

UNIVERSITY OF WISCONSIN-LA CROSSE

Graduate Studies

CHARACTERIZATION OF A UNCLASSIFIED VIRUS AND  
SURVEY FOR ITS PRESENCE IN WISCONSIN BLUEGILL POPULATIONS

A Manuscript Style Thesis Submitted in Partial Fulfillment of the Requirements for the  
Degree of Master of Science in Biology

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College of Science and Health

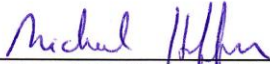
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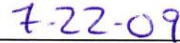
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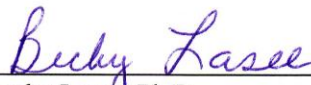
By Marisa Barbknecht


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


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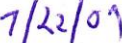
  
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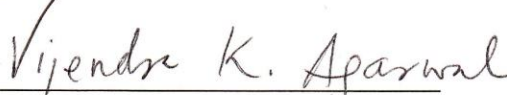
  
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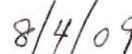
  
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## ABSTRACT

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Bluegills (*Lepomis macrochirus*) are important both ecologically and as sport fish. Viral disease is a threat to bluegills and other fish species. Six viruses of bluegills have been previously described, many which are able to infect other fish species. In 2001, the La Crosse Fish Health Center (LFHC) isolated a novel bluegill virus (BLGV) collected from a bluegill kill in Montana Lake in northeastern Wisconsin. Since then, the LFHC has reported multiple presumptive BLGV isolations from 14 Wisconsin locations during routine inspections of hatchery fish and wild fish health surveys, but has had no confirmatory assay. Using BLGV genomic sequence obtained during this study, a diagnostic RT-PCR for BLGV was developed. The assay confirmed BLGV isolation from 5 of 17 locations included in a preliminary survey for the virus in Wisconsin bluegills, and the LFHC has used it to confirm more recent cell culture isolations from bluegills and black crappies from Wisconsin, Illinois, and Ohio. Analysis of the genomic sequence shows BLGV is most closely related to members of the *Parechovirus* genus within the family *Picornaviridae*. However, more analysis must be performed before we can determine if BLGV should be placed within the *Parechovirus* genus or within a new genus.

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## INTRODUCTION

Bluegills (*Lepomis macrochirus*) are members of the family *Centrarchidae*. Other members of this family include smallmouth and largemouth bass (*Micropterus* spp.), rock bass (*Ambloplites rupestris*), and crappies (*Pomoxis* spp.) (7, 56). The common name bluegill reflects the blue coloration of the lower jaws and operculum of these fish. Bluegills are relatively small fish, with an average length of 15-20 cm and average depth of 7.5-10 cm. They are laterally compressed, or pan-shaped, so even larger bluegills barely reach 2.5 cm wide (7, 32, 56).

The native range of the bluegill in North America includes both the Great Lakes and Mississippi River basins. However through deliberate stocking programs, the current range has expanded to include most of the continental U.S. and Hawaii, Puerto Rico, southern Canada, and Mexico (21). In Wisconsin, bluegills are the most abundant panfish, and often sport fish, in many lakes. Because they are fairly easy to catch, young and first-time anglers gain interest and experience by targeting bluegills, and enjoy eating their catch. Bluegills provide nourishment for other animals as well. They are hosts to the glochidial stage of many important native freshwater mussels (7), and young bluegills are forage for largemouth bass (*Micropterus salmoides*), northern pike (*Esox lucius*), bullheads (*Ameiurus* spp.), and other panfish (7, 56). For these reasons, it is important to maintain healthy bluegill populations.

## **Viruses of Bluegills**

Fish viruses pose a threat to aquatic ecosystems and the fishing industry. Viruses are spreading to new geographic locations by way of fish transports and invasive species. As viruses evolve they may increase their impact on fish health by becoming more virulent or by expanding their host specificities. As a result, the impact of fish virology is extensive.

The described viruses of bluegills include hepatic necrosis reovirus, lymphocystis disease virus, largemouth bass virus, spring viremia of carp virus, bluegill virus, and viral hemorrhagic septicemia virus. The United States Fish and Wildlife Service La Crosse Fish Health Center (LFHC) has isolated an uncharacterized virus of bluegills that has been associated with fish disease on multiple occasions. Understanding the physical characteristics, host range, and virulence of these six described viruses and how they have evolved could help to better understand the LFHC-isolated bluegill virus.

### **Bluegill Hepatic Necrosis Reovirus**

Bluegill hepatic necrosis reovirus (13p<sub>2</sub>) is in the family *Reoviridae* (41-44, 65). Reoviruses are non-enveloped and icosahedral, and average between 70 and 85 nm in diameter (46). The 13p<sub>2</sub> genome is composed of 11 segments of double-stranded (ds) RNA. Its icosahedral capsid is dual-layered, with four structural proteins forming the outer capsid and three forming the inner capsid. Of these seven structural proteins, five can be easily visualized by SDS-PAGE and are approximately 135, 128, 70, 45, and 33 kD (46, 65).

In the late 1970s, 13p<sub>2</sub> was first isolated from juvenile American oysters (*Crassostrea virginica*) from a Long Island, New York hatchery. The oysters were alive

and appeared healthy, and were not suspected to be the primary host for the virus (41). Because the virus was first propagated in bluegill fry-2 (BF-2) cells, bluegill fingerlings were first to be experimentally inoculated with 13p<sub>2</sub> alongside oysters. Infected bluegills developed acute hepatitis with a high mortality rate, while the virus failed to replicate in the experimentally inoculated oysters (42). Later studies showed 13p<sub>2</sub> is also infectious but less virulent in rainbow trout (*Oncorhynchus mykiss*), where it causes a chronic form of hepatitis (43).

These studies provide solid evidence to suggest that the host for 13p<sub>2</sub> is a teleost, but because both bluegills and rainbow trout are freshwater fish, they seem to be unlikely hosts in the wild. However, because no 13p<sub>2</sub>-associated disease has been reported in either marine or freshwater teleosts isolated from the wild, little subsequent 13p<sub>2</sub> research has been done.

### **Lymphocystis Disease Virus**

Lymphocystis disease virus (LCDV) is a member of the *Lymphocystivirus* genus in the family *Iridoviridae*. Lymphocystiviruses are non-enveloped and icosahedral, with a diameter between 200 and 300 nm. Their genomes are composed of dsDNA ranging from 103 to 186 kbp (63, 66).

Lymphocystis disease was first reported in the late 1800s, though the virus was not determined to be the etiologic agent of the disease until the first half of the 20<sup>th</sup> century (62, 66). LCDV has an extensive host range, with over 100 marine and freshwater teleost species susceptible to infection, including bluegills. Bluegills were the preferred specimens used to study *in situ* LCDV replication and pathology (62, 67). These pathological studies revealed the main clinical sign of lymphocystis disease is the

development of wart-like benign tumors on the surface of infected fish. Histology showed these tumors are composed of hypertrophied mononucleated fibroblasts (62, 68).

Because the wart-like tumors are found on the skin, LCDV can be spread through both direct physical contact and shedding into the water when tumors rupture. The disease is self-limiting but still could be problematic within the commercial fishing industry, as diseased fish are visually undesirable. LCDV infection is confirmed by histological examination of tumors (66).

### **Largemouth Bass Virus**

Largemouth bass virus (LMBV) is also in the family *Iridoviridae*, but in the *Ranavirus* genus. Ranaviruses are smaller than lymphocystiviruses, averaging 150 nm in diameter, and have dsDNA genomes averaging 105 kbp. Largemouth bass virus particles are icosahedral and appear to be variably enveloped, which suggests an envelope is not required for infectivity (63).

LMBV was first isolated in 1991 in largemouth bass from Lake Weir, Florida; however, the first largemouth bass kill attributed to LMBV infection occurred later in Santee-Cooper Reservoir, South Carolina, in 1995 (26, 28, 50). This was the first report of virus-associated death in centrarchids (50). Since then, LMBV has been associated with localized largemouth bass kills involving up to 3,000 fish in a single event (37). LMBV isolations have been reported from midwestern, northeastern, and throughout southern regions of the United States, and the virus continues to spread (38).

Clinical signs of LMBV infection include formation of hemorrhagic skin lesions and accumulation of fluid in the swim bladder, loss of equilibrium, gill necrosis, and death (28, 50). LMBV is only known to cause disease in largemouth bass. However,

asymptomatic infections are also common in this species. The variable virulence of LMBV is affected by fish age and environmental stressors, such as elevated water temperatures, low dissolved oxygen, and fishing pressure. Localized immunity or genetic variability within largemouth bass populations may also affect LMBV virulence (28).

Although LMBV-associated disease has only ever been reported in largemouth bass, common prey and cohabitants including bluegills, crappies, smallmouth bass, and chain pickerel (*Esox niger*) can carry the virus (28, 37). Because LMBV can be transmitted horizontally either in the water column or by eating infected prey, the presence of LMBV is monitored in all potential carriers (28). Presumptive tissue culture isolation from fathead minnow or BF-2 cells followed by PCR-based diagnostic testing is the routine process for confirming LMBV infection (4, 28).

### **Bluegill Virus**

Bluegill virus (BGV) has not been fully classified, but is suspected to be a member of a novel virus family (10). BGV is enveloped, has a diameter between 70 and 120 nm, and is presumed to have an icosahedral capsid (8, 10, 31, 52). Its genome is composed of non-segmented single-stranded (ss) RNA (52, 53). BGV was first isolated from a bluegill with epitheliocystis, a chlamydial disease that causes large white cysts on the infected fins and gills. BGV was passaged in the BF-2 cell line long after the bacterial agent was lost (31), and was then studied for the next 15 years (66).

BGV grows in several cell lines but replicates best in centrarchid-derived cell lines like the BF-2 cell line, causing a cytopathic effect (CPE) resembling a wagon wheel (radial extension of elongated cells from a central point) (31). Yet, experimental infection of juvenile bluegills with BGV has been unsuccessful, and no determination of

BGV pathogenicity in bluegills of any age has been reported. Only two isolations of BGV have been reported, the last occurring in 1979. Because BGV was isolated so rarely and appeared to have no virulence in bluegills, interest in the virus faded in the 1980s (66).

### **Spring Viremia of Carp Virus**

Spring viremia of carp virus (SVCV), or *Rhabdovirus carpio*, is a member of the *Vesiculovirus* genus in the family *Rhabdoviridae* (6, 22, 61). SVCV is an enveloped, bullet shaped virus that averages 75 nm by 135 nm (66). The SVCV genome is composed of approximately 11 kb of ssRNA coding for five structural proteins that are approximately 240, 57.5, 47, 35.5, and 25.5 kD (6, 61, 66).

SVCV was first isolated in Yugoslavia in 1971 (20). Since then, SVCV isolations have been reported from other regions of Europe, the Middle East, China, and the Americas (2). SVCV causes an acute hemorrhagic disease most often affecting common carp (*Cyprinus carpio*), although the virus can cause disease in other cyprinids, including koi (*C. carpio koi*), grass carp (*Ctenopharyngodon idella*), fathead minnows (*Pimephales promelas*), and golden shiners (*Notemigonus crysoleucas*) (24). Other fish experimentally infected with SVCV, which could thus act as carriers, include northern pike, perch (*Perca* ssp.), and pumpkinseed (*Lepomis gibbosus*) (2). In June 2008, SVCV was isolated for the first time from bluegills and largemouth bass; however, neither species showed clinical signs of SVCV infection (38).

SVC can cause extreme morbidity and mortality in susceptible fish populations. External clinical signs of SVCV infection include abdominal distension, pale gills, exophthalmia, darkened skin, inflamed vent, and petechial hemorrhaging of the skin,

gills, and eyes. Internally, diseased fish have extensive edema, focal lesions on the swim bladder and other organs, inflamed intestines and swollen spleen (2, 6). SVCV is most virulent at water temperatures between 5 and 20 °C, with disease and mortality most commonly observed in young fish. SVCV is transmitted horizontally via mucous secretions, urine, and fecal casts, and via invertebrate vectors (2, 6, 66).

SVCV has most severely impacted farmed fish (6, 14) but does threaten wild fish populations. Recent wild carp kills due to SVCV have occurred in several locations in the United States, including Cedar Lake, Wisconsin (24) and Pool 8 of the Upper Mississippi River (38). Because sport and bait fish other than carp are susceptible to infection and/or disease, more problems could arise if SVCV becomes increasingly virulent in these species. Therefore, surveillance and control of SVCV spread is important. Presumptive tissue culture isolation followed by PCR-based diagnostic testing is the routine process for confirming SVCV infection (2, 4).

### **Viral Hemorrhagic Septicemia Virus**

Viral hemorrhagic septicemia virus (VHSV), or Egtved virus, is a member of the *Novirhabdovirus* genus in the family *Rhabdoviridae*. VHSV is an enveloped, bullet shaped virus that averages 60 nm by 180 nm. The VHSV genome is composed of 11-12 kb of ssRNA. VHSV has five structural proteins that are approximately 172, 80.5, 45, 27.5 and 22.5 kD (66).

VHSV may be the most serious of all described fish viruses because it causes severe disease in many fish species worldwide. Different genogroups of this virus have been responsible for extreme devastation of European trout farms and have caused numerous mass mortality events in marine fish (55). Recently, a new sublineage of the

North American genogroup (genogroup IVb) emerged in the Great Lakes region and has been responsible for a number of large fish kills of many fish species (3, 64). A notable mortality event involving bluegills, freshwater drum (*Aplodinotus grunniens*), smallmouth bass, and crappies occurred in Lake Ontario in the spring of 2006 (3), which placed bluegills on the ever-growing list of fish species susceptible to VHSV infection (1, 3, 30, 55).

Historically, the virus is presumed to have been maintained in native European marine salmonid populations for centuries without reported virulence. Soon after the introduction of rainbow trout into Europe near the end of the nineteenth century, scientists began noticing farmed trout with clinical signs of disease resembling viral hemorrhagic septicemia, which include darkened skin, enlarged kidneys, and hemorrhaged muscles, eyes, and internal organs (55). Although presumed for decades, the viral etiology of the disease was not confirmed until the 1960s. Soon after, efforts to visualize the virus by transmission electron microscopy (TEM) were successful and thus, TEM was used for early detection of VHSV (66). In the late 1980s, the first isolation of VHSV outside of Europe was reported. The virus was isolated from Atlantic salmon (*Salmo salar*) off the coast of Washington state in North America. Near the turn of the twentieth century, VHSV was isolated off the Atlantic coast of North America, near Greenland, and off the Pacific coasts of Japan and Korea (55). Isolations of VHSV from the Great Lakes region soon followed in 2005 (3).

Authorities fear VHSV could threaten sport, bait, and food fishing industries in the Great Lakes and throughout North America. Despite their efforts to prevent the spread of the virus to regions outside of the Great Lakes, it has been isolated from

northern pike from Clear Fork Reservoir near Mansfield, Ohio. Presumptive tissue culture isolation followed by PCR-based diagnostic testing is the routine method used to confirm VHSV infection (4, 64). With increased surveillance of VHSV comes a greater chance for isolating other viruses, including some that may be previously unclassified.

### **Routine Bluegill Viral Diagnostics**

When adult bluegills are brought into the LFHC, equal amounts of spleen, kidney, and swim bladder tissues are removed to screen for VHSV, SVCV, and LMBV. Tissues from five fish are pooled, diluted in HBSS, and homogenized. Diluted homogenate is used to inoculate two cell lines, each in duplicate: BF-2 cells for propagation of LMBV and epithelial papillosum cyprinid (EPC) cells for propagation of SVCV and VHSV. The cells are incubated for 21 days and observed for CPE twice a week. To rule out low virus titer and/or the presence of slow growing virus, culture media from cells in one well of each duplicate showing no CPE are pooled, filtered, and re-passaged onto both cell lines at two-weeks post-inoculation. Media from cells that exhibit CPE are filtered and re-passaged in the cell line in which CPE was observed. If CPE is observed after re-passage, nucleic acid is extracted from the media and diagnostic PCR is performed on each of the nucleic acid extracts (4).

### **Uncharacterized Virus of Bluegills**

In 2001, the LFHC isolated a virus from bluegills collected from a bluegill kill at Lake Montana, Wisconsin, that will be referred to as bluegill virus, BLGV. During routine diagnostic screening, the virus could only be propagated in BF-2 cells, and isolates tested negative for both VHSV and LMBV using diagnostic PCR. When a similar virus was isolated from Lake Montana bluegills in 2003, infected cells were sent

for TEM. The intracellular virus particles appeared to be non-enveloped, icosahedral, and approximately 30 nm in diameter (Figure 1). No additional characteristics of the virus had been determined by the LFHC; however, based on TEM alone, the virus was suspected to be a novel virus of bluegills.

Before this study, the LFHC presumptively isolated BLGV from bluegills from 14 sites in Wisconsin (Figure 2) as well as several locations within Illinois and Ohio. Presumptive isolation of BLGV is based on recovery of a filterable agent causing CPE in BF-2 cells and testing negative for LMBV, SVCV, and VHSV using diagnostic PCR. There appears to be no optimal season for isolation, because isolations from fish have occurred within nine different months (Table 1). However, infected bluegills have displayed clinical signs of viral infection (hemorrhaging of skin and fins (Figure 3), erythema, pale and swollen internal organs, and swollen vent) more often in spring and fall months than during other times of the year. Notably, bluegills exhibiting these clinical signs of infection frequently have been collected from bluegill kills, with the most recent presumptive BLGV-associated mortality event occurring in May 2009 on Lake Monona (38).

