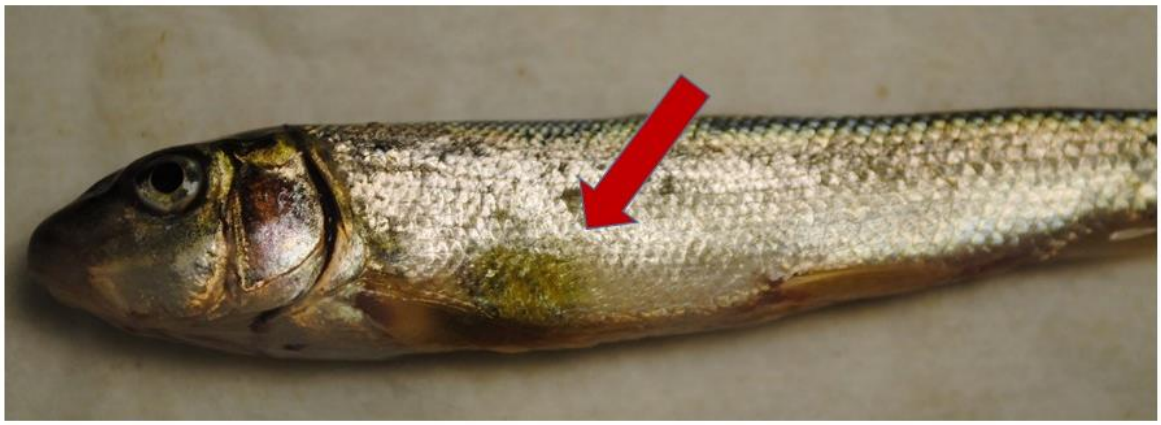

Figure 14. WSBV infected white sucker demonstrating gallbladder staining due to internal gallbladder damage.

Homogenates of all the WSBV infected fish tissues were plated individually on EPC cells in 24-well tissue culture plates and all of these samples produced CPE within a week of being plated (Table 5). The control fish were pooled into groups of five-fish pools and plated on EPC cells in 24-well tissue culture plates and none of these samples produced CPE over the 28 day incubation period (Table 5). The longer incubation of the control samples was done to check for the unknown virus previously isolated in these fish. All of the WSBV infected fish were found positive via tissue culture and RT-PCR assay. All of the control fish, including the mortalities, were found negative for WSBV via tissue culture and RT-PCR assay (Table 5).

Table 5. Spring 2015 WSBV experimental infection tissue culture and RT-PCR results.

Number of WSBV infected fish processed	Days post infection	Tissue culture result	Diagnostic RT-PCR result
2	5	Positive	Positive
4	10	Positive	Positive
1	11	Positive	Positive
1	12	Positive	Positive
9	14	Positive	Positive
2	15	Positive	Positive
6	16	Positive	Positive
5	17	Positive	Positive
2	18	Positive	Positive
4	19	Positive	Positive
1	20	Positive	Positive
3	21	Positive	Positive
Number of mock infected fish processed			
2	10	Negative	Negative
1	18	Negative	Negative
4	20	Negative	Negative
14*	21	Negative	Negative
Number of untouched fish processed			
2	10	Negative	Negative
1	20	Negative	Negative
17*	21	Negative	Negative

*These remaining living fish were euthanized and processed for diagnostic assay

DISCUSSION

A novel virus of white suckers was partially characterized and its geographic range and pathogenicity was studied. Based on its genome sequence, the virus can be presumptively placed in the family *Bunyaviridae*, and appears to be most closely related to viruses in the *Orthobunyavirus* genus. A diagnostic one-step RT-PCR was developed and used to confirm infections with WSBV during a preliminary survey and experimental challenge. This assay will be used by the LFHC and other fish health labs to confirm future isolations. The survey did not detect the virus in the 12 watersheds sampled. An experimental challenge of juvenile white suckers with WSBV showed its virulence when injected intraperitoneally at high doses (10^7 PFU).