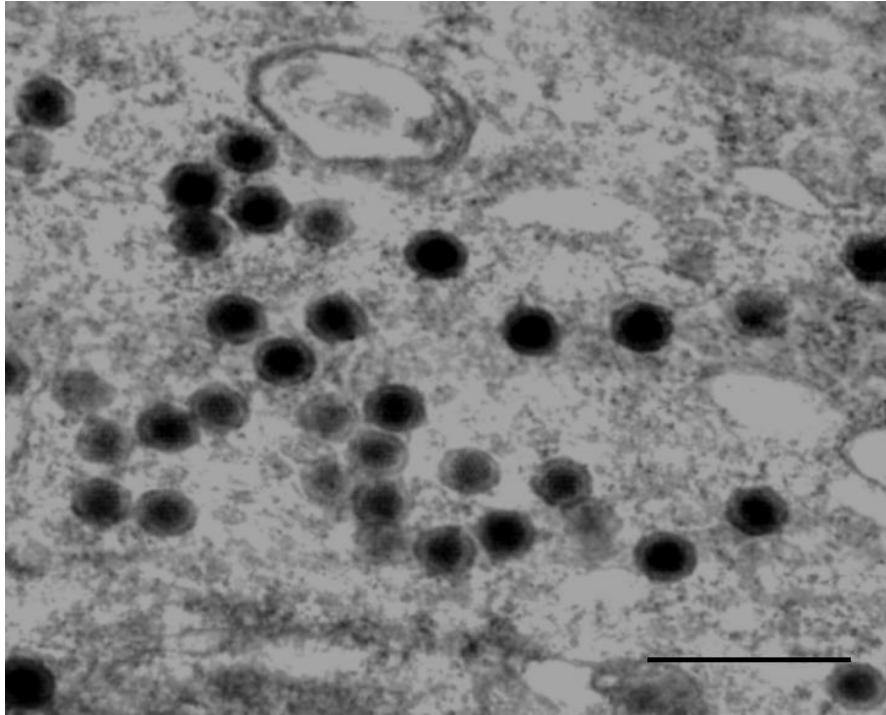
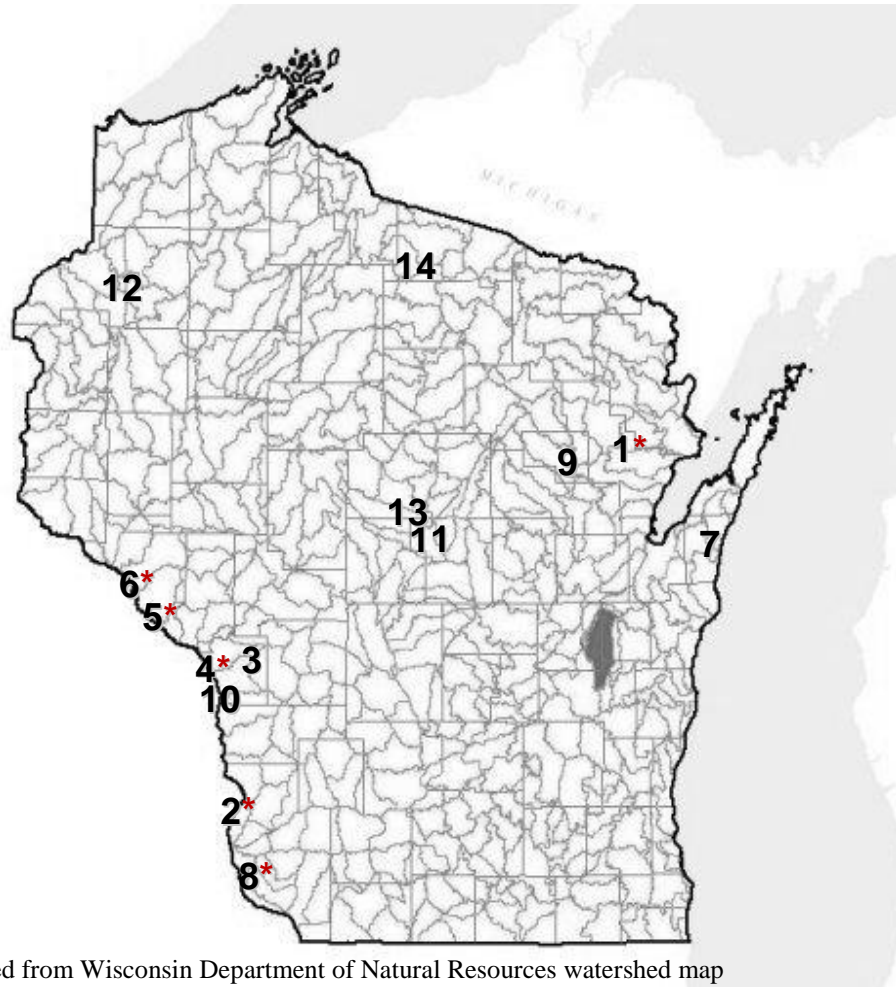


Figure 1. Transmission electron micrograph of virus particles within BF-2 cells. Virus was isolated from infected bluegills from Montana Lake in 2003 (La Crosse Fish Health Center case number 04-032). Scale bar is approximately 100 nm.



- |                     |                   |
|---------------------|-------------------|
| 1- Montana Lake     | 8- UMR Pool 10    |
| 2- UMR Pool 9       | 9- Legend Lake    |
| 3- Lake Neshonoc    | 10- UMR Pool 8    |
| 4- UMR Pool 7       | 11- Second Lake   |
| 5- UMR Pool 5a      | 12- Spooner Lake  |
| 6- UMR Pool 4       | 13- Lake DuBay    |
| 7- East Alaska Lake | 14- Madeline Lake |

Figure 2. Locations of presumptive BLGV isolation from bluegills in Wisconsin before August 2008. Red asterisk indicates sites of multiple isolations.

Table 1. Dates of presumptive BLGV isolations from each Wisconsin water body (December 2001 to August 2008).

Site	Isolation date(s)	
Montana Lake	December	2001
	November	2003
UMR <sup>a</sup> pool 9 (Reno Bottom) <sup>b</sup>	January	2003
UMR pool 9 (Genoa Fish Hatchery)	March	2003
	April	2007
	August	2007
Lake Neshonoc	April	2003
UMR Pool 7 (Lake Onalaska)	June	2003
	June	2005
UMR Pool 4 (Lake City, MN) <sup>b</sup>	July	2003
UMR Pool 5A (Minnesota City, MN) <sup>c</sup>	July	2004
East Alaska Lake	May	2007
UMR Pool 10 (Wyalusing State Park)	June	2007
UMR Pool 10 (Prarie du Chien)	April	2008
Legend Lake	October	2007
UMR Pool 8 (Lauderdale Bay)	November	2007
Second Lake	April	2008
Spooner Lake	April	2008
Lake DuBay	April	2008
Madeline Lake	May	2008

<sup>a</sup> UMR = Upper Mississippi River

<sup>b</sup> sampled from IA side of UMR

<sup>c</sup> sampled from MN side of UMR



Figure 3. Bluegill harvested from 2001 bluegill kill at Montana Lake, Wisconsin with hemorrhaging at fin bases (arrows), a clinical sign of viral infection. Ruler below fish is in cm.

## **RESEARCH OBJECTIVES**

The main objective of this study was to characterize BLGV and begin to determine its geographic distribution within Wisconsin. Because no assay to confirm BLGV infection existed before this study, only presumptive isolations could be reported. In order to develop a diagnostic PCR to confirm BLGV isolation, we first needed to learn more about the characteristics of BLGV. We believed that characterization of BLGV, focusing heavily on genomic sequencing, would allow us to begin classification of this virus and develop a PCR-based diagnostic test that could be used to confirm presumptive positive isolations from a preliminary statewide BLGV survey. Therefore, the following three specific research objectives were designed to effectively complete this study:

1. Characterize BLGV to obtain genomic sequence and classify the virus.
2. Develop a PCR-based confirmatory diagnostic assay for BLGV.
3. Conduct a preliminary survey of the BLGV distribution in Wisconsin bluegills.

## **METHODS**

### **Cells and Virus**

BF-2 and EPC cells were cultured in Eagle Minimum Essential Medium (MEM) supplemented with 10 % fetal bovine serum (FBS). BF-2 and EPC cells were incubated at 25 °C and 15 °C, respectively.

The BLGV isolate that was characterized and used for diagnostic PCR development came from Montana Lake bluegills collected in November 2003 (LFHC case number 04-032).

### **Microscopic Observation of Viral Infection**

Two separate methods were used to study CPE associated with BLGV infection of BF-2 cells. The first method analyzed the progression of BLGV infection throughout a 14-d infection using microscopy at a magnification that is likely to be used in a diagnostic lab, 10X. BF-2 cells (95 % confluent) growing in 6-well tissue culture plates were inoculated with clarified medium from either BLGV-infected BF-2 cells or uninfected cells as a negative control. Growth medium in each well was replaced with either 800 µl of a  $5 \times 10^{-4}$  dilution of inoculum in Hank's balanced salt solution (HBSS) or HBSS (negative control). Cells were incubated at 25 °C for 1 hr to promote viral adsorption. Following adsorption, the inoculum was replaced with 3 ml of MEM (10 % FBS). Cells were incubated at 25 °C and monitored for CPE on 3, 7, 10, and 14 d post-infection (p.i.).

The second method of examination used phase contrast and differential interference contrast (DIC) microscopy at higher magnification to analyze CPE observed

in cells after a 14-d infection. BF-2 cells (30 % confluent) growing on sterile cover slips in separate 6-well tissue culture plates were inoculated with clarified medium harvested from BLGV-infected BF-2 cells or uninfected cells (negative control). Growth medium was removed from cells and 250  $\mu$ l of inoculum and 250  $\mu$ l phosphate-buffered saline (PBS) were added to each well. Cells were incubated at 25 °C for 1 hr to promote viral adsorption. Following adsorption, the inoculum was replaced with 2 ml of MEM (10 % FBS). Cells were incubated at 25 °C for 14 d. Cover slips were submerged in fixative (3 % paraformaldehyde and 0.1 % glutaraldehyde in PBS) for 10 min, rinsed 3 times with PBS, and then mounted onto slides for observation.

### **Concentration of Virus Particles**

To propagate BLGV, cell culture medium was first removed from BF-2 cells (80 % confluent) seeded in 175 cm<sup>2</sup> dishes. Cells were inoculated with 2 ml clarified medium harvested from BLGV-infected BF-2 cells and an additional 1 ml of HBSS to ensure cell coverage. Cells were incubated for 1 hr at 25 °C with gentle rocking every 15 to 20 min to promote viral adsorption. Following adsorption, 17 ml of MEM (10 % FBS) was added to cells. Approximately 10 d p.i. (when cell detachment was first observed) the growth medium was replaced with FBS-free MEM. When only 10 to 20 % of cells remained attached to the tissue culture surface (often observed 12-14 d p.i.), medium was harvested and clarified via low-speed centrifugation (1,500 X g) for 10 min at 4° C. The supernatant was then centrifuged (131,000 X g) through a 40 % (w/w) sucrose/PBS cushion (25 ml supernatant atop 5 ml cushion) for 3 hr at 4 °C. The sucrose cushion allowed virus particles to pellet at the bottom of the centrifuge tube while less dense cell debris remained on top of the cushion. Pellets were resuspended in sterile PBS (20-25  $\mu$ l

per 175 cm<sup>2</sup> cells inoculated). As a control for downstream characterization, growth media from uninfected cells was treated in an identical manner, except cells were inoculated with clarified medium harvested from uninfected BF-2 cells. Concentrated samples were frozen at -80 °C for protein and nucleic acid analysis.

In addition to concentration by high speed centrifugation, infected and uninfected cells from these preparations were also scraped from tissue culture dishes, pelleted via low speed centrifugation (1500 X g) for 10 minutes at 4 °C, and treated with cell lysis buffer (CLB) at a 3:1 CLB to pellet ratio. Lysates were clarified by low-speed centrifugation (2500 X g) for 10 minutes at 4 °C and frozen at -80 °C.

### **Physical Characterization of BLGV**

#### **Transmission Electron Microscopy (TEM)**

Microscopy was performed by Dr. Sol Seppenwol at the University of Wisconsin-Stevens Point, Stevens Point, Wisconsin. TEM was performed on negative stained, concentrated extracellular virus particles. Particles were diluted 1:10 in HEPES-NaCl buffer (50 mM HEPES, 100 mM NaCl, pH 7). Ultra-thin Formvar-carbon grids (Pella) were pretreated with 254 nm UV illumination for at least 20 min, Formvar-side-up. Grids were then placed Formvar-side-down onto 2.5 µl of virus suspension for 2 min and then onto four 50 µl drops of HEPES-NaCl buffer to rinse, blotting onto #1 filter paper between each rinse. Grids were transferred to 30 µl of uranyl acetate, incubated 5 min, blotted, and allowed to dry for at least 30 min. All incubations were performed at room temperature. Grids were examined with a Hitachi H-600 transmission electron microscope operating at 75 kV. Images were recorded on Kodak EM film and negatives

were scanned at 1200 dpi resolution on an Epson V-750 scanner. Images were digitally enhanced for optimum contrast and brightness.

### **Sodium Dodecyl Sulfate-Polyacrylamide Electrophoresis (SDS-PAGE)**

Concentrated virus particles and negative control pellets (9  $\mu$ l) were each added to 9  $\mu$ l of a 2X SDS loading buffer (54). Samples were heated at 90 °C for 3 min, then loaded onto an SDS-PAGE gel (12% resolving/5% stacking) and subjected to electrophoresis. The resulting gel was silver stained as described previously (58), with a few modifications. Gels were incubated in destain (10 % methanol, 7.5 % acetic acid) on a slow rocker for 2 hr and then rinsed 3 times (30 s each) with rehydration solution (10 % ethanol, 5 % acetic acid). The rehydration solution was replaced with potassium dichromate solution (3.4 mM potassium dichromate, 0.014 % nitric acid) for 5 min. Gels were then rinsed 4 times (30 s each) with distilled water and incubated in 12 mM silver nitrate on a light box for 5 min, then under room lighting for 25 min. Following incubation, gels were submerged for 20 s in distilled water, briefly rinsed (5 s) with sodium carbonate solution (280 mM sodium bicarbonate, 0.025% formaldehyde) and then submerged in sodium carbonate solution until bands became clearly visible. Color development was stopped by adding enough 20 % glacial acetic acid to make a final concentration of 4 %.

### **Nuclease Treatment**

Nucleic acid was extracted from concentrated virus particles using the QIAamp UltraSens Virus kit (Qiagen) according to manufacturer specifications. The extract was separately treated with 1 unit of DNase I (New England Biolabs) for 15 min at 37 °C and 0.75  $\mu$ g RNase A (Sigma) for 30 min at 45 °C, alongside pGEM-3Zf DNA and BF-2

rRNA controls. Samples were transferred to a native 1.2 % agarose gel, electrophoresed alongside a double-stranded DNA ladder (Minnesota Molecular) under RNase-free conditions, and visualized by ethidium bromide staining.

## **Viral Genome Sequencing**

### **Random RT-PCR Amplification**

RNA was extracted from concentrated BLGV particles with TRIzol (Invitrogen) according to manufacturer specifications. Varying amounts of extract were reverse-transcribed using 200 units of Moloney murine leukemia virus (M-MuLV) reverse transcriptase (New England Biolabs) in standard buffer plus 0.01 M dithiothreitol (DTT), with 0.5 mM dNTPs and 0.4  $\mu$ M primer, in a total volume of 25  $\mu$ l for 2 to 4 hr at 43 °C. The reverse transcription primer, courtesy of Nick Knowles (Institute for Animal Health, Pirbright Laboratory, UK), was a modified oligo-dT<sub>21</sub> primer containing an *Xho*I site, 5'-CGATCGCTCGAGAATAGCCCTTTTTTTTTTTTTTTTTTTTTTTT-3'. PCR was performed with 2  $\mu$ l of cDNA, 2.5 units *Taq* polymerase in standard buffer (New England BioLabs), 0.2 mM dNTPs, and either 0.2  $\mu$ M reverse transcription primer and 50 ng of random hexamers (Invitrogen) or random hexamers only, in a total volume of 50  $\mu$ l. The following two sets of PCR cycle parameters were separately attempted:

1. 94 °C for 5 min; 40 cycles of 94 °C for 30 s, 45 °C for 1 min, and 72 °C for 1 min; and a final extension at 72 °C for 5 min.
2. 94 °C for 5 min; 5 cycles of 94 °C for 45 s, 45 °C for 1 min, 72 °C for 2 min; 30 cycles of 94 °C for 30 s, 45 °C for 1 min, and 72 °C for 1 min; and a final extension at 72 °C for 5 min.

Products were transferred to a 0.8 % agarose gel, electrophoresed, and visualized by ethidium bromide staining.

### **Cloning and Sequencing of Random RT-PCR Products**

Resulting PCR products from random RT-PCR were cloned into the pCR8/GW/TOPO vector (Invitrogen) and transformed into Mach1-T1 chemically competent *Escherichia coli* cells (Invitrogen) according to manufacturer specifications. Plasmids from individual colonies were extracted using the Eppendorf Fast Plasmid Mini Kit (5 Prime) and screened for the presence of insert by *EcoRI* restriction enzyme digestion. Inserts were sequenced by Eton Bioscience, Inc. (San Diego, California) using the GW1 and GW2 TOPO sequencing primers (Invitrogen).

### **Sequence Analysis of Cloned Random RT-PCR Products**

Insert sequences were analyzed using tools within the San Diego Supercomputer Center (SDSC) Biology Workbench 3.2 (<http://workbench.sdsc.edu/>). Sixframe analysis was used to identify the longest open reading frame (ORF) of each sequence. If an insert sequence lacked a long ORF, it was directly compared to entries within the non-redundant nucleic acid database using the nucleotide Basic Local Alignment Search Tool (BLAST). Predicted amino acid sequences from long ORFs were first compared to entries within the non-redundant protein database and then more specifically within the GenBank Viral database using protein BLAST. Nucleotide sequences with translated peptides matching closest to viruses were manually assembled into larger, overlapping regions and re-translated into peptides using Sixframe. Protein BLAST was used again to compare these larger peptides to the GenBank Viral protein database. Pair-wise and group ClustalW alignments with peptide sequences from three different virus matches

were constructed to estimate where each BLGV nucleotide sequence was located within the genome. This allowed for the design of primers for RT-PCR amplification between each segment.

### **Second Round of Amplification and Sequencing Using Sequence-specific Primers**

To sequence more of the BLGV genome, primer pairs were designed to amplify between each previously sequenced region of the BLGV genome. Primer pairs were designed to amplify products that include at least 150 bp of known sequence at both ends to simplify assembly of sequenced fragments.

For RT-PCR, nucleic acid was extracted from concentrated BLGV particles with the UltraSens Virus kit (Qiagen). Reverse transcriptions with 3.2  $\mu$ l RNA extract and 2.0  $\mu$ M reverse primer were performed using the Transcriptor High Fidelity cDNA Synthesis kit (Roche) according to manufacturer specifications. PCRs were performed with 2.5  $\mu$ l cDNA and Expand Long Polymerase (Roche) according to manufacturer specifications. PCR parameters were 94 °C for 2 min; 35 cycles of 94 °C for 10 s, 60 °C for 30s, 68 °C for 3 min; and a final extension at 68 °C for 7 min. Products were transferred to a 1.6 % agarose gel, electrophoresed, visualized by ethidium bromide staining, and extracted from the gel using the MinElute kit (Qiagen). Gel extracts were sequenced directly using forward and reverse PCR primers by Eton Bioscience, Inc.

## **BLGV Diagnostic RT-PCR Development**

### **Diagnostic Primer Design**

The National Center for Biotechnology Information Primer-BLAST program was used to design four primer pairs amplifying within regions of BLGV nucleotide sequence obtained from random amplification, all with a  $T_m$  near

60 °C to make direct specificity comparisons possible.

### **Testing Specificity of Potential Diagnostic Primer Pairs**

To determine which potential diagnostic primer pair was most specific to a single region of the BLGV genome, RT-PCR products using each pair were compared. RNA was extracted from concentrated virus particles using the UltraSens Virus kit (Qiagen). Reverse transcriptions were performed with 5.0 µl RNA extract, 200 units of M-MuLV RT (New England Biolabs) in standard buffer plus 0.01 M DTT, 0.5 mM dNTPs and 0.4 µM reverse primer in a total volume of 25 µl for 1 hr at 43 °C. PCRs were performed with 5 µl cDNA, 2.5 units *Taq* polymerase in standard buffer (New England Biolabs), 0.2 mM dNTPs, and 0.2 µM forward and reverse primers in a total volume of 50 µl. PCR cycle parameters were 94 °C for 4 min; 35 cycles of 94 °C for 20 s, 58 °C for 30s, 72 °C for 25 s; and a final extension at 72 °C for 5 min.