Preliminary Classification of WSBV in Genus *Orthobunyavirus*

WSBV seems to be most closely related to viruses in the genus *Orthobunyavirus* based on BLAST searches conducted with the translated L segment (RDRP) of the WSBV genome. Because the RDRP is often the most highly conserved protein of RNA viruses, analysis of the sequence is most likely to reveal the similarity between distantly related viruses. Global pairwise alignments were conducted with the WSBV partial L segment protein sequence and five known Orthobunyavirus L segment protein sequences. These alignments revealed that WSBV has between 22.4-24.3% similarity with these known Orthobunyaviruses. Furthermore, WSBV partial L segment has 43.8% similarity to the LMBBV partial L segment (Table 2). To determine if there were any conserved sequences between WSBV and other viruses in this genus, a multiple sequence alignment

was done with WSBV and five known orthbunyaviruses (Khurdun virus, Tete virus, Shuni virus, Pacui virus and Douglas virus) and novel fish virus LMBBV. The multiple sequence alignment revealed that the most conserved residues are found between amino acid numbers 900-1145 in all seven viral sequences (Figure 5). This section of the RDRP has many highly conserved residues known as the "polymerase module" which function for the catalytic activity of the polymerase (44). WSBV and LMBBV seem to be more closely related to each other through this analysis and both viruses are derived from fish hosts. The other five orthobunyaviruses are derived from birds and mammalian hosts with arthropods as vectors. To date, this genus has no documented fish viruses and WSBV may be one of the first to be documented.

Many viruses in this genus are vectored by arthropods. How WSBV is transmitted in nature is to be determined, but the possible role of a vector should be examined. White suckers feed primarily on invertebrates in their environment and may contract the virus in this way. Parasites of fish are also invertebrates and fish may contract viruses when these invertebrates feed off of them. Many other fish viruses are related to viruses vectored by arthropods, however no studies have been done to experimentally test this (15). Also, the rapid identification of RNA viruses in insects, due to platforms such as next generation sequencing, has recently detected many viruses in the *Orthobunyavirus* genus that are carried by many different arthropods, mainly mosquitos (5). A survey looking for WSBV in aquatic invertebrates or other waterborne arthropods could be conducted to explore this possibility. Knowing the classification of WSBV will help fish health professionals in identifying cases and containing diseased fish.

WSBV Diagnostics and Fish Health

Recognizing CPE under low power inverted phase magnification is a necessary skill for WSBV screening in diagnostic settings. WSBV cytopathic effects start when the cells become crenated and all cells eventually detach from the flask. Cytopathic effects caused by WSBV are different from those of other characterized fish viruses. Other fish viruses such as IPNV, Fat head minnow Nidovirus and GSV all form syncytia in EPC cells. VHSV and other rhabdoviruses of fish form raised clumps of EPC cells (1).

The standard process for diagnosis of viral infection of fish used by fish health personnel consists of first culturing the virus from tissue samples in tissue culture cells and then confirming the identity of virus by PCR, fluorescence antibody testing or histology (40). Fish are tested when there is a large fish kill, when fish are being transported to or from hatcheries, when fish are acquired due to illegal activities or during routine pathogen surveys of wild fish. With hatcheries, it is critical that positive identification of pathogens be made. Fish must be certified free from pathogens such as parasites, bacteria, and viruses before they can be transported. If a pathogen is detected, it must be reported to the proper agencies. Depending on the type of pathogen, it is categorized as high or low risk. If it is high risk, the facility is placed under quarantine and fish are not allowed to be moved to or from the facility for an entire season (1). Presumptive findings of a virus from hatchery fish, based on cytopathic effects, make it difficult to be certain about the identity of said pathogen. By developing a diagnostic one-step RT-PCR assay specific for WSBV and sensitive enough to detect virus from tissue culture isolates, this virus can now be identified with a high level of certainty.

Conducting risk assessment analysis of the virus can further help fisheries managers to appropriately respond when WSBV is identified.

In this study, no WSBV-positive samples were detected from the 12 watersheds in seven different counties in Wisconsin. Fish were sampled throughout the spring and summer months and consisted of mainly adult fish. Two watersheds sampled by the WIDNR (North Twin Lake and Lac Vieux Desert) that had been previously positive for WSBV via tissue culture in 2011-2012 were negative during this survey (Table 3, Table 6). With the development of the diagnostic RT-PCR, the LFHC was able to test 11 presumptive isolations (based on CPE) of WSBV. By extracting nucleic acids from the saved tissue culture isolates, all 11 presumptive isolations were confirmed positive for WSBV. WSBV was confirmed in wild fish and baitfish in Wisconsin. Fish from two baitfish dealers in Minnesota and one baitfish dealer in Wisconsin also tested positive for WSBV (Table 6).

Table 6. Presumptive WSBV isolations confirmed with WSBV one-step RT-PCR at the La Crosse Fish Health Center.

Case Year	Source	Species	Sample Type	County, State
2010	Baitfish dealer	white sucker	Kidney/spleen	Otter Tail, MN
2010	Baitfish dealer	white sucker	Kidney/spleen	Carver, MN
2010	Baitfish dealer	white sucker	Kidney/spleen	Otter Tail, MN
2010	Baitfish dealer	Mix*	Kidney/spleen	Portage, WI
2011	Nelson lake	white sucker	Ovarian fluid	Sawyer, WI
2011	Lac Vieux Desert	white sucker	Ovarian fluid	Vilas, WI
2011	Turtle Flambeau Flowage	white sucker	Ovarian fluid	Iron, WI
2011	Baitfish dealer	white sucker	Kidney/spleen	Portage, WI
2011	Baitfish dealer	northern dace	Kidney/spleen	Portage, WI
2012	North Twin Lake	white sucker	Ovarian fluid	Vilas, WI
2014	Rat Lake	white sucker	Ovarian fluid	Forest, WI

* Mix includes white suckers and other species of fish

Many factors could contribute to not finding the virus during the survey including: tissue selection, age of fish, sample size, and fish health. Selection of kidney and spleen tissue for this survey was based on the organs being important in fish immune function. The spleen filters out immune cells from the blood that the body uses to combat viral infections and the kidneys also act as a filter for the blood. These are the typical tissues tested when fish are being surveyed for the presence of viruses (40). Ovarian fluid was not tested in this survey, but has been found positive for WSBV. In 2014, ovarian fluid samples from Rat Lake were tested by the LFHC (Table 6) and were positive for WSBV by tissue culture CPE and via RT-PCR assay. My sampling of spleen and kidney tissue from white suckers from this lake did not detect WSBV, however, previous isolations of WSBV were made from kidney and spleen tissue from young fish being sold

as baitfish. It is possible that infection of juvenile white suckers results in widespread dissemination of virus in the fish, but as the fish ages the virus becomes restricted to the reproductive tissue.

A sample size of 360 fish may not have been a large enough sample size to detect WSBV. In another study looking at viruses in baitfish 1,204 white suckers were tested and viruses were detected in 8% of these fish via tissue culture isolation (33). Virus prevalence may be lower in wild fish compared to baitfish because stressors placed on baitfish may lead to increased infections and spread of the viruses.

Fish health (and therefore increased susceptibility to infection) may be influenced by environmental changes and mating activities. Environmental stressors include high water temperatures, low dissolved oxygen levels and freeze/thaw cycles (22). When water temperatures rise and dissolved oxygen levels drop, this affects the ability of the fish to fight disease and can result in large fish kills. Baitfish are continually stressed due to crowded housing conditions and extensive handling (48). Wild fish, as sampled in this survey, are not placed under these additional stressors. During mating, wild fish gather in large densities increasing the likelihood for viral transmission (22). Fish from Vilas County and Jersey Valley Lake were actively spawning when caught. Male fish collected in this survey displayed dark lateral striping which is an indicator of spawning activity. However, because spawning activity occurs in the fishes' natural environment it is much less stressful to fish than being caught and handled like baitfish routinely are.

Geographic Range

The geographic range, host specificity and tissue distribution of WSBV are yet to be concretely determined. Based on the previous isolations of WSBV, the virus seems to

be most often detected in northern Wisconsin and central Minnesota (Table 6). However, white suckers have a large geographic range across the Midwest and central states. Further surveys should be conducted on baitfish stocks and wild fish in different bodies of water, in which the tissue collected should include kidney, spleen and ovarian fluids. WSBV was detected in ovarian fluids of adult fish and kidney/spleen tissue of juvenile fish. Collecting samples from previously positive lakes every year at different times of the year could relate a seasonality to WSBV infections. Also, since several baitfish sources were found positive for WSBV, other surveys could focus on baitfish suppliers and different species of baitfish. Many baitfish dealers mix different species of baitfish together in holding tanks, which can increase transmission of viruses between species of fish. A single confirmed case in which northern dace (*Chrosomus eos*) tested positive for WSBV (Table 6), suggests that this virus may infect this species of fish as well.