To assess the specificity of the best BLGV diagnostic primer pair candidate, RT-PCR reactions were performed using nucleic acid extracts from other fish viruses and uninfected cells. Media was harvested from BF-2 cells infected with LMBV and BLGV; EPC cells infected with VHSV, SVCV, and infectious pancreatic necrosis virus (IPNV); and uninfected BF-2 cells. Nucleic acid was extracted from 1 ml of each medium sample using the UltraSens Virus kit (Qiagen) according to manufacturer specifications. To ensure extracts contained copies of each viral genome, diagnostic PCR-based assays for LMBV, VHSV, SVCV, and IPNV were also performed (4).

Once the presence of viral templates was confirmed, BLGV RT-PCRs were performed with all seven nucleic acid extracts as described previously. Products were

transferred to a 1.6 % agarose gel, electrophoresed, and visualized by ethidium bromide staining.

### **Preliminary Wisconsin BLGV survey**

#### **Bluegill Collection and Processing**

Between August and November 2008, 30 bluegills from each of 16 sites and 20 bluegills from 1 site in Wisconsin were captured by hook-and-line angling, fyke-netting, or electrofishing. Captured bluegills were observed for clinical signs of viral infection and euthanized in a lethal concentration of tricaine methane sulfonate (MS-222).

Approximately 0.2 g of kidney and spleen tissue was removed from each of 30 fish with forceps. Five-fish pools of tissue were diluted 1:10 in 3 ml HBSS, homogenized in a stomacher, and clarified via low-speed centrifugation (2300 X g) for 15 min at 4°C.

#### **Tissue Culture**

Clarified tissue homogenates (1 ml) from each pool were diluted 1:2 in HBSS and incubated overnight at 4 °C or filtered with a 0.2 µm filter and processed the same day. Duplicate wells of BF-2 and EPC cells in 24-well plates were inoculated with 0.1 ml of dilute homogenate from each pool and incubated at room temperature for 1 hr on a plate rocker to facilitate adsorption. Following adsorption, complete bicarbonate media (0.5 ml) was added to each well. BF-2 and EPC cells were incubated at 25 °C and 15 °C, respectively, for 21 d and examined for CPE twice a week (Figure 4). Negative controls consisted of uninoculated cells.

If CPE were observed any time within 21 d of primary inoculation, a reset was performed to determine if CPE was a result of a filterable infectious agent. Media from

both wells of a duplicate exhibiting CPE were filtered through a 0.2 µm filter and diluted 1:6 in HBSS. Duplicate wells of 24-well tissue culture plates, seeded with the cell line in which CPE was observed, were inoculated with 0.1 ml of diluted filtrate as previously described.

If no CPE was observed within 14 d post-inoculation, a blind passage was performed on both cell lines in order to rule out low virus titer and/or the presence of slow-growing virus. Growth medium from one well of each duplicate well lacking CPE was pooled, filtered with a 0.2 µm filter, and diluted 1:6 in HBSS. Pools consisted of media from three wells. Cells in 24-well tissue culture plates were inoculated with 0.1 ml of diluted pooled filtrate in duplicate as previously described. These cells were incubated for another 14 d and examined twice a week for CPE. The second duplicate well from each primary inoculation continued to be monitored for CPE for the entire 28 d (4).

### **Diagnostic RT-PCR**

Media from cells exhibiting CPE after re-passage were analyzed by diagnostic PCR assay. Nucleic acid was extracted from 1 ml clarified medium harvested from infected cells using the UltraSens Virus kit (Qiagen). Separate routine diagnostic PCR-based assays for LMBV, SVCV, and VHSV (4), and BLGV diagnostic RT-PCR were performed on all extracts as described previously. Two negative control RT-PCRs, one using extract from uninfected medium and the other with no template, were also performed. Products were transferred to a 1.6 % agarose gel, electrophoresed, and visualized by ethidium bromide staining. Positive PCR products were sequenced with forward and reverse PCR primers by Eton Bioscience, Inc.

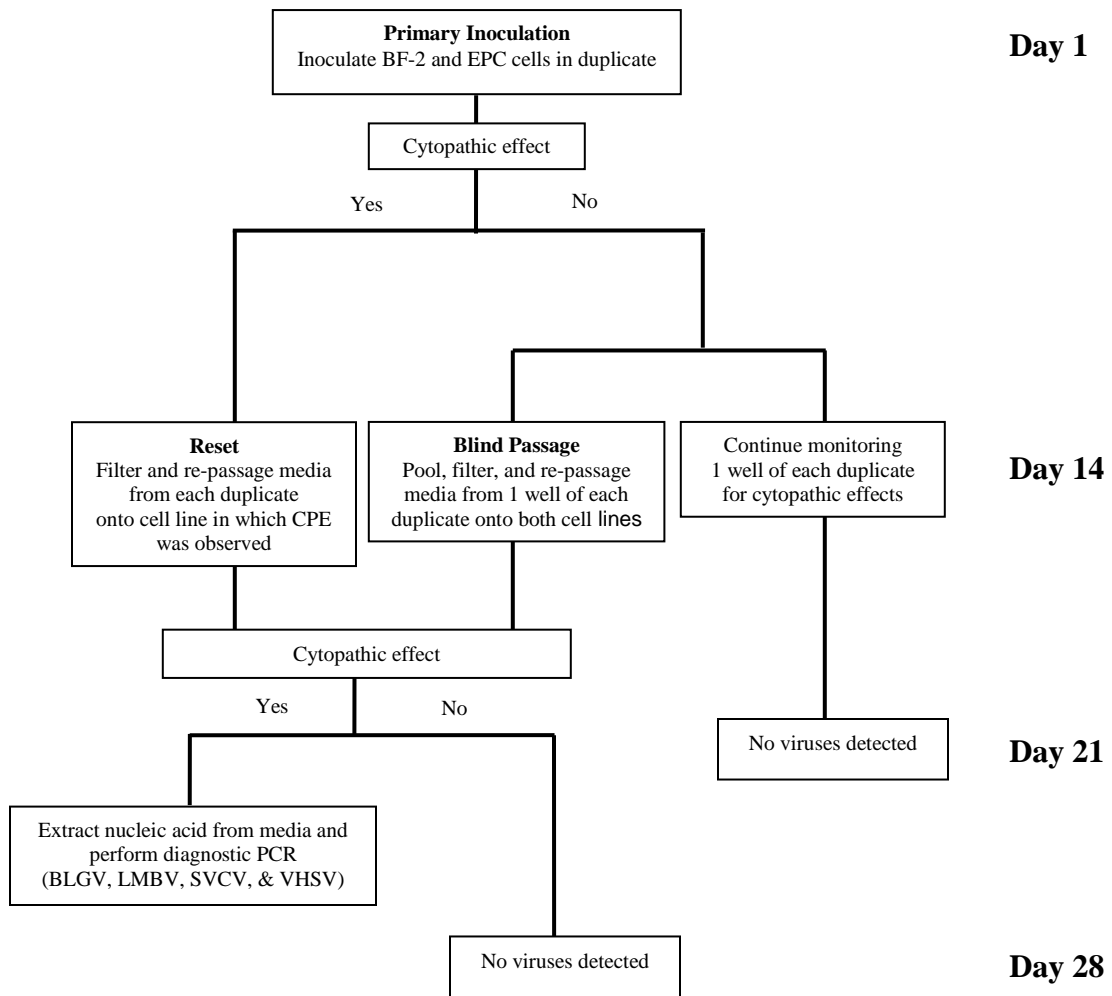


Figure 4. Laboratory reference flow chart for identification and confirmation of BLGV, LMBV, VHSV, and SVCV from kidney and spleen tissues of bluegill.

## **RESULTS**

### **Molecular Characterization of BLGV**

#### **Morphology of Extracellular Virus Particles**

TEM was performed on negatively stained extracellular BLGV particles to confirm they were non-enveloped (Figure 5). Virus particles were, round to icosahedral, and averaged 30 nm in diameter. BLGV particles resembled crumpled pieces of paper due to their rough surface appearance.

#### **Protein Profile**

To estimate the purity of concentrated virus particles and sizes of viral proteins, concentrated media harvested from infected and uninfected BF-2 cells were analyzed SDS-PAGE and silver staining (Figure 6). Infected and uninfected preparations only shared one common protein, having a molecular weight consistent with bovine serum albumin, which is found at high levels in cell culture medium containing FBS. Although complete medium was replaced with serum-free medium late in infection, cells were not rinsed in order to minimize cell detachment, leaving residual FBS behind. The four most prominent bands observed in concentrated medium from infected cells were estimated to be 24.6, 32.9, 39.5, and 45.8 kD. These proteins could either be viral capsid proteins or virus-associated cellular proteins. MALDI-TOF analysis of in-gel digested 24.6 kD and 32.9 kD proteins failed to retrieve any database matches despite successful mass spectroscopy, suggesting these proteins are not of eukaryotic origin or have not been

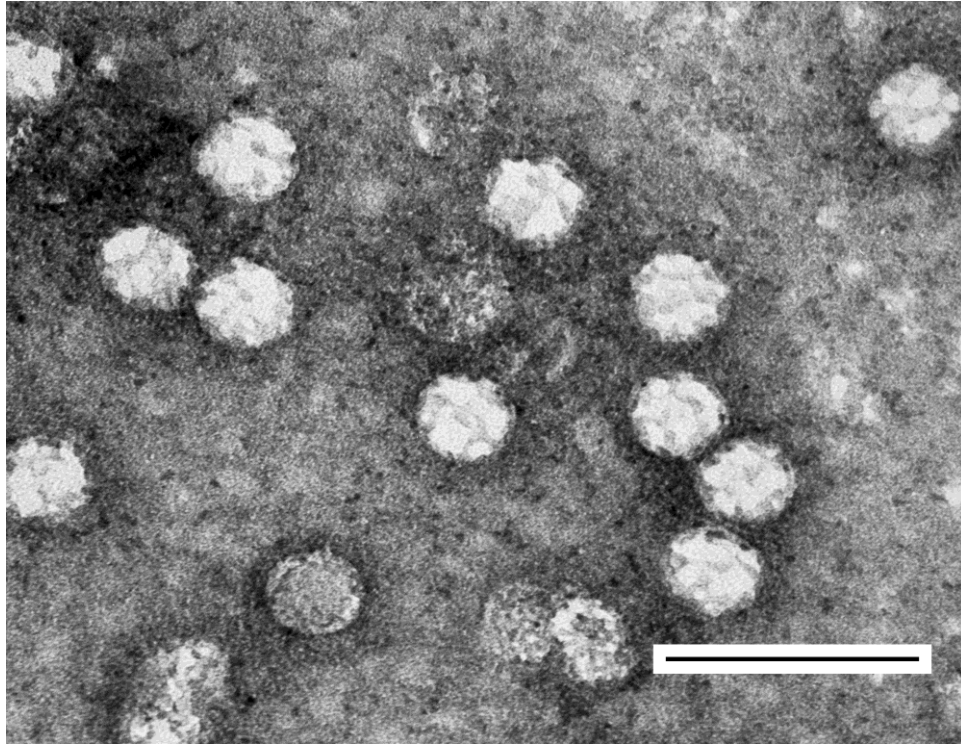


Figure 5. Transmission electron micrograph of negatively stained, concentrated extracellular BLGV particles. Particles were stained with uranyl acetate and examined using a Hitachi-600 TEM, operating at 75 kV. Scale bar = 100 nm.

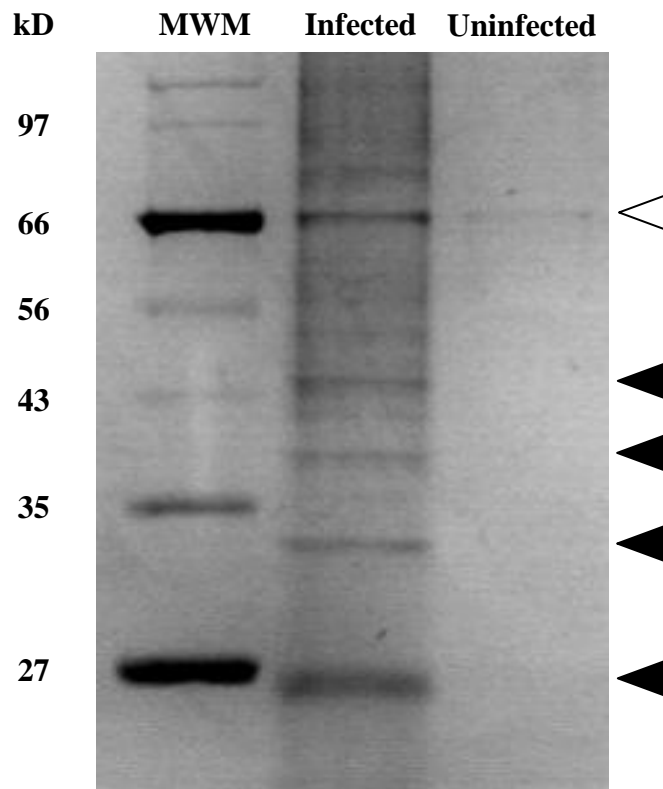


Figure 6. Protein profile of BLGV. Concentrated media from infected and uninfected BF-2 cells were analyzed by SDS-PAGE and silver staining. Bovine serum albumin (white arrow); four potential viral proteins (black arrows).

previously sequenced. Protein profiles of four other presumptive BLGV isolates were very similar (data not shown).

### **Genome Type**

To confirm BLGV is an RNA virus and to determine if its genome is segmented, viral nucleic acids were treated separately with DNase I and RNase A and visualized on an agarose gel (Figure 7). DNase I degraded the pGEM-3Zf control, but failed to digest the BLGV genome. RNase A degraded both the uninfected BF-2 rRNA control and the BLGV genome, confirming the genome is RNA. Extract from concentrated virus particles ran as a single band on the agarose gel. This same band was present in infected cell lysates, along with an additional band that ran higher on the gel. Both bands were degraded by RNase A. This second band suggests BLGV may have a double-stranded genome-length RNA replication intermediate within its life cycle, like positive-sense (mRNA-sense) RNA viruses. Unfortunately attempts to see if this second band disappeared under denaturing conditions, which would confirm its double-stranded nature, were unsuccessful. Another interesting observation was the appearance of a distinct ladder of small dsDNA bands visible within the cell lysate extracts after treatment with RNase A (not shown). The presence of this fragmented DNA pattern suggests infected cells undergo apoptosis (70).

### **Genome Sequencing and Analysis**

Sequencing of the BLGV genome began with random RT-PCR amplification. A modified oligo-dT<sub>21</sub> reverse transcription primer was used, hoping that the BLGV genome contains a 3' poly-A tract like many small, icosahedral, positive-sense RNA viruses. RT-PCR with the modified oligo-dT<sub>21</sub> and random hexamers produced many

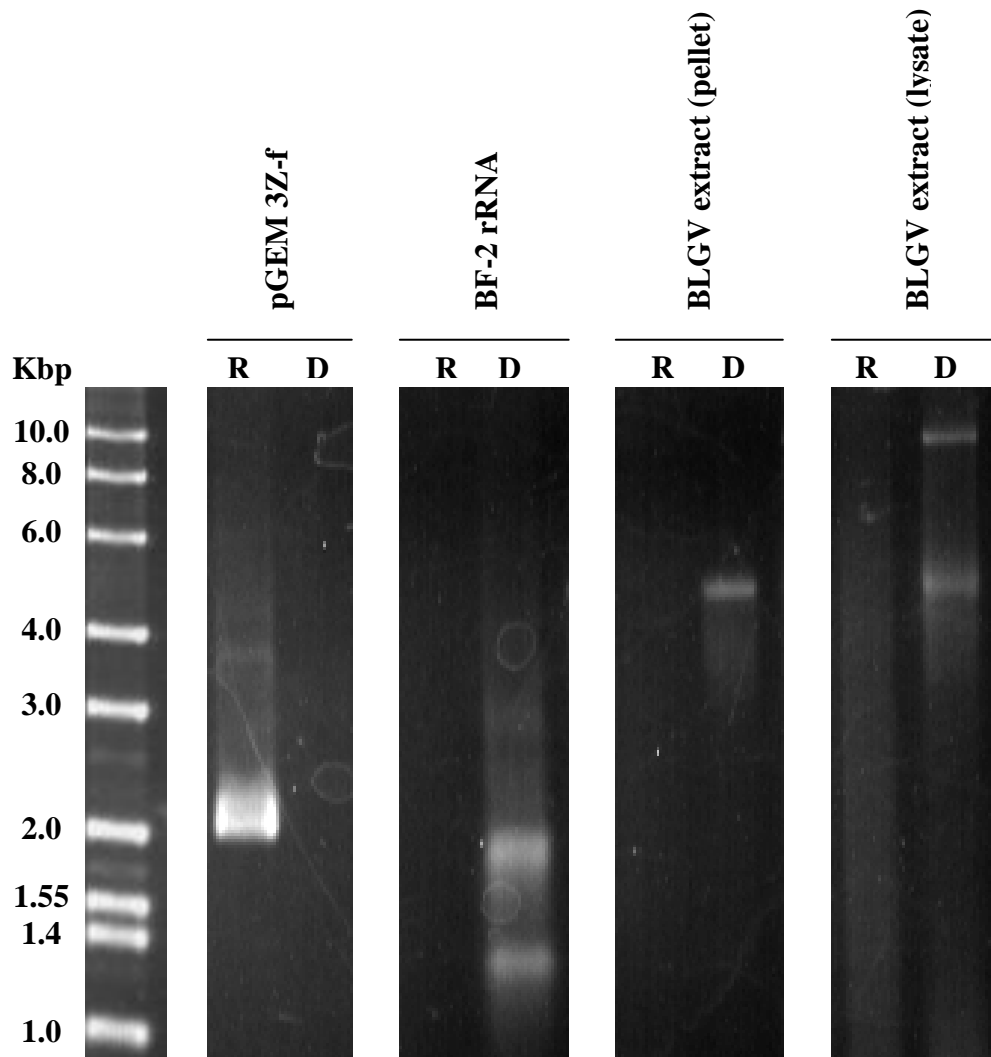


Figure 7. Nuclease treatment of BLGV genome. Nucleic acids were extracted from concentrated virus particles and infected BF-2 cell lysates and treated separately with RNase A (R) and DNase I (D) alongside DNA and RNA controls.

different sized products. Upon sequencing PCR products cloned into the pCR8/GW/TOPO vector, all cloned products appeared to have been amplified by the modified oligo-dT<sub>21</sub> annealing to both ends of the amplified regions. Even when the oligo-dT<sub>21</sub> primer was omitted from PCR, only the residual oligo-dT<sub>21</sub> from the reverse transcription appeared to amplify regions of the genome. The oligo-dT<sub>21</sub> may have bound non-specifically to adenine-rich regions of the template at the low annealing temperatures used to facilitate hexamer binding, and the hexamers most likely did not yield products with ends that would allow for ligation. Nevertheless, multiple PCR products were sequenced, manually assembled into three larger segments (1174 nt, 1190 nt, and 576 nt), and analyzed using SDSC Biology Workbench.

Random oligo-dT<sub>21</sub> amplification yielded three sequenced regions of the BLGV genome. To determine their order and location within the genome, sequences were compared to a suitable reference genome. The three nucleotide sequences were translated, and protein BLAST was used to compare resulting peptides to Genbank viral databases. BLGV peptides matched protein sequences within the family *Picornaviridae*.

Picornaviruses are small, icosahedral, single-stranded positive-sense RNA viruses. Their genomes contain a single polyprotein gene flanked by 5' and 3' untranslated regions (UTR). To determine the location of each of the three nucleotide sequences, translated BLGV peptides were aligned to polyproteins of human parechovirus (HPeV), Ljungan virus (LV), and duck hepatitis virus 1 (DHV-1), the closest matches to BLGV protein sequence. It was necessary to align amino acid rather than nucleic acid sequences because more conservation among viral protein sequences was observed. The 1174 nt segment fell within the first half of the polyprotein gene, the

1190 nt segment within the second half of the gene, and the 576 nt segment included the very end of the polyprotein gene and a large portion of the 3' UTR.

Determining the location of these three segments within the BLGV genome allowed for the design of primers to amplify and sequence regions between each segment. Primer pairs were designed to cover regions between the 1174 and 1190 nt and 1190 and 576 nt segments (Table 2, Figure 8). F1 primers in primer pairs were designed to amplify larger products with more overlapping sequence, making future alignments easier. AF2 and BF2 were designed to reduce the amount of known sequence included in each product by 350 nt, allowing more new sequence to be obtained.

Each of the four primer pairs yielded RT-PCR amplification products. Products were successfully sequenced first with forward and reverse PCR primers and then with second and third sets of internal primers to sequence the entire PCR product. By sequencing these PCR products, a 6,235 nt region of the BLGV genome was sequenced.

The picornavirus open reading frame codes for a single polyprotein that is approximately 2200 amino acids long. The polyprotein is post-translationally cleaved into segments P1, P2, and P3, which are further processed into 10 to 12 smaller viral proteins, much of which is completed by the viral 3C protease that recognizes conserved cleavage sites located at protein-protein junctions (Figure 9). P1 contains capsid proteins VP1 through VP4. Non-structural proteins involved in polyprotein processing and genome replication are found within P2 (2A-2C) and P3 (3A-3D) (51). BLGV nucleotide sequence was translated in order to better determine which region of the polyprotein gene was sequenced (Figure 9). Stop codons only resided at the carboxy-terminus of the translated sequence, which also marks the beginning of the 3' UTR. Notably, the

Table 2. Primer pairs designed for the second round of BLGV genome amplification and sequencing.

Primer pair	Forward primer <sup>a</sup>	Reverse primer <sup>a</sup>
A	1: GTTTCACACACCGCTGTATCACCTC 2: GAGGAGGAAGGTCACAGACAGACAG <sup>b</sup>	AATGTTGGCCCAGCAGTCCTG
B	1: GGAGTTTGAGAGTGGATTGAGGAGC 2: GACGGAAACATTCAAGGCATGAGG <sup>b</sup>	AACGGTCCGAGTCATACTATCGAG

<sup>a</sup> Primers are in 5' to 3' orientation

<sup>b</sup> Resulting PCR product is approximately 350 bp shorter than F1 counterpart

34

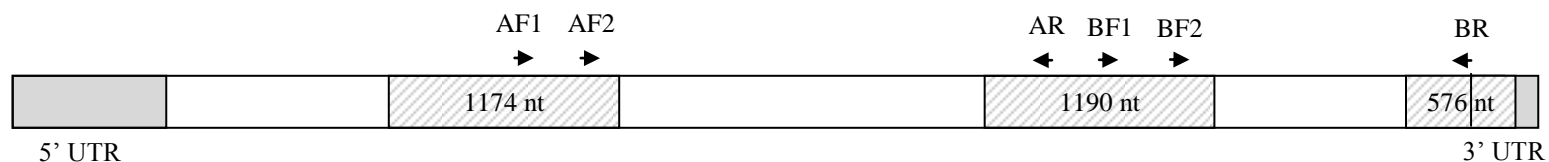
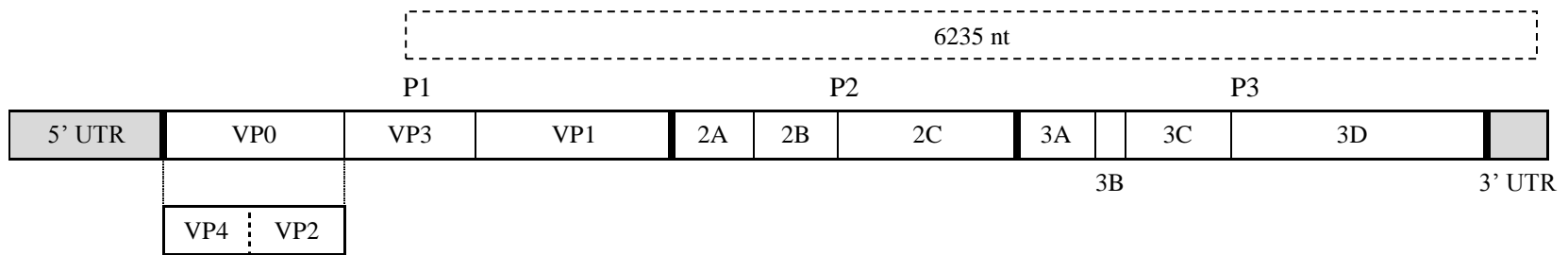


Figure 8. Location of forward (F) and reverse (R) primers designed for the second round of BLGV genome amplification and sequencing. Locations of sequenced regions (diagonal shading) were estimated by performing ClustalW alignments with Ljungan and human parechovirus polyproteins and BLGV genome length was modeled off of the genomes of these two viruses.



35

Figure 9. General organization of a picornavirus genome, including regions of the single open reading frame that give rise to all viral proteins. The P1 region is cleaved into capsid proteins, and regions P2 and P3 are cleaved into non-structural proteins necessary for viral replication. Final maturation cleavage of structural protein VP0 into VP4 and VP2 is variable among genera. The dashed border outlines the sequenced region of the BLGV genome. Predictions of the location of this region were based on pair-wise global sequence alignments with picornavirus capsid and non-structural proteins using ClustalW.

sequenced portion of the 3' UTR is 319 nt long and contains 14 consecutive cytosine residues at its 3' terminal end. The translated BLGV peptide was aligned with HPeV, LV, and DHV-1 polyproteins and individual peptides using ClustalW to estimate which viral proteins were included within the BLGV sequence. The sequence appears to begin within P1, including a portion of the VP3 and complete VP1. A sequence at the amino terminus of the peptide, VLNPLTYT, is very similar to a conserved sequence, VLNRLTYN, positioned near the carboxyl end of *Parechovirus* VP3 capsid proteins. The amino terminus of the BLGV peptide also displayed other regions of similarity with VP3, suggesting the sequence begins within this protein. The exact VP3 | VP1 junction could not be estimated. However, the predicted BLGV VP1 region most closely matched the DHV-1 VP1.

The sequenced region also covers the entire P2 and P3 regions. There is considerable variability observed within much of the picornavirus P2 region. Some picornaviruses have multiple 2A proteins. Even within the *Parechovirus* genus, HPeV codes for a single 2A protein (33), while LV has two 2A proteins, 2A1 and 2A2 (35). Other closely related viruses, SPV (36) and DHV-1(15, 60), may encode for up to three 2A proteins. Sequence found just after the predicted BLGV VP1 region, CGDVESNPGPDI, has strong similarity to the LV 2A1 | 2A2 junction, CGDVETNPG | PDI. Downstream from this homologous region, a second region identical to the SPV 2A1 | 2A2 junction GDVEQNPG | P was observed. No sequence similarity was observed between downstream BLGV sequence and LV 2A2 or SPV 2A2 and 2A3. No clear 2A | 2B junction could be identified. However, a 124 amino acid

region lies in between the second “NPG | P” junction and the predicted beginning of 2B. Together, this information suggests BLGV may have three 2A proteins.

Several non-structural proteins, primarily 2C, 3B, 3C, and 3D, contain several highly conserved sequences, either within specific genera or throughout the picornavirus family. As a result, identifying proteins included within the second half of the BLGV peptide was easier. The BLGV 2C region shares a conserved sequence, EPGQGK, with members of the *Parechovirus* genus, which is part of the larger (G/S)XXGXGK(S/T) element conserved throughout the family. A sequence matching the conserved RAYNP(T/Q) of *Parechovirus* 3B (or VPg) proteins, RAYNPT, was found within a larger region displaying strong sequence similarity with 3B proteins within the genus. Within the BLGV 3C region, the sequence GDCGS aligned with the GXCG(G/S), a conserved sequence within many picornavirus 3C proteins. Although this second half of the BLGV peptide does not contain a highly conserved 3A sequence, a small stretch of about 100 to 120 amino acids, approximately the size of HPeV and LV 3A proteins, is located between the predicted 2C and 3B regions, suggesting a less conserved BLGV 3A is present. At the carboxyl end of the peptide are five highly conserved 3D sequences found within all picornavirus genera; KDE(I/L)R, DYS, PSG (except DHV-1; CSG), YGDD, and FLKR. The PSG, YGDD, and DYS sequences are part of the 3D polymerase active site.

To determine the beginning of the BLGV 3D sequence, the BLgV peptide was aligned with complete LV, HPeV-1, and HPeV-2 3D sequences (Figure 10). From this alignment, it was determined that the BLGV 3D sequence begins with GLVT, which is very similar to the G(V/I)V(T/V) at the beginning of the other *Parechovirus* 3D

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HPeV-2      1  GIVTEVTPIQ-PMYINTKTSQIHKSPVYGAVEVKMGPAVLSKSDTRLEFPVDCLVKKSAASKYRVNKFQVNNELWQGV
HPeV-1      1  GIVTEITPIQ-PMYINTKTQIHKSPVYGAVEVKMGPAVLSKSDTRLEFPVECLIKKSAASKYRVNKFQVNNELWQGV
LV          1  GVVVAKEKLEKLPIMLPSKKTALNPSPLNGVVPVKMEPAVLSPHDTRLEVIMPVVVKTAAAKYRVNIENPDFEILSERV
BLGV       1  GLVTSKTPSDDDVFFQPPKSAIHKSPFYGIQEQATMQPAPLRATDQRITVPIENLITKEAAEKYRVDDQEDVDLNTFAVA
consensus  1  GiVtektepiq-pmyi-tks-ihkSpvyGavevkMgPAvLsksDtrLe-pveclvKk-AaKYRVnkFqv-nelwqgv

HPeV-2      80 KSKFREIFGVNGIVDMKTAAILGTSHVNSMDLSTAGYSFVKS GYKKKDLICLLEP---FSVSPMLEKLVQEKFHNL
HPeV-1      80 KSKFREIFGMNGIVDMKTAAILGTSHVNSMDLSTAGYSFVKS GYKKKDLICLLEP---FSVAPLLERLVQDKFHNL
LV          81 KSKFERTKLGILHKhVSEFQKAVQGFSSLSLSDLSTSPGQKYVEKEMKKRDLLESTEP---EWMHPQLEGDVKDILGAVY
BLGV       81 LERIRPHVKVGRSIPMEEAITE-AGTNPIDKNTSPGLKYTRDHLKKSDFLFTIDEKGNVAVSDRLRADVEEQBELLC
consensus  81 kskfReifgvngivdmktAilGtshvnsMDlStsAgysfvksGyKkKDLiclep---fsvspmLe--Vqekfhnl

HPeV-2      157 ITTIFNTCLKDELRLKLDKIATGKTRCIEACEIDYCIIVYRMIIMEIYDKIYQTPCYYSGLAVGINPYRDWHFMINAI
HPeV-1      157 ITTIFNTCLKDELRLKLDKIATGKTRCIEACEVDYCIIVYRMIIMEIYDKIYQTPCYYSGLAVGINPYRDWHFMINAI
LV          158 PHTFFAAHLKDELRLKKEKIAQGKTRCIEACSIDYVIAYRVVMSLVEAIYQTPAQELGLAVGMNPWTDWDPMINVI
BLGV       160 PTTTETAACKDELRADEKVALGKTEVIEAGELDYVILYRHHMNSIYRDLYNAYS GDVGVAAAGINPLAEARLREDI
consensus  161 -tTtFn-aLKDELRLkldKIaTGKTRaIEACEiDY-iVYRmiMm-iYdkIyqtPcyySgLAvgiNpYrdwhfMinaI

HPeV-2      237 --YEMDYSQYDGSLSMMLLWEAVQVLAYCHDSPDLVMQLHKPVIDSDHVVFNERWLIHGGMPSCSPCTTVLNSLCN
HPeV-1      237 --YEMDYSQYDGSLSMMLLWEAVEVLAYCHDSPDLVMQLHKPVIDSDHVVFNERWLIHGGMPSCSPCTTVLNSLCN
LV          238 --YGLDYSNFDGSLSEQLMRYGVEILAYCHEQPEAVMILHEPVIINSQHLVMDIWHVNGGMPSCGAPCTTVLNSICN
BLGV       240 SFLALDYSRFDGSLSEKLMRAAVDILADLHEDPDLVRRLEPWWISKHLVDEDWIVTGGMPSCSPCTTVLNCICN
consensus  241 --yemDYSqyDGSLS-mLl-eaVeVlAyChdsPdlVmqlhP-VidSdhVvF-eRwliHGGMPSCsPCTTVLNsLCN

HPeV-2      315 IYTTNLIS-----PGIDCLP IVYGDDVILSLDKIEPERLQSIMAESFGAEVTGSRKDEPPSLKPRMEVSEFI
HPeV-1      315 IYTTNLIS-----PGIDCLP IVYGDDVILSLDKIEPEKLSIMADSFSGAEVTGSRKDEPPSLKPRMEVSEFI
LV          316 TVLAYEQS-----LDIEVLP IVYGDDVIFSVNSPLDABVIVQSAAMQFCMEVTSSEKSGPPKLLKMDIESEFI
BLGV       320 DYAMLVHHDVYEDGVGLEQCDYLSVYIGDDCVVAYNGMRMGLDFAQTIEDTEGMTVTPASKLGDFNVVELHEVSEFI
consensus  321 iYttnlis-----pgidclPiVYGDDvilsldkieperlq-imadsFG-eVTgSRKd-ppslkprMEvSEFI

HPeV-2      386 GYFP-ESTFIVGKLDTEN--MIQHLMWMKNFSTFKQQLQSYLMECLH GKDTYQHVVKILNPYLKQWN--ITVDDY
HPeV-1      386 GYFP-ESTFIVGKLDTEN--MIQHLMWMKNFSTFKQQLQSYLMECLH GKDTYQHVIKILEPYLQWN--ITVDDY
LV          387 KFFP-GSTYKVGALS LDT--MEQHIMWMKNLETFPEQLVSEFENSLV LHGKEIIDDVKNRFNPIINQWR--VCMQDY
BLGV       400 MAFETSEG YKVAIILALNENVIVQHLMWMRNLTTLPPQCIQLSMMEYAAAYGKEKYDKLRDTMKRRLAKQNLQITVPGY
consensus  401 gyFp-estf-VgkLd-en--miQHlMwMKn-sTf-qQlqSyLmElclhGkdtY-hy-kilnpyLqewn--itvddy