Experimental Infection and Disease Signs

WSBV is highly virulent to juvenile white suckers injected with high concentrations of the virus. The fish in this study appeared healthy with no signs of disease before the experiment started. It remains uncertain whether or not the unknown viral agent could have had an effect on these fish in this study. This unknown agent may have led to higher mortalities in the infected fish or even the mortalities in the control fish. However, because the tissue culture results from the control fish in this study remained negative for unknown virus, it seems to have had little impact on the results but should remain a consideration. All infected fish were found positive for WSBV and 39 of the 40 fish died presumably from the infection with WSBV. Initial signs of infection were subtle changes in behavior such as, erratic swimming and/or lethargy. Upon noting

fish that exhibited behavioral changes in a certain tank, it was also noted that within three to five days there would be dead fish in that same tank. The fish were not individually identified so a direct correlation with behavior and eventual death could not be established. External signs of infection included loss of juvenile spots and overall pallor, (Figure 13) as well as hemorrhaging at fin bases and gills (Figure 12). Internal signs of disease consisted of damage to the liver, spleen and gallbladder. The liver and spleen tissues became very pale and mushy and the gallbladder damage caused bile to leak into the abdomen, leaving a visible external stain (Figure 14).

If infection with this virus was to occur in wild fish, this pathology could lead to large fish kills involving white suckers. According to the WIDNR, most of the documented fish kills in Wisconsin that involve white suckers are suspected (confidence code) to be due to environmental conditions (Table 7). In 2012, fish kill investigations from January through September conducted by the WIDNR could not link any white sucker deaths to a particular virus. However, a bacterial disease called Columnaris does occasionally contribute to massive death of white suckers and other species of fish when water temperatures rise (Table 7). Looking for the WSBV disease signs in white suckers when a fish kill occurs will help in identifying the cause of future fish kills.

Table 7. Wisconsin fish kill investigations in 2012 that involved white suckers (adapted from the WIDNR website).

Start Date	County	Waterbody	Confidence Code	Cause of Kill
May 17	Sauk	Delton Lake	Known	Columnaris
June 20	Dodge	Rock River	Suspected	Low dissolved oxygen
June 21	Dodge	Rock River	Suspected	Low dissolved oxygen
June 22	Rock	Beckmans Millpond	Suspected	Low dissolved oxygen
July 6	Dodge	E. Branch Rock River	Suspected	Temperature
July 6	Fond Du Lac	W. Branch Rock River	Suspected	Low dissolved oxygen
July 7	Jefferson	Lake Koshkonong	Suspected	Temperature
July 7	Sheboygan	Sheboygan River	Suspected	Temperature
July 9	Marquette	Harris Pond	Suspected	Temperature
July 11	Columbia	Lazy Lake	Suspected	Temperature
July 19	Outagamie	Black Otter Lake	Known	Low dissolved oxygen
July 26	Sauk	Delton Lake	Suspected	Unknown
August 16	Milwaukee	Oak Creek	Unknown	Unknown

Management Implications and Further Risk Assessment

A risk assessment of WSBV was done with a survey of wild white sucker fish in Wisconsin and an experimental infection of juvenile white suckers. This assessment, along with previous surveys, indicated that WSBV can be found in wild fish and baitfish in Wisconsin and can cause disease in juvenile white suckers. Management agencies should remain cautious about this virus while further risk assessment is performed. As of now, the risk to wild sucker populations appears to be low, while the white suckers used as baitfish seem to be at a higher risk of infection. White suckers are a popular baitfish in Wisconsin and can potentially transmit viruses to other bodies of water when anglers use

them in multiple lakes and rivers. Because WSBV is found in this highly marketable baitfish, education for anglers and baitfish dealers about viral pathogens should continue. Furthermore, because the transmission and route of infection for WSBV is still unknown, decontamination protocols should be put in place when this virus is detected and further studies to find the route of infection should be conducted. Decontamination protocols usually consist of disinfecting water and surfaces with quaternary disinfectant or bleach (1, 41). WSBV could possibly spread via contaminated water or from direct contact with infected fish. Water borne transmission as a route of infection could be tested by placing fish in water containing the virus and then monitoring fish for disease signs. Contact transmission could be tested by housing infected fish with uninfected fish to see if the virus transmits between the fish and disease signs manifest.

This study demonstrated that high doses of virus delivered directly to highly susceptible fish does lead to disease progression. However, natural disease in adult fish and wild fish populations is still unknown. Further studies which include different infectious doses and different species of fish could be conducted. Various amounts of virus injected directly into fish could help find the lowest infectious dose for WSBV. There is some anecdotal evidence that other species of fish (northern dace) could contract this virus, so experimental infections with other species of baitfish should also be conducted (Table 7).

Overall, the risk to white sucker populations across the geographic range of this species remains unknown. WSBV does cause disease and some of the signs of disease have been documented here. Now that some disease signs are known and a reliable

diagnostic test has been developed, further study can proceed with this virus and the U.S. Fish and Wildlife Service can reliability identify this virus as a pathogen in the future.

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