HPeV-2      461 GKLVPMVFD--
HPeV-1      461 TKLMPMVFD--
LV          462 HRMLRYVFD--
BLGV       480 TMLNSVMGDE
consensus  481 tklvpmVfd--

```

Figure 10. ClustalW alignment of predicted BLGV 3D with *Parechovirus* 3D protein sequences. Residue color scheme: red with black text = completely conserved; dark gray with white = identical; light gray with black text = similar.

sequences, and results in a protein that is 490 amino acids long. This complete predicted BLGV 3D protein sequence was used to begin classifying BLGV, by aligning it to other picornavirus 3D sequences.

### **Preliminary Classification**

Protein BLAST searches performed with several BLGV peptides matched members of the family *Picornaviridae*, with the best matches occurring with members of the *Parechovirus* genus. Consequently, BLGV 3D sequence was aligned with 3D sequences of three viruses within the *Parechovirus* genus, fifteen viruses representing all nine established picornavirus genera, and the two unassigned viruses; duck hepatitis virus 1 (DHV-1) and seal picornavirus (SPV) (Table 3). An unrooted tree was generated from the alignment using DrawTree and bootstrapped using ClustalTree, correcting for multiple substitutions (Figure 11). BLGV was positioned closest to members of the *Parechovirus* genus within the tree, followed by DHV-1 and SPV, and was clustered this way in 951 of 1000 possible trees. Gonnet matrix scores for all pair-wise alignments are present in Figure 12.

The level of identity observed between BLGV and other picornavirus 3D protein sequences was determined by performing pair-wise global alignments using the Align program, which calculates the percent identity between BLGV 3D and each other picornavirus 3D sequence used to generate the tree (Table 4). Not surprisingly, BLGV 3D is most identical to sequences within the genus *Parechovirus*, averaging 38.8 % identity. Comparatively, LV 3D is approximately 50% identical to the HPeV sequences. BLGV 3D sequence is 29.2 and 27.4 % identical to DHV-1 and SPV, respectively.

Table 3. Picornavirus 3D sequences used for preliminary BLGV classification.

<b>Genus</b>	<b>Virus</b>	<b>Abbreviation</b>	<b>Accession Number</b>
<i>Parechovirus</i>	Human parechovirus 1 (Harris)	HPeV-1	AAA72291
	Human parechovirus 2	HPeV-2	NP_740737
	Ljungan virus (87-012)	LV	NP_647602
<i>Hepatovirus</i>	Hepatitis A virus	HAV	ABX27953
	Avian encephalomyelitis virus	AEV	NP_705605
<i>Kobuvirus</i>	Aichi virus	AiV	NP_047200
	Bovine kobovirus	BKV	NP_740257
<i>Erbovirus</i>	Equine rhinitis B virus	ERBV	NP_653077
	Equine rhinovirus 3	ERV-3	NP_201566
<i>Aphthovirus</i>	Foot-and-mouth disease virus	FMDV	P03306
	Equine rhinitis A virus	ERAV	AAB61952
<i>Cardiovirus</i>	Encephalomyocarditis virus	EMCV	P03304
	Theiler's murine encephalomyelitis virus	TMEV	NP_740434
<i>Teschovirus</i>	Porcine teschovirus	PTV	NP_653143
<i>Enterovirus</i>	Human rhinovirus 1	HRV-1	ACK37367
	Human rhinovirus 2	HRV-2	P04936
	Human enterovirus A	HEV-A	NP_740536
	Human poliovirus 1 (Mahoney)	PV-1M	P03300
Unassigned	Seal picornavirus	SPV	YP_001497184
	Duck hepatitis virus 1	DHV-1	ABI23434

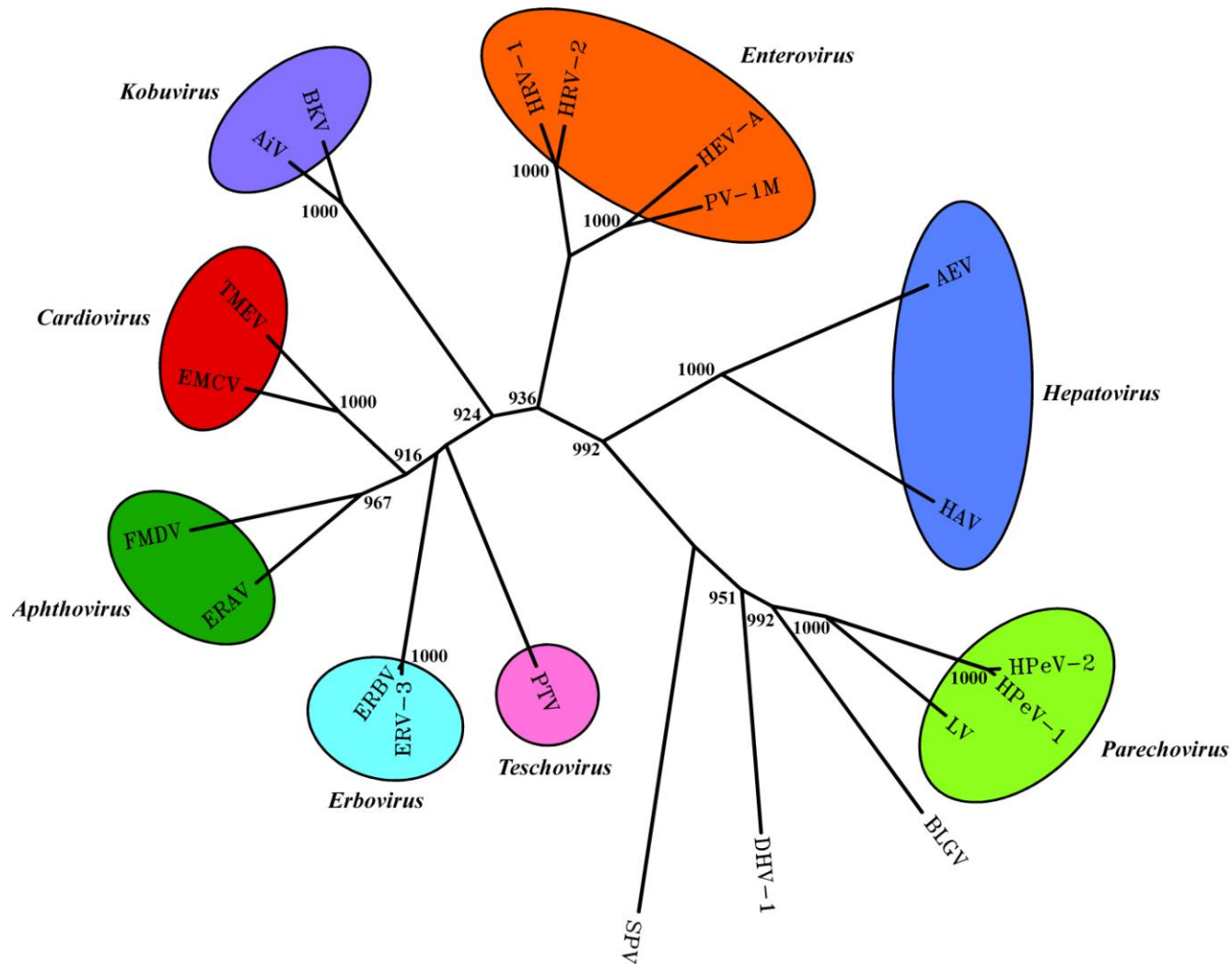


Figure 11. Unrooted tree generated from pair-wise global alignments (Gonnet matrix) of predicted bluegill virus (BLGV) 3D sequence and complete 3D sequences of 20 other picornaviruses. Tree was constructed using DrawTree and bootstrapped using ClustalTree, correcting for multiple substitutions.

	DHV-1	SPV	LV	HPeV-1	HPeV-2	HAV	AEV	HEV-A	PV-1M	HRV-1	HRV-2	BKV	AiV	TMEV	EMCV	FMDV	ERAV	ERBV	ERV-3	PTV	
BLGV	34	24	37	39	37	21	24	23	23	28	26	24	21	21	21	17	25	23	18	17	
DHV-1		31	37	34	35	23	20	25	25	25	23	20	19	23	20	16	22	17	15	22	
SPV			31	27	26	17	19	24	21	23	24	15	17	20	23	21	21	22	21	21	
LV				49	49	24	21	25	27	28	26	20	20	23	23	22	21	23	22	22	
HPeV-1					95	21	25	24	24	23	24	23	23	21	22	20	23	22	22	21	
HPeV-2						21	24	24	23	24	24	23	22	22	21	20	23	22	21	20	
HAV							38	25	23	25	25	23	23	22	22	20	28	22	22	25	
AEV								25	27	26	25	22	26	24	25	20	23	24	24	23	
HEV-A									68	54	53	34	31	27	28	28	26	31	30	32	
PV-1M										57	57	31	33	29	29	29	28	31	30	32	
HRV-1											82	31	29	29	30	28	27	25	35	32	
HRV-2												31	30	29	30	30	28	33	33	32	
BKV													75	30	31	34	33	33	33	30	
AiV														31	32	33	32	32	32	30	
TMEV															65	40	48	41	40	38	
EMCV																42	45	45	45	38	
FMDV																	49	37	37	37	
ERAV																		40	41	36	
ERBV																			96	39	
ERV-3																				40	
PTV																					40

Figure 12. Gonnet matrix scores for pair-wise global alignments of picornavirus 3D protein sequences used to generate the unrooted tree.

Table 4. Percent identity observed between picornavirus and predicted BLGV 3D protein sequences

<b>Genus</b>	<b>Virus</b>	<b>Percent identity</b>
<i>Parechovirus</i>	HPeV-1	39.5
	HPeV-2	38.9
	LV	37.9
<i>Hepatovirus</i>	HAV	29.2
	AEV	27.1
<i>Kobuvirus</i>	AiV	24.8
	BKV	25.3
<i>Erbovirus</i>	ERBV	25.7
	ERV-3	25.5
<i>Aphthovirus</i>	FMDV	24.8
	ERAV	21.5
<i>Cardiovirus</i>	EMCV	25.0
	TMEV	24.6
<i>Teschovirus</i>	PTV	26.3
<i>Rhinovirus</i>	HRV-1	26.3
	HRV-2	26.9
<i>Enterovirus</i>	HEV-A	29.2
	PV-1M	25.4
Unassigned	SPV	27.4
	DHV-1	29.2

## **BLGV Viral Diagnostics**

### **Microscopic Observation of BLGV-induced CPE**

Low-power inverted phase contrast microscopy was used to follow the course of a 14-day BLGV infection in BF-2 cells. Infected and uninfected cells were compared at days 0, 3, 7, 10, and 14 days p.i. (Figure 13). Little noticeable difference between infected and uninfected cells was observed for the first three days. Beginning on day seven, viral plaques began to form as circular clusters of phase-bright cells within infected wells. As they expanded by day 10, plaques contained elongated cells in the center that were surrounded by rounded, phase-bright, cells on the periphery. By day 14, almost all cells had detached from the plate surface. Infected and uninfected cells that were fixed and analyzed by phase contrast and DIC microscopy under higher magnifications showed infected cells were slightly more elongated than uninfected cells and contained many large vesicles (Figure 14).

### **Diagnostic RT-PCR Development**

BLGV sequence information was used to develop four primer pairs to use for diagnostic RT-PCR. The primer pairs amplified within regions of the BLGV genome that were sequenced after random primer amplification (Table 5, Figure 15) because there was little homology between these BLGV sequences and other viral genomes. Primer pair A appeared to bind more specifically to the BLGV genome than the other primer pairs, producing the least amount of background (Figure 16). To make sure pair A would not amplify any false positive products from other nucleic acid templates, RT-PCR with the primer pair A was performed on LMBV, VHSV, SVCV, and IPNV, and BLGV genome extracts and an extract from uninfected BF-2 cell culture medium (Figure 17A).

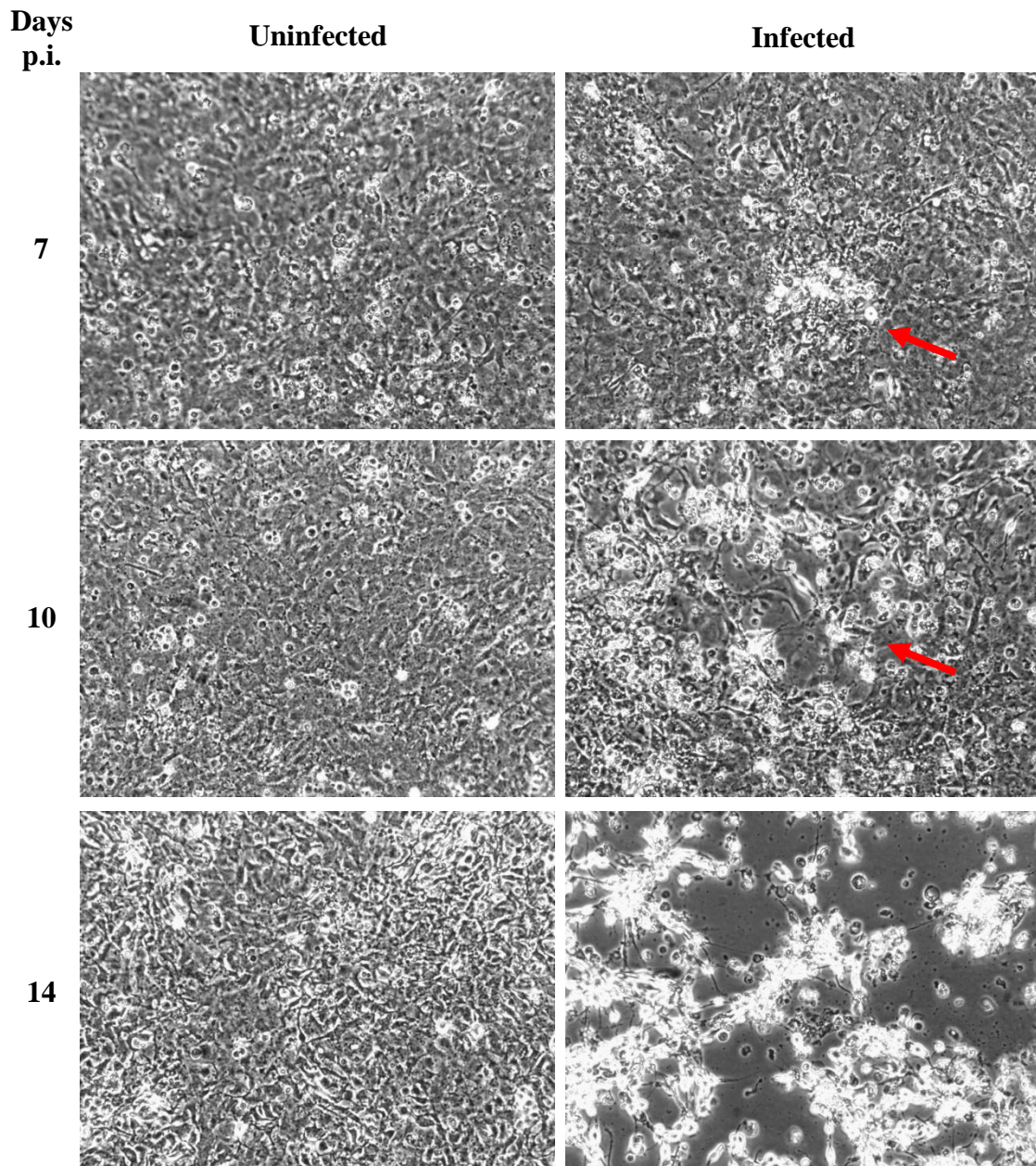


Figure 13. Development of cytopathic effects in BF-2 cells infected with BLGV. Cells were observed on 0, 3, 7, 10, and 14 d post-infection (p.i.) with an inverted phase contrast microscope (10X). Arrows indicate plaque formation.

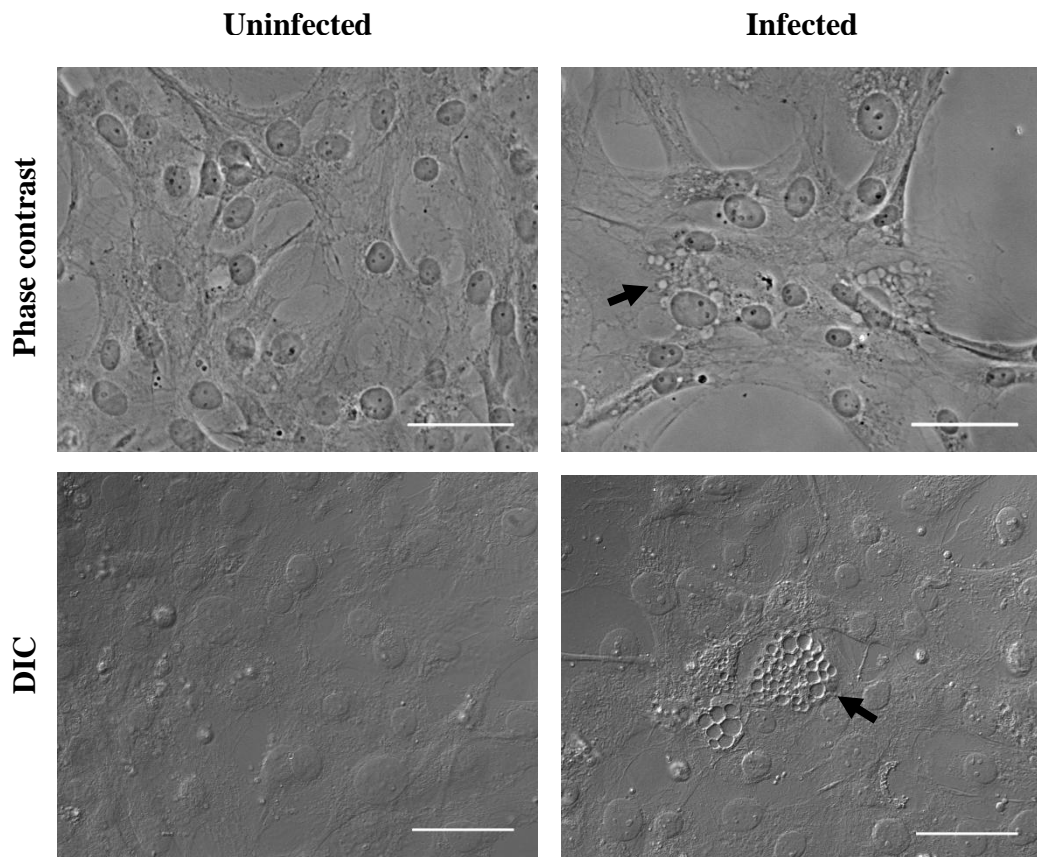


Figure 14. Cytopathic effects associated with BLGV infection BF-2 cells. Note large vesicles (arrows) in infected cells. Cells were fixed (3 % paraformaldehyde and 0.1 % glutaraldehyde in PBS) and examine using phase-contrast and differential interference contrast (DIC) microscopy on 14 d p.i. Scale bar = 30  $\mu$ m.

Table 5. BLGV diagnostic RT-PCR primer pair candidates and expected size of products.

Primer pair	Forward primer <sup>a</sup>	Reverse primer <sup>a</sup>	Product size (bp)
A	CTCGATAGTGTATGACTCGGACC	CATGGGGTTCAACACTCACA	180
B	GTTTCACACACCGCTGTATCACCTC	CTGTCTGTCTGTGACCTTCCTCCTC	373
C	GTGAAGAGCAACCAACTGGTGCAC	AATGTTGGCCCAGCAGTCCTG	288
D	GGAGTTTGAGAGTGGATTGAGGAGC	CCTCATGCCTTGAATGTTTCCGTC	379

<sup>a</sup> Primers are in 5' to 3' orientation

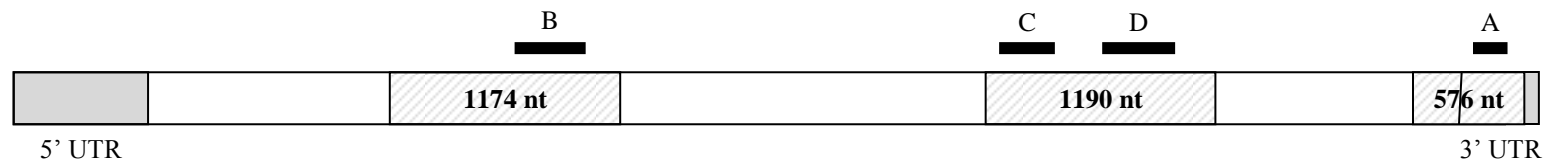


Figure 15. Locations of target regions amplified by candidate diagnostic BLGV RT-PCR primer pairs. Primers were designed to amplify within the three regions sequenced after random primer RT-PCR amplification.

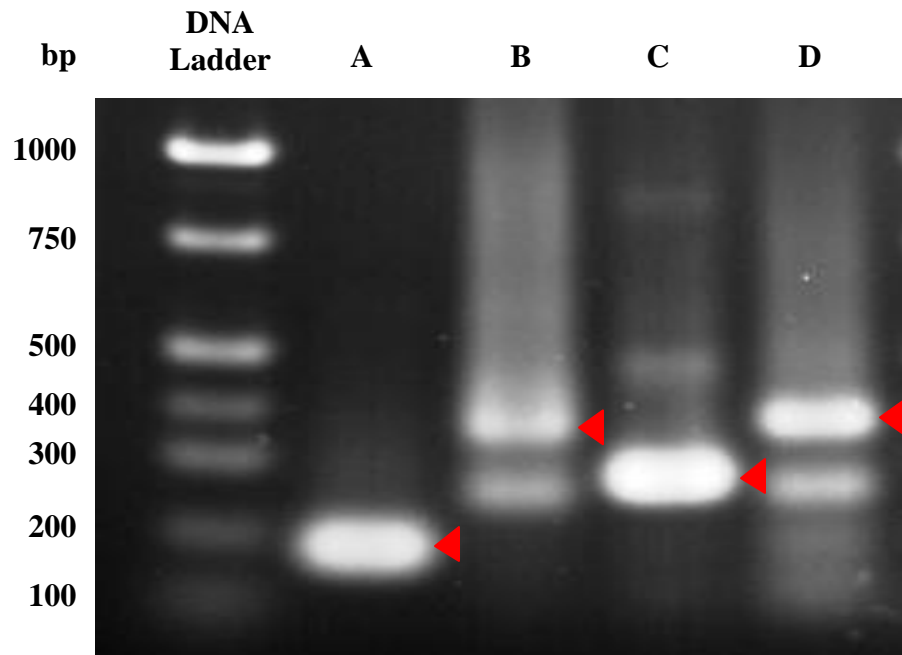


Figure 16. End products of BLGV RT-PCR using primer pairs A through D. Desired positive products are indicated by red arrows; A (180 bp), B (373 bp), C (288 bp), and D (379 bp).

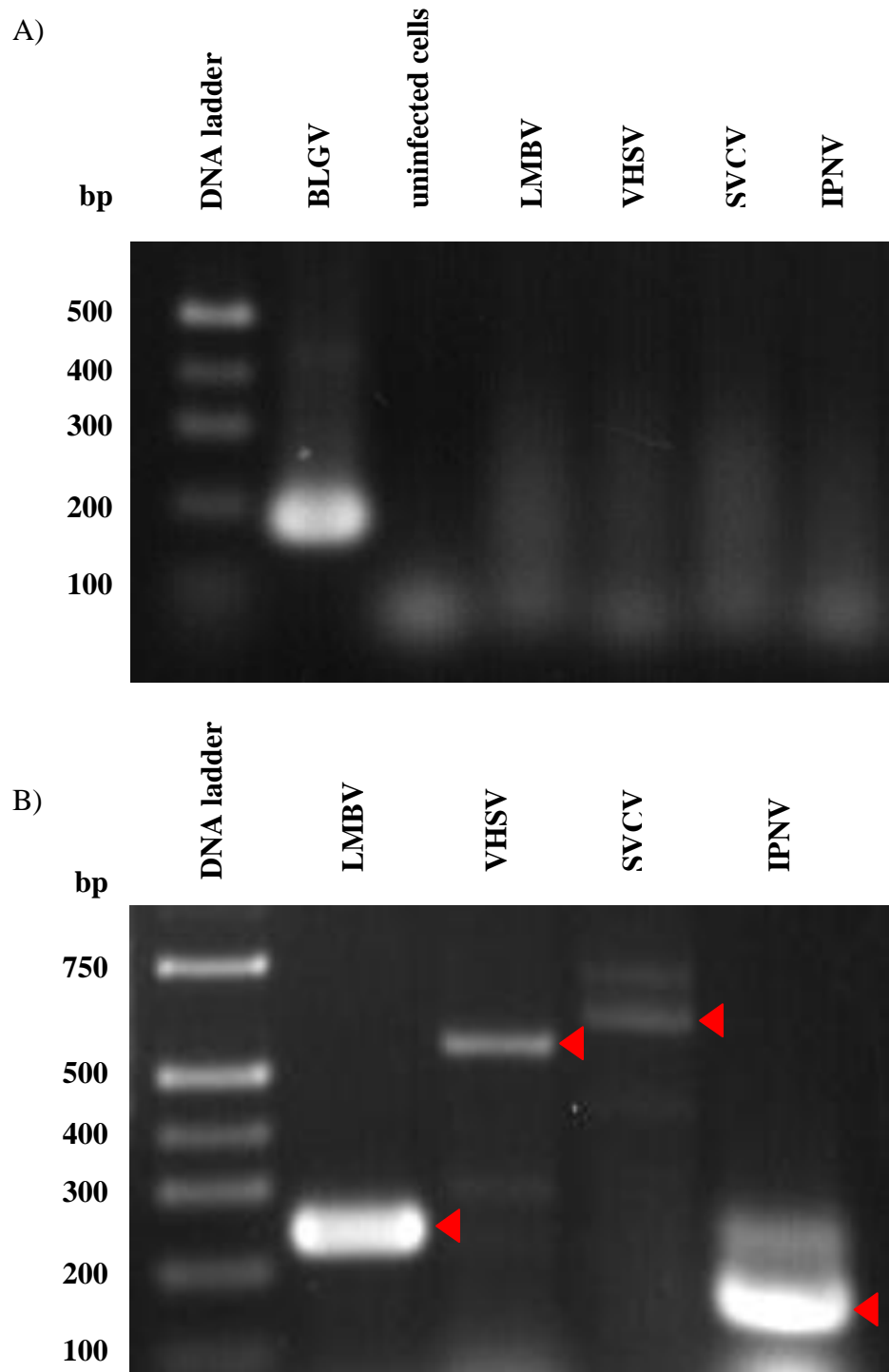


Figure 17. Viral specificity of BLGV diagnostic primer pair. A) RT-PCR performed on viral nucleic acid extracts. B) Control diagnostic PCRs performed on viral extracts to confirm the presence of nucleic acid. Arrows in B indicate positive PCR products; LMBV (248 bp), VHSV (558 bp), SVCV (606 bp) and IPNV (174 bp).

IPNV has not been isolated from bluegills, but was included because it has a wide host specificity and could potentially be isolated from bluegills. Primer pair A only amplified a 180 bp product from the BLGV extract, and was therefore specific to BLGV. In order to verify extractions yielded all viral genome templates, routine diagnostic PCRs for each virus were performed on respective nucleic acid extracts (Figure 17B). Primer pair A also failed to amplify a 180 bp product from uninfected BF-2 RNA extract and produced little background (data not shown). As a result, primer pair A was determined a suitable primer for BLGV diagnostic RT-PCR.

### **Preliminary Survey for BLGV in Wisconsin Bluegills**

None of the 500 bluegills examined in the preliminary BLGV survey showed clinical signs of viral infection. Samples from five of the 17 Wisconsin locations exhibited CPE in BF-2 cells: Montana Lake, Pearl Lake, Big Sand Lake, Lake Waubesa, and UMR Pool 8 (Table 6). No CPE was observed in EPC cells. Nucleic acid extracts from all five extracts tested negative for VHSV, LMBV, and SVCV but tested positive for BLGV using PCR-based assays (Figure 18).

To confirm 180 bp product sequences matched that of the target region, all diagnostic BLGV PCR products, including the positive control product, were sequenced and aligned with the original target sequence (Figure 19). PCR products were 99 to 100 percent identical to the target sequence, confirming that all five presumptive survey isolates are BLGV (Figure 20). Of the five confirmed BLGV isolates, three were from new locations: Pearl Lake, Big Sand Lake, and Lake Waubesa. BLGV was only isolated from two sites of past presumptive isolations; UMR Pool 8 and Montana Lake.

Table 6. Number of five-fish tissue pools producing CPE in BF-2 cells from each BLGV- positive sampling location.

<b>Water body (Wisconsin county)</b>	<b>Number of five-fish tissue pools<sup>a</sup> producing CPE</b>
Pearl Lake (Waushara)	6
UMR <sup>b</sup> Pool 8 (La Crosse)	2
Lake Waubesa (Dane)	6
Montana Lake (Marinette/Oconto)	5
Big Sand Lake (Burnett)	4

<sup>a</sup> Samples from each location consisted of a total of 6 5-fish pools of kidney and spleen tissue

<sup>b</sup> UMR = Upper Mississippi River

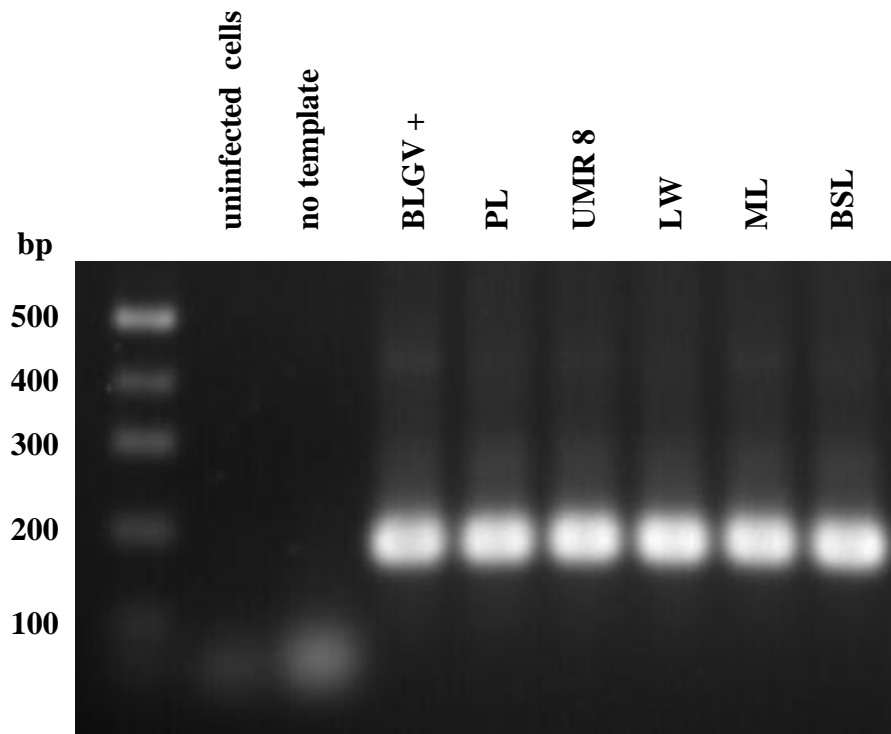
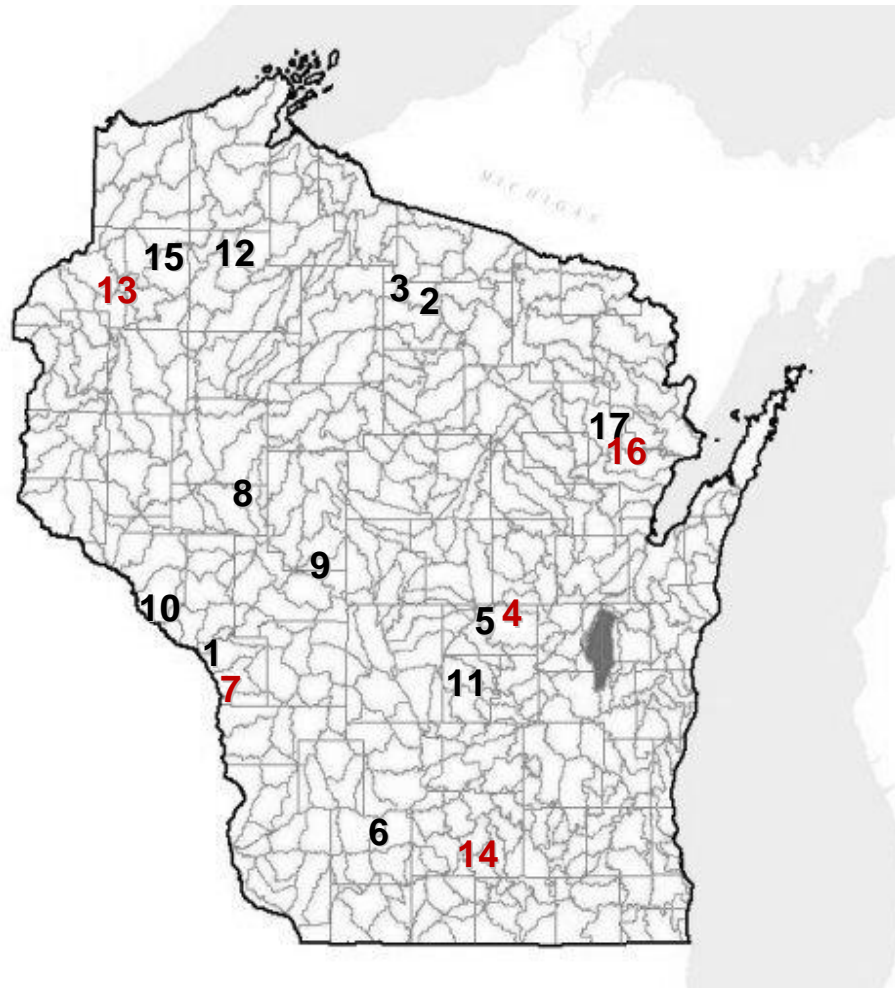


Figure 18. BLGV confirmatory diagnostic RT-PCR of five presumptive BLGV survey isolates. Negative control reactions were performed with nucleic acids extracted from uninfected cell culture media and with no template. Positive control reaction was performed with BLGV nucleic acid extract. Positive products are 180 bp. Isolates were from Pearl Lake (PL), Upper Mississippi River Pool 8 (UMR8), Lake Waubesa (LW), Montana Lake (ML), and Big Sand Lake (BSL).





- |                          |                          |                       |
|--------------------------|--------------------------|-----------------------|
| 1. UMR Pool 7            | 7. UMR Pool 8            | 13. Big Sand Lake     |
| 2. Wind Pudding Lake     | 8. Lake Eau Claire       | 14. Lake Waubesa      |
| 3. Lower Kaubashine Lake | 9. Saddle Mound Lake     | 15. Silver Lake       |
| 4. Pearl Lake            | 10. UMR Pool 5A          | 16. Montana Lake      |
| 5. Alpine Lake           | 11. Fox River (Montello) | 17. White Potato Lake |
| 6. Twin Valley Lake      | 12. Barker Lake          |                       |

Figure 20. Preliminary Wisconsin BLGV survey. Red numbers indicate RT-PCR confirmed BLGV positive sites, and black numbers indicate sites where BLGV was not isolated from tissue culture.

## DISCUSSION

In this study, a novel virus of bluegills was characterized. Based on its physical characteristics and genomic sequence, the virus can be placed in the family *Picornaviridae*, and appears to be most closely related to members of the *Parechovirus* genus. A diagnostic RT-PCR for BLGV was developed, and confirmed isolation of BLGV from 5 of 17 water bodies included in a preliminary survey for the presence of virus in Wisconsin bluegills. The LFHC has begun to use this test to confirm all tissue culture isolations and will continue to routinely use it as a viral diagnostic tool. This test will also be useful during further studies of BLGV geographic range, host specificity, and virulence.

### **Molecular Characterization and Classification of BLGV**

#### **Physical Characteristics**

The observed physical characteristics of BLGV are entirely consistent with members of the *Picornaviridae* (51). BLGV particles are round to icosahedral and average 30 nm in diameter. The protein profile of concentrated BLGV particles suggests BLGV has four capsid proteins, 24.6, 32.9, 39.5, and 45.8 kD, and nuclease treatment and visualization of the BLGV genome on an agarose gel confirmed it is composed of RNA. Because a single RNA band was visualized from extracts coming from concentrated extracellular virus particles, the genome was also determined to be non-segmented.

## **Preliminary Classification**

The translated BLGV protein is most similar to a picornavirus polyprotein. Almost all protein BLAST matches to BLGV sequence were in the *Picornaviridae*, with the most similar matches being within the *Parechovirus* genus. When the complete BLGV 3D sequence was compared to 20 other picornavirus 3D sequences, BLGV was positioned most closely to members of the *Parechovirus* genus and the two unassigned viruses, duck hepatitis virus 1 (DHV-1) and SPV.

BLGV 3D sequence is nearly 40 % identical to the *Parechovirus* 3D proteins and nearly 30 % identical to those of DHV-1 and SPV. The *Parechovirus* genus consists of human parechovirus (HPeV) and Ljungan virus (LV) species. HPeV types 1-14 have been most often associated with mild gastroenteritis and respiratory illness in young children, but few types have also been associated with acute flaccid paralysis, meningitis, encephalitis (57), neonatal sepsis (39, 69), and otitis media (59). LVs have a natural reservoir in bank voles (*Myodes glareolus*) (48), but are suspected to contribute to increased incidence of a variety of diseases in humans, such as myocarditis (48), Type I diabetes (47), and fetal central nervous system malformations resulting in terminated pregnancy (49). DHV-1 causes acute, fatal hepatitis in young ducklings (25) and SPV has been associated with a number of seal deaths though its virulence has not been determined (36).

Classification of BLGV should be based upon morphology, genome sequence similarity, species specificity, and the type of disease it causes. Pair-wise alignments of the BLGV and parechovirus 3D sequences score high using matrices that take sequence identity, similarity, and gapping into account. These values are very similar to those

observed from alignments between hepatitis A virus (HAV) and avian encephalomyelitis virus (AEV), which are both hepatoviruses. Interestingly, HAV and AEV belong to the same genus but have different host specificities, which may account for reduced similarity. Conversely, pair-wise alignments between *Aphovirus* and some *Cardiovirus* 3D proteins score higher than the pair-wise alignments between BLGV and any of the parechoviruses. These findings indicate that 3D sequence similarity alone cannot be used to place BLGV into a genus.

Although its virulence has not been confirmed, BLGV appears distinct from the parechoviruses based on host species-specificity. In fact, BLGV is the first picornavirus known to infect fish. Viruses resembling picornaviruses that have been reported from fish have been given the term “picorna-like.” Significantly, none of their genomes have been sequenced. Picorna-like viruses have been reported from smelt (*Osmerus mordax* (45) and *O. eperlanus* (5)), grouper (*Epinephelus malabaricus*) (12), turbot (*Scophthalmus maximus*) (11), barrumundi (*Dicentrarchus labrax*) (23) European sea bass (*Dicentrarchus labrax*) (13), steelhead (*Oncorhynchus mykiss*) (19), sandbar shiners (*Notropis scepcticus*) (34) and grass carp (40). Most cases were associated with fish disease and mortality (11-13, 23, 45). Pathology of brain, retinal, and sometimes intestinal tissues from affected fish in multiple studies revealed a large number of vesicles within infected cells (11-13, 23). These vesicles stained bright red with acridine orange, indicating a concentration of single-stranded nucleic acid on their surface (45). Both of these characteristics suggest these isolated fish viruses are positive-sense ssRNA viruses. Similar examinations of BLGV-infected bluegill tissue samples could reveal if BLGV infection causes similar histopathology.

Since the genomes of the various fish picorna-like viruses have not been sequenced, tentative classification has been based on physical and chemical characterization. Examined characteristics have included pH (19), and chloroform (45) resistance; ability to replicate during 5-iodo-2 deoxyuridine (a thymine analogue) treatments (34, 45), and virus particle size and morphology using transmission electron microscopy (11-13, 19, 23, 34, 45). Picorna-like particles were found organized individually within the cytoplasm of infected tissue culture cells and tissue samples taken from diseased fish. Virus particles within diseased fish tissue were also found arranged in a paracrystalline array within large cytoplasmic inclusions, very characteristic of picornaviruses (11-13, 19, 23). It would be interesting to assess how closely these viral genomes are related to BLGV and other picornaviruses to determine if they belong within a new genus with BLGV, other genera within the *Picornaviridae*, or if they belong in other virus families that possess similar physical characteristics.

The International Committee on Taxonomy of Viruses standard classification protocol states that if classifying a virus to the genus level is uncertain, but classification of the family is clear, a virus should be classified as an unassigned species within that family. Although assigning BLGV to a genus requires further research, BLGV could currently be termed an unassigned virus of the family *Picornaviridae*, and named bluegill picornavirus (BGPV).

### **Genome Organization**

The BLGV genome appears to share common features of picornavirus genomes, but also contains some distinct features. My preliminary analysis of translated portions of the BLGV genome identified proteins homologous to picornavirus proteins VP3

through 3D. Furthermore, most of the protein junctions within the polyprotein could be determined by performing sequence alignments with related virus polyproteins.

However, the VP3 | VP1 junction could not be clearly determined. Amino-terminal sequencing of BLGV structural proteins could help clearly define the cleavage site used. Amino-terminal sequencing could also help determine if the VP0 protein is cleaved into VP2 and VP4, a process that leads to particle maturation for many picornaviruses. Because BLGV particles appear to contain four capsid proteins, BLGV particle maturation may involve cleavage of VP0, resulting in VP4, VP2, VP3, and VP1 comprising the BLGV capsid. However, the VP0 proteins of DHV-1 (15), SPV (36), LV (48), and HPeV (33) do not appear to be cleaved.

Two conserved 2A1 | 2A2 junctions located within the P2 region of the BLGV polyprotein suggest post-translational cleavage may yield three 2A proteins. The 2A proteins of picornaviruses appear to have diverse functions among the different genera. The 2A trypsin-like proteases of enteroviruses and rhinoviruses inhibit cap-dependent host cell translation by cleaving initiation factor 4. Other picornavirus 2A proteins are not believed to exhibit protease activity, but are proposed to either slow down cell proliferation or regulate apoptosis (29). The two conserved BLGV 2A sequences are very similar to 2A1 | 2A2 junctions of LV and SPV. A closer look at these two junction sequences reveals a conserved DXEXNPG | P motif described in the aphtho-, cardio-, erbo- and teschoviruses, which is proposed to mediate primary polyprotein cleavage at its 2A carboxy terminus (29). Multiple 2A proteins may increase the efficiency of the primary cleavage of the BLGV polyprotein or interact with host cell proteins in order to maximize virus replication.

The long 3' untranslated region of the BLGV genome exceeds the length of the longest recorded picornaviral 3'UTR, belonging to DHV-1, which is only 314 nt (60). The modified oligo-dT<sub>21</sub> primer annealed adjacent to this sequence, but because it annealed to multiple sites within the genome, it is difficult to determine if the entire BLGV 3' UTR has been sequenced. RT-PCR amplification using oligo-dT<sub>21</sub> and sequence-specific primers at higher annealing temperatures could amplify a single PCR product that can be sequenced in order to determine if the complete BLGV 3' UTR sequence has been obtained. Picornavirus 3' UTRs contain secondary structure that plays a role in genome replication (51). While the reason for its long 3' UTR is not clear, some of the sequence certainly would be expected to have a role in BLGV genome replication. The function of the stretch of 14 cytosines located at the 3' end of the sequenced BLGV 3' UTR region is also unknown. Homopolymeric regions, aside from the 3' polyadenylated tracts of positive-sense genomes, are unusual for viruses, which suggests the poly(C)<sub>14</sub> tract of the BLGV UTR has some role within BLGV replication cycle. The murine cardioviruses, such as Mengo virus and encephalomyocarditis virus, and aphthoviruses, such as foot-and-mouth disease virus, have homopolymeric poly(C) regions within their genomes (51). The length of the poly(C) tract of Mengo virus has been shown to significantly correspond to lethality of viral infection in mice (17). Mice were able to mount a more efficient immune response to attenuated Mengo virus mutants with shortened poly(C) tracts even though growth of mutant and wild-type viruses were indistinguishable in tissue culture. These findings suggest the poly-C reacts with immune modulators to help the virus evade host immunity. The BLGV poly(C)<sub>14</sub> may have a

similar function. However, cardiovirus and aphthovirus poly-C tracts are found within the 5' UTRs and can be over 250 nt long (17, 18, 51).

Although approximately 75 % of the BLGV genome has been sequenced, the 5'-proximal portion still remains. Sequencing the BLGV 5' UTR will allow analysis of secondary structures that could be important in controlling genome replication and translation, while sequencing the complete VP0 and VP3 regions of the polyprotein gene would allow for alignments with complete picornavirus polyproteins to be analyzed.

### **BLGV Diagnostics and Fish Health**

#### **Cytopathic Effects**

Isolation of BLGV in tissue culture is the first step in detecting the virus in fish. Recognizing CPE under low magnifications using an inverted phase microscope is a necessary skill for BLGV screening. BLGV plaques begin as small clusters of phase-bright cells. As they spread, plaques contain elongated cells in the center, surrounded by rounded, phase-bright cells at the periphery. BLGV-induced CPE is very different from CPE observed in BF-2 cells infected with LMBV, which exhibit hypertrophy and round up before detaching from the cell culture surface (38).

At higher magnifications, BLGV-infected BF-2 cells appear slightly more elongated than uninfected cells; however, the most notable characteristic of infected cells is that they possess a large number of cytoplasmic vesicles. Positive-sense RNA viruses like the picornaviruses trigger the formation of vesicles. These vesicle surfaces are the sites of viral genome replication (9, 51). The accumulation of vesicles within BLGV-infected BF-2 cells strongly suggests vesicle surfaces are the site of BLGV replication as

well. However, the vesicles associated with BLGV-induced CPE are substantially larger than those often associated with other picornavirus infections.

### **Diagnostic RT-PCR**

Fish health viral diagnostic protocols are based on an initial screening using fish tissue culture methods followed by a confirmatory test such as PCR, fluorescent antibody testing, or histological examination. A fish health report stating that fish are free of select pathogens, including many viruses, is required to stock and/or transport fish from a hatchery. If one of these viruses is isolated from hatchery fish, infected fish must be euthanized and contaminated areas of the hatchery must be disinfected. It is very difficult to implement these control measures or impede fish movements based on presumptive findings. Before this study, the LFHC did not have an assay to confirm presumptive BLGV isolations from tissue culture. Fish hatchery managers and DNR fisheries managers did not know how to respond to a report of presumptive virus isolation, which increased the need for a confirmatory BLGV assay. The BLGV genome sequencing and analysis performed in this study has led to the development of a PCR-based method of confirming presumptive BLGV isolations, a much needed diagnostic tool. The BLGV diagnostic RT-PCR uses a primer pair that anneals within the 3' UTR of the genome and amplifies a 180 bp product. This assay appears to be virus-specific and sensitive enough to detect BLGV propagated in tissue culture.

Diagnostic PCRs used to confirm virus isolation in fish infection are performed on nucleic acid extracted from cell culture medium. This method is considered the gold standard for fish viral diagnostics, because screening samples in tissue culture will occasionally lead to the isolation of a novel virus. Such findings would be missed if PCR

was performed on nucleic acid extracted directly from kidney and spleen tissues because no CPE would be observed prior to PCR. However, performing BLGV PCR using nucleic acid extracts from kidney and spleen homogenates may be a more cost-effective choice when screening only for a specific virus, such as BLGV. Furthermore, some evidence suggests that performing diagnostic PCR on tissue homogenate extracts is more sensitive. LMBV diagnostic PCR has been shown to detect virus in up to four times as many samples when nucleic acid was extracted directly from largemouth bass kidney, spleen, and swim bladder homogenates instead of infected tissue culture media (27). A VHSV study comparing the two PCR methods reported PCR using nucleic acid extracted from tissue homogenates (liver, spleen, kidney, heart, and brain) was more sensitive than when extracts from tissue culture media were used (16).

In addition to being cost-effective, performing PCR on viral nucleic acid extracted from bluegill tissue homogenates may be a more sensitive way to screen for BLGV for future surveys. The increased sensitivity could be because many virus particles harvested from tissues are non-infectious. The ratio of total picornavirus particles to infectious particles, or particle to plaque-forming-unit ratio, in a given sample ranges from 30 to 1,000 (51), meaning that as few as one in every 1,000 virus particles extracted from bluegill kidney and spleen tissue may be viable in tissue culture. A larger number of those same 1,000 virus particles harvested from tissues would still contain an RNA template for diagnostic RT-PCR. Therefore, screening virus using only tissue culture methods may underestimate total number of positives by missing samples that are not viable in tissue culture.

## **Geographic Range and Host Specificity**

The distribution of BLGV is widespread in Wisconsin. Between December 2001 and August 2008, the virus was presumptively isolated from 14 different water bodies within the state. In this study, BLGV was confirmed from 5 of the 17 Wisconsin water bodies using the newly developed RT-PCR. BLGV had not been previously reported in 3 of the 5 locations. The LFHC can now use the RT-PCR to test tissue culture isolates that have been archived at -80 °C. This will most likely expand the known range of the virus in Wisconsin and the Upper Midwest.

Routine viral testing of bluegill as part of the United States Fish and Wildlife Service's National Wild Fish Health Survey has indicated that BLGV may have an extensive range throughout the Upper Midwest. During routine health inspections, BLGV was presumptively isolated in November of 2007 from bluegills from North Spring Lake (Manito, Illinois) and in March and April 2008 from bluegills from Hebron State Fish Hatchery Pond (Licking, OH), Salt Fork Lake (Cambridge, OH), and Clear Fork Reservoir (Mansfield, OH) (38).

The RT-PCR developed in this study was recently used by the LFHC to confirm BLGV isolations from bluegills from locations in Wisconsin, Illinois, and Ohio. In the spring and early summer of 2009, BLGV was isolated from Little Grassy State Fish Hatchery (Carbondale, IL) during a routine health inspection and UMR Pool 10 (Prairie du Chien, WI), a site of previous presumptive isolation, during a routine wild fish survey. Near the same time, BLGV was isolated during VHSV screening in Ohio, again from bluegills from Clear Fork Reservoir (Mansfield) and from Pleasant Hill Lake

(Perrysville), which is part of the same watershed as the Clear Fork Reservoir. No fish from any of the sites exhibited clinical signs of viral infection (38).

BLGV isolation has since been confirmed from diseased fish using diagnostic RT-PCR. In May 2009, a large bluegill kill involving thousands of bluegills occurred in Lake Monona (Madison, WI). Bluegills exhibited clinical signs of viral infection and tested positive for BLGV using diagnostic PCR. After the confirmed isolation, frozen bluegills and black crappies that had been found dead in Monona Bay during the previous winter were also screened for virus, and BLGV was confirmed by RT-PCR in both fish species. This is the first confirmed isolation of BLGV from a different fish species, but the virus has been presumptively isolated in 2008 from black crappies collected from Spooner Lake (Spooner, WI) and in 2007 from largemouth bass sampled from the Genoa Fish Hatchery (Genoa, WI) during a routine fish inspection (38). Because black crappies, largemouth bass, and bluegills are all members of the family *Centrarchidae*, it may be expected that BLGV could infect other centrarchid species as well. Isolation of BLGV from these three centrarchid species, and association with disease of both black crappies and bluegills, gives reason for conducting a more extensive survey to determine the host specificity of BLGV(38).

### **Virulence**

Field observations suggest BLGV is virulent in bluegills. To confirm this, the virulence of BLGV should be studied in the laboratory, and seek to fulfill Koch's postulates. Disease-free bluegills must be experimentally infected with purified BLGV and then monitored for the development of clinical signs of viral infection. Groups of bluegills with specific ages should be inoculated and subjected to environmental

stressors. Rapid temperature change may be enough to cause disease in experimentally inoculated fish due to the fact that BLGV is most often isolated from diseased fish during late fall, early winter, and late spring. If clinical signs of infection are observed, the virus should then be re-isolated from diseased fish.

In order to study infection levels and persistence in inoculated fish, a virus titering protocol for BLGV must be developed. Recently, members of the LFHC have successfully quantified VHSV preparations using a methylcellulose overlay (38), and based on results from a few plaque assay attempts performed during this study, I believe their protocol could be slightly modified to work for titering BLGV as well.

### **Management Implications**

With continued BLGV association with fish disease and confirmed virulence, BLGV will become recognized as a clinically significant fish virus. Preliminary confirmed isolations of BLGV suggest the virus is already widespread, but because the full geographic range of the virus has not been determined, precautionary measures should still be taken when BLGV isolation has been reported during routine health inspections of hatchery fish and during wild fish health surveys. Recognizing BLGV as a potential pathogen will result in the implementation of standard control measures in fish hatcheries, thus preventing the movement of infected fish to other bodies of water. Continuing to educate anglers about what they can do to help control the spread of VHSV and LMBV will go a long way to prevent the spread of BLGV as well. Anglers should remember to drain all water from boats before leaving a water body, disinfect live wells, and limit the use of baitfish in multiple water bodies. Anglers should also be informed

about where they can take dead or diseased fish they find, so that they can continue to help in the determination of BLGV geographic range, virulence, and host specificity.

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APPENDIX A

INSPECTION PROCEDURES FOR BLUEGILL VIRUS (BLGV)

[FORMATTED TO BE INCLUDED IN USFWS AND AFS-FHS STANDARD  
PROTOCOLS FOR AQUATIC ANIMAL HEALTH INSPECTIONS (USFWS AND  
AFS-FHS 2007)]

## Bluegill Virus (BLGV)

Bluegill virus (BLGV) is a non-enveloped, icosahedral, single-stranded RNA virus belonging to the family *Picornaviridae*. The known BLGV geographic range extends throughout much of the Upper Midwest. BLGV is primarily found in bluegills, but has also been isolated from black crappies. Transmission of the virus is still not understood, but because BLGV particles are hardy in the laboratory setting, particles are suspected to remain infectious in the water column for a long time. Under natural conditions, BLGV has most often been associated with fish disease during periods of rapid temperature change observed in late spring, late autumn, and early winter months. Most notable diseased fish have exhibited hemorrhaging of the skin and fin bases and vent swelling.

### 1. Screening Method

- a. Cell culture on BF-2 cell line incubated at 25 °C.
- b. Monitor for CPE at least twice per week for an initial incubation period of 14 days.
- c. Re-inoculations are made from representative wells exhibiting CPE during the initial incubation and from at least one well of all samples not exhibiting CPE (blind passage) and monitored for an additional 14 days.  
**If no CPE is produced by the end of the re-inoculation incubation, the sample is considered negative for BLGV.**
- d. If CPE typical of BLGV is produced at any time during the incubation, that sample is considered **PRESUMPTIVELY** positive for BLGV, LMBV, and VHSV, and confirmatory tests must be completed to distinguish among these viruses.
  - i. The appearance of CPE typical of BLGV is described as the initial development clustered, phase-bright cells. As the plaques spread, they are composed of elongated cells in the middle surrounded by clustered phase-bright cells on the periphery, which contain many large vesicles. At higher magnifications, these vesicles can be visualized more easily. See Figures 21 and 22. Typically, CPE are slow to develop, often after 5-7 days. Eventually, nearly all cells lyse and detach from plate surface.
- e. RT-PCR may be used to confirm the cause of the CPE.

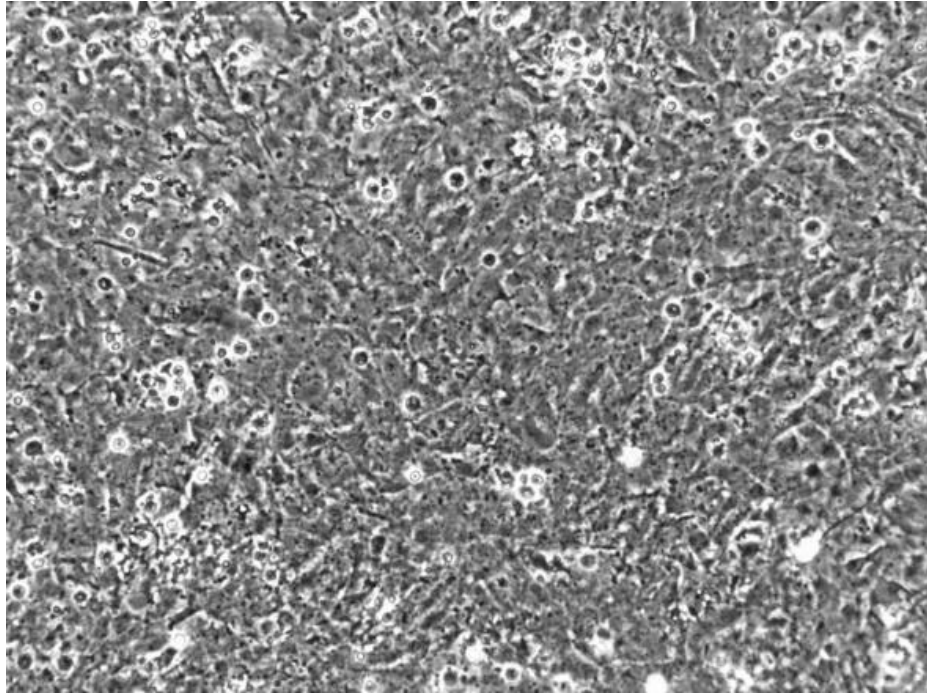
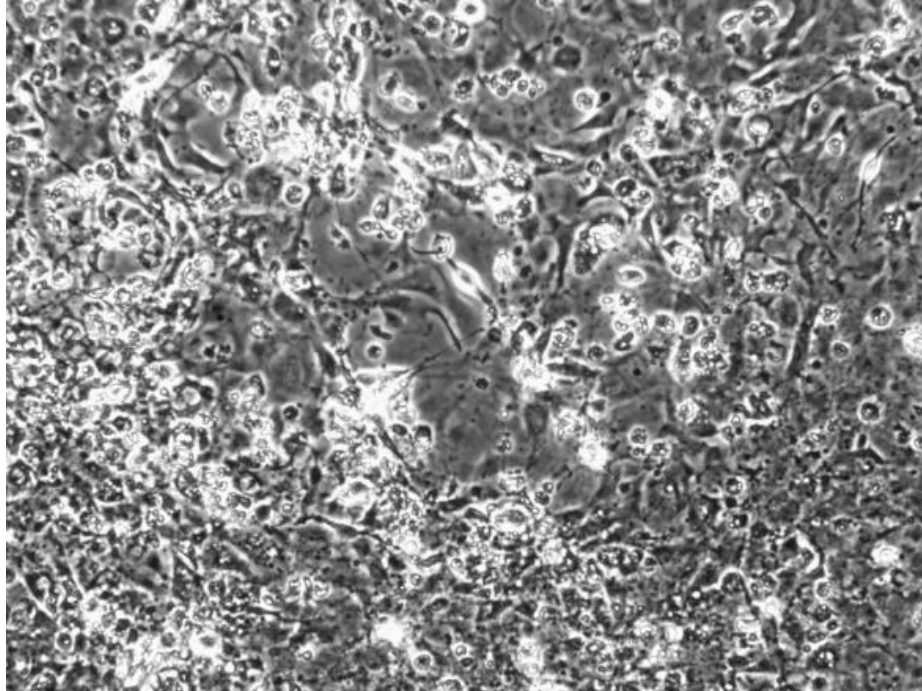


Figure 21. Normal BF-2 monolayer (10X magnification). Photo courtesy of Marisa Barbknecht, University of Wisconsin-La Crosse.

A)



B)

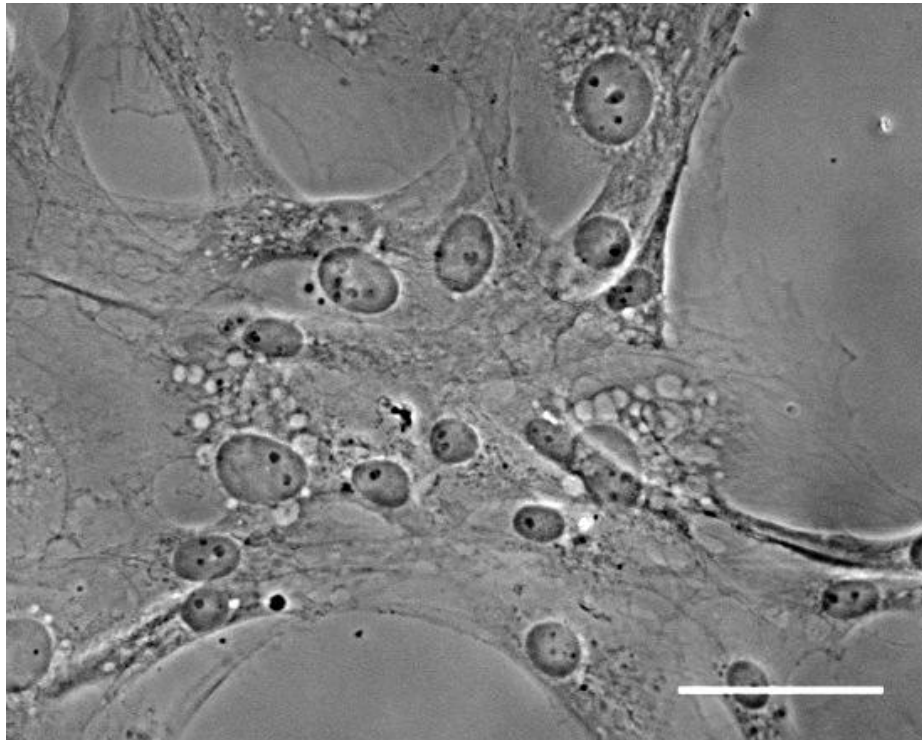


Figure 22. CPE typical of BLGV on BF-2 cells; A) 10X magnification and B) 60 X magnification; scale bar is 30  $\mu\text{m}$ . Photos courtesy of Marisa Barbknecht, University of Wisconsin-La Crosse.

## 2. Confirmation Method for BLGV

### a. Polymerase Chain Reaction (PCR) Method for Confirmation of BLGV

The Reverse Transcription Polymerase Chain Reaction (RT-PCR) technique employs oligonucleotide primers to amplify segments of the complementary DNA (cDNA) produced in RT reactions containing the target virus. Total RNA is extracted from cell culture supernatant, subjected to RT for production of the appropriate cDNA, which is then amplified with forward and reverse primers. The double-stranded DNA product from RT-PCR amplification is then visualized by agarose gel electrophoresis.

#### i. Extraction of RNA from Cell Cultures

Total RNA from infected cells is extracted using an RNA affinity spin column (Qiagen UltraSens, RNeasy Total RNA kit) according to manufacturer specifications.

#### ii. Production of cDNA by Reverse Transcription and Amplification by PCR

1. QA/QC (See Section 2, 6.2 PCR – Quality Assurance/Quality Control for specific QA/QC considerations for PCR)
2. Using Section 2, 4.A1.A Worksheet A – PCR Sample Data/Log Sheet, record appropriate data for each sample to be tested by PCR
3. Using Section 2, 4. A1.B.1 Worksheet B.8 – Bluegill Virus (BLGV), record date of assay and then calculate the amount of each reagent to go into the “Master Mix” (MM) according to the number of samples to be processed. (Add four to the number of samples so that there is enough to run controls.)
4. Primers for BLGV
  - a. Forward: 5' CTCGATAGTGTATGACTCGGACC 3'
  - b. Reverse: 5' CATGGGGTTCAACTCACA 3'
5. Reverse Transcription
  - a. Incubate 43 °C for 1hr
6. Thermal Cycler Program for Polymerase Chain Reaction
  - a. Denature sample at 94 °C for 4 minutes
  - b. 35 cycles as follows:
    - i. Denaturing at 94 °C for 20 seconds

- ii.* Annealing at 55 °C for 30 seconds
- iii.* Extending at 72 °C for 25 seconds
- c.* Final Extension at 72 °C for 5 minutes

**Hold samples at 4 °C after cycling is complete. PCR products can be refrigerated for one month or frozen at -20 °C for long-term storage.**

iii. Visualization of PCR Product by Electrophoresis (Section 2, 6.3.C “Detection of Product”)

*1.* Visualize the Band

Carefully record location of bands on positive control samples in relation to DNA ladder bands. Band locations of positive controls should be at anticipated locations according to primers used in PCR assays.

- a.* **Bands occurring at the 180 bp location are confirmatory for BLGV and are reported as POSITIVE.**
- b.* **If there is an absence of appropriate bands with no indication of problems with the assay, consider testing for other viruses or consult an appropriate reference library.**

*2.* Photograph the Gel (Section 2, 6.3.G “Visualize the DNA”)

**Photo document all gels** and attach the photo to the case history information (Section 2, 4.A1.C Worksheet C – Photo documentation of the PCR Product Gel)

## Bluegill virus (BLGV)

Case Number \_\_\_\_\_

Date \_\_\_\_\_

### Reverse Transcription

Reagent	Lot #	Stock Conc.	Final Conc.	Volume (μl)	Volume for _____ samples
Sterile dH <sub>2</sub> O		--	--	6.0	
M-MuLV buffer		5X	1X	5.0	
dNTPs		5 mM	0.5 mM	2.5	
Dithiothreitol (DTT)		0.1 M	0.01 M	2.5	
RNase inhibitor		40 U/μl	0.8 U/μl (20 U/rxn)	0.5	
Reverse primer		20 μM	2 μM	2.5	
M-MuLV reverse transcriptase		200 U/μl	8 U/μl (200 U/rxn)	1.0	

- Transfer 20 μl of Master Mix and 5 μl of RNA template to each tube and mix.
- Incubate at 43 °C for 1hr. Proceed to PCR.

### Primer Set for BLGV

Forward	Reverse
5' CTC-GAT-AGT-GTA-TGA-CTC-GGA-CC 3'	5' CAT-GGG-GTT-CAA-CAC-TCA-CA 3'

**PCR**

Reagent	Stock Conc.	Final Conc.	Volume Added (μl)	Volume for ____ samples
Sterile dH <sub>2</sub> O	--	--	36.5	
Taq Buffer	10X	1X	5.0	
dNTPs	5 mM	0.2 mM	2.0	
Forward Primer	20 μM	0.2 μM	0.5	
Reverse Primer	20 μM	0.2 μM	0.5	
Taq Polymerase	5 U/μl	0.05 U/μl (2.5 U/rxn)	0.5	

- Transfer 45 μl Master Mix and 5 μl of RT reaction per tube and mix.

**Control Information**

Positive Control (s)		Negative Control (s)	
Extraction	PCR	Extraction	PCR

**Amplification (Thermal Cycle Process)**

Date & Time	Program #	NOTES

**Gel Preparation**

Gel Concentration	Apparatus and Gel Size	Weight of Agarose (grams)	Volume of buffer (mL)

**Gel Template (Sample Placement Map)**

Ladder Brand/Lot # \_\_\_\_\_

Loading Buffer Brand/Lot # \_\_\_\_\_

APPENDIX B

PARTIAL BLUEGILL VIRUS (BLGV) GENOMIC SEQUENCE FROM THE  
LA CROSSE FISH HEALTH CENTER CASE HISTORY NUMBER 04-032 ISOLATE  
(MONTANA LAKE, WISCONSIN)

1 ACTTCTTGTG GTCTTTTATC CTGGAGAGGA TAATAAAGAC AAGGATGTGA CAATGGACAA  
61 GGTGAACAAT GCGTTCACAC AGATTCTGGA CTTGGGATCA AAAACAACAG TCAGATTACAC  
121 ATTGCCGTTT GTGAATCAGC AACCATACAG ACCAATGTCA AACGTGCACG GCCGCTTTGC  
181 TGTATTTGTG CTTAACCCGC TAACATATAC CCCCGCATGC CCAAGTGCTG TTCGCATTTT  
241 GTTTTACATT GGTGCTGGAT CGTCATTCAA CTTTGTGTAC CCAAACAAT CTGCTGTAAA  
301 ATTTCAAGCA CCAGAACGGA ATCAGGTGGG CGATGCTACA TCGGCTGATG GAGCACCGAT  
361 CACCAACATT GAAGATGAGA CATCGAACAC TCGTGTTCAA AAACGCCAG TGTTTAGGAA  
421 TGTTAAAGCG ACACTTCCGC GCCAGATTAA ATCAAACCAC ATGCTTCTGT CCAACCTAGT  
481 TGGACGTGCC TTTGCTCTTG AACCGTACAC TTCAACAAGT ACGGGAGGAC AAAACATTTT  
541 GCTGAAGGAA AACAACTTGT CTTTTTTGAA GGTGTTTAAG CTCCTTTTGT ATCGGAGTGG  
601 AGACATGACT CTTTATGTTT CACACACCGC TGTATCACCT CTTTACATGG CACACAGTTA  
661 CATCCAGACA GCAATTGCAG GAACAACATC TGCGGACCGC TTGTCCCAGA TAATGTCACA  
721 AGGATCTGTA TGCCTGATGT CGAACTCAGC AGCAAGCCTT TGTGTGCCTT TTTACGTTAT  
781 GACACCATTG GTGAAGACCG CAGACTTTGG CAGACTGTTT GTGGAAGGAC AAAATGACTC  
841 AACATGGATC ATGTGGATAG GAGCAACATT TGACACAGAC ACCAGATTTT ACTTTTTGCA  
901 GTCCCCCCCC GACATTGCTC TTGGAGAGAA CGAGAAAGAA GAACCAGAAG ACTTGGAAATG  
961 GGTGAGGAG GAAGGTCACA GACAGACAGA AGTTGAAACC ATTCAAAGGT TCTTGAAGG  
1021 AAGAGGACTC TTCCCAGTAC ACAAGACATG TGACATTAAT GACAGAGATG GCATGTACAT  
1081 GACTTGTTAT TCAGAGGTGA CCATTGGAGA CAAAACATTC AGGGCAAACCT CTTTTTGGAG  
1141 AACAAAGTCC TTACTIONAAG CTTACAGGAT GTGCAAAGAA TTTGTAAGAG AATCAGACAA  
1201 CCAGGAGCTA CTCAAGTGTG GAGATGTGGA GTCTAACCCA GGTCCAGATA TTTGGAGAAA  
1261 CCGCATCACA GGATGCATGG CAGTTCAAGA AGGGGCAGCG CTCTATTACA GCCCAAACAA  
1321 ACAGATTGTG GATAACATTT ATGTGCTGCC AGTACAAAAT CACATTGAGG ACTGGGAGAG  
1381 ATATTCCCAT TTTACCATGG CTTGTCTTGA ACCAATCTGG ACAGCCCAGA TGCCCCGACT  
1441 TCAACACACA AACACTATAG CTGTAAACAA CCCAGCACCA AACACCGTTG CTGCTGCCAT  
1501 TACTGATG GCAATGAGGG TCAATGGACC TAGATGGTTG GTGAAATTCA TTTTCCCTT  
1561 CCTTCACGCT GCGAATATGT GGGACCTTTC AACAGGATGG TTTCACTTTT TCCGTTTGT

1621 GAGGAGCGGA GATGTTGAAC AGAACCTGG ACCAATTAAC ATCCGGTACT GCACTGATGA  
1681 AGAACACATT AGATATGCGT TGAAAGTGCA TAACCTGAAG TCCATTGAGA TAGTGAACCG  
1741 GGATTTTTTT CAAGAAATTG GGGAGAAAAC CATTCACTTG CAGATTGTTG ACACAAAGCA  
1801 TTGGAGAAAT TTGACACTTA CTGATTGCGA AGAGAAAACA CTGATGAAAA TTGGTAAGGA  
1861 CTTGATCACA AATGATATGA AATTGACAAC ACCCGTTGGA CTTGGGAGAG TGGAACATTT  
1921 GATCGAAAAG ACACACCTTC TGACGGGACT AAGGTTCTTG AAGATTGTGA ATCCTGAAGG  
1981 TCGCATATCT GACACACTTG CTTGGTTCTT GGGTTGGGAG AAATCTGGAC TCGAGAGAGT  
2041 ACATGTGCTG ACGACAATGA TGGCAGATGG TAACATCACC CGCATTGTTG ATGGGATCAC  
2101 CGATTCCATT GCCCGCACAT GTGTTAAAGG AATGATAAGA GCGGTGTTAT ACACATTGAT  
2161 TTTTTCTTTG ACACCAAATG CTGTTGCAAG AGGAGCAATA GCAGCCCTAG CGGCCTTTGA  
2221 TTTGACCACT AGCATCCTAG ATGCGTTTCG AAACATGAGC CTGAAAAGAC TAGCAGAGTA  
2281 CTTGATACCA GAGATGGAGA ACAAGCAGGA GTACACCAGA GAGAGGACCA ATCTCCTAGC  
2341 AAAAGTGAAC AAAGTTATGC GACATAATGA GATGAAGCAA GATGAGTGGT TTGATGCAGT  
2401 TGATGACACA CAGGAGGTTG TCACAGAACA AGGACAACCT GGGTTTAAACA CATTACCTT  
2461 GGTATCAAAG AACCTTGGGT GGTGGATAAC AAAGATTCAA GAATTGGCTG ACAAACCTACA  
2521 ACAGTGGTAT AACCCAGACA GAGTCCAAGT TGCTAAGACA ACGTTGGAAG AGGTTAAGGA  
2581 GAAGATCATT GATGCTTTGA CAGAGACTGA AGCCCTTATG GCCAAAGTGA AAGTACAAGG  
2641 GGTACCAACA CAAGCAGACA GAGATGAGTG GCAACAGAAA TTTGACACCT TGACCAAGTT  
2701 TTACAAACTG AGCCTGGTCG TTCCAAATTG CCCAGTGTCA AGAAACATTG GGATTGTGCT  
2761 AAACAACATG AAGAGAATGC AACTTGGGCA CCAAAAACCT AAGGATCCAA TTCGAGTTGA  
2821 TCCGGTGGCA ATTCATTTTT GTGGAGAGCC AGGACAGGGA AAGACAGTTC TGATGACTAG  
2881 ATTGACAAAC GCATTGGCTA AGGCCCTGAA CACAGAGGTG TACACACACG CAACAGGAAG  
2941 CAATTATTTT GATGGTTATT GTGGACAAGG TGTGCACTAC ATTGATGAGT TCATGGCTAA  
3001 CACAGATGAG CAAGAGGCTT CACTGGCTTT GCAATTGATG GGCTCGTCCC AAGTGATTTT  
3061 GCCAATGGCA GATGTTGACC AGAAAGGGAT GTACTATAAA GGAAAAGTGT TGATAACAAC  
3121 TTCAAATACA AGTCCAGTGA GTGACAGCAA ACTAAAAGCA CCAAAGCAT TGAAAAGAAG  
3181 GTTTATGGTA GTCGGCTTTC GAGCTGCAAC CTATTACCAG AAGATCACCA ATGGTGCAAT  
3241 GTGCATTGAT TTGGCTAAGG CAACGAAGAC AGGAGCACTT CAAGATGGAT CTTGTTGGGA  
3301 GTGCGAGATT GATGGCAAAT GGAGAACATT TAGTTTTTAT GACTTTGTTG ACATGATTTT

3361 GGCAAAAGTG AAGAGCAACC AACTGGTGCA CCAAGCACTT CTTGAACAAA TGAAGCAAGA  
3421 TGAAGAACAG ATGAAGAAGT ATGCTGTGCT AGCCCAAGGG AGCGAAGATG AGGAGAAACA  
3481 AATGACTGAT GATGAATTTG ACCAATTGCT GAGTGAGATG AGAGATTTGA CTAAAGAGAT  
3541 GACACAAATG AGCAGCGATC TCGAGATTTT TCACGAGGCG CGAGAGACAG CTGTTGTTCC  
3601 GCCTGACATT CGGAGCAAAG TTGTAAAGGC CATGCAGGAC TGCTGGGCCA ACATTGTTTC  
3661 AACAGACATT AAGACTGTC TAGAGAGAGT GTCTTTGGCG GTCACTGTGA TCAGAGTAAG  
3721 CACCACCATT TATGACATGA TTAAAGGGTT CCGTGCCACA GAAGAGCAAA GAGCCTACAA  
3781 CCCAACAACA AAACCTGGAC TGACAAGAGT GCTGAAGAAG ACAGCAAGAG CAGGACCCAT  
3841 TCAAGTTGAA GGAGGAAACA ACAGTACAGA GTACAAACAC ATTCTTGATT CAATGGTACA  
3901 TTTGGAGTTT GAGAGTGGAT TGAGGAGCAC TGGATTTTGC GTGGGACAAC GCGACATTTT  
3961 TGTGTACGCA CACGGGGTCG GAGAAGGATC CATGATGTTT GAACACAGGT CTGTTGCATA  
4021 TGAAATTCCA GAGGACATGT ATGAAGTGG AACAATTTGTA ACTGGACAAG GGAAGATGGA  
4081 CATGGCTCGC ATTGTGATTG ATTCGTCGAT CGGATTGGAG TTTAAAGGAA TGTGGAAACA  
4141 CATAGCCAAG CCCATTTTTT CCCGCGATGC TTTGCTAATA AAAAAATGG ACAACTTGTT  
4201 CAGAGAGAGA GAAGCAGGGA CAGTCAAGGA CGCTGGAATG ATTGTGATGA AGGATTTTGA  
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4321 AGATTGTGGA TCATTGCTGC TTCAGAAGCA GTATGGAACT TGGAAAGATCA TAGCGATGCA  
4381 CAACGGATCT AGGCAAGGAC TTGCCTACGG CGTGAGGCTT GATGTGTGCA TAGCAAAGTA  
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4501 TGCCATACAT AAATCACCTT TTTATGGTAT TCAAGAAGCA ACAATGCAAC CAGCGCCACT  
4561 GCGAGCAACT GACCAACGGA TAACTGTTCC TATTGAAAAT TTGACAAAAG AGGCAGCTGA  
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5461 TGACTIONTTG TCGGTGGTGT ATGGAGATGA TTGCGTTGTA GCCTACAATG GCATGCGCAT  
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5581 AAAACTTGGT GACCACTTTA ACGTTGAACT GCATGAAGTG GAGTTTTTGA AGAGAAAGTT  
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5941 GCGATGACCT TGGACTGGAG ACTATGATTT TGGATATCTT TGAAATACTC GATAGTGTAT  
6001 GACTCGGACC GTTTGACAAT GCTGATGCGG ATATTGACTT TTAGACACAT TATTAGCCAC  
6061 CTTTAGTTGG AGCGTTCAGG CACTAGACCT GTAATGTGTT GATATATATA ATTTATGCTT  
6121 GCAATAAATA GATGTAATGT TAGTTTTTGT GAGTGTTGAA CCCCATGTAT TTGTATGTGG  
6181 TTATAACTAG TTTTLAGGGA AACTAGTTT TTAATGTTTA CCCCCCCCC CCCCT