In vitro culture of lake trout cells (*Salvelinus namaycush*)

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ABSTRACT

The lake trout (*Salvelinus namaycush*) is an ecologically and economically important fish species in the Great Lakes basin. Primary cultures of lake trout cells were produced following manual and enzymatic digestion of tissue and incubation at 15°C with Earle's salt-based minimal essential medium (MEM) supplemented with 15% fetal bovine serum (FBS). Primary cultures of both yearling gonad tissue and sac fry body cell types were readily established and subculturing occurred within 2-4 weeks of initial seeding. Repeated passaging of cells resulted in gonad cells reaching subculture number 35 and fry cells reaching 52. Additional primary cell cultures were produced from yearling fin and broodstock liver tissues. Morphologically, both fry and gonad cells started out as mixed populations with a substantial percentage of fibroblast-like cells, however as passages went on, cell populations became increasingly epithelial-like. Species origin was confirmed using DNA barcoding. Infection of novel lake trout cell lines with pathogenic aquatic viruses VHSV, IPNV and EEDV suggested the cells are capable of supporting viral replication.

Key words: lake trout, cell culture, *Salvelinus namaycush*, virus susceptibility

INTRODUCTION

The lake trout (*Salvelinus namaycush*) is an extremely important native fish species in the Laurentian Great Lakes Basin (GLB), and in fact, Lake Michigan once held the world's largest population of this species (Bronte et al. 2008). This coldwater, apex predator is well adapted to life in the Great Lakes, has a steadying effect on local ecosystems and is prized by the sports and commercial fishing industries alike (Redick 1967; Bronte et al. 2008). Tragically, fishery

managers and enthusiasts have been confronted with significant population threats and declining numbers over the past 60 years due to a conglomerate of ecological and anthropogenic factors including invasive species invasion (i.e., sea lamprey (*Petromyzon marinus*) and alewife (*Alosa pseudoharengus*)), habitat destruction, over fishing, and emerging infectious diseases (Hile et al. 1951; Eschmeyer 1957; Wells and McLain 1973; Hansen et al. 1995; Holey et al. 1995; Eshenroder and Amatnagelo 2002; Bronte et al. 2008; Cline et al. 2013). Rehabilitation programs focused on the recovery of this important fish rely heavily on the use of captive breeding programs, unfortunately, the intensive nature of salmonid aquaculture serves as a ripe location for the eruption of infectious diseases (Redick 1967; Bronte et al. 2008).

Lake trout, particularly in the Great Lakes, are susceptible to a number of viral diseases. In the mid 2000s, Infectious Pancreatic Necrosis Virus (IPNV) was detected in the Allegheny National Fish Hatchery in Warren, Pennsylvania, leading to the culling of all lake trout and brook trout (*Salvelinus fontinalis*) on site (Faisal et al. 2013). Lake trout are also especially sensitive to infection with the Epizootic Epitheliotropic Disease Virus (EEDV), which led to the death or culling of more than 15 million lake trout in the mid 1980s (Bradley et al. 1988; McAllister and Herman 1989; Kurobe et al. 2009) and has recently re-emerged as a pathogen of particular interest in the Great Lakes (Shavalier 2017). Additionally, lake trout are susceptible to the OIE-reportable pathogen Viral Hemorrhagic Septicemia Virus (VHSV) which has been detected throughout the Great Lakes basin and led to mortality events in multiple species of fish (Faisal et al. 2012; Standish et al. 2016; International Office of Epizootics. Aquatic Animal Health Standards Commission. 2017).

One key aspect of aquatic animal health programs is the diagnosis, prevention and study of viruses such as these, tasks that often utilize cell culture techniques and assays. Cell culture

has become ubiquitous in many fields of study, including toxicology, immunology and others (Fryer and Lannan 1994; Lakra et al. 2011), and tissue culture *in vitro* models often also serve as an acceptable alternative to whole animal models in research study design (Lakra et al. 2011). Unfortunately, while American Type Culture Collection (ATCC) carries over 3,400 commercially available distinct cell lines, less than 20 of those are derived from fish tissues, leaving many researchers and diagnosticians no choice but to develop their own cell lines for particular projects. The development of primary cell cultures has inherent difficulties as normal somatic cells are not immortal and will eventually trigger senescence if not immortalized (Lakra et al. 2011). Additionally, as primary cells are passaged and subcultured, original characteristics may be altered over time, affecting their usefulness to virological, pathological or toxicological studies (Lakra et al. 2011). Attempts were made several decades ago following the initial outbreak of EEDV, to produce a cell line of lake trout origin, unfortunately, the cell cultures were not preserved (Cheng et al. 1993). To date, there are no established cell lines originating from lake trout tissues. However, primary cultures have been successfully created from rainbow trout gills and head kidney for use in immunology and pharmacology studies (Stott et al. 2015; Khansari et al. 2017). In the present study we describe the establishment and characterization of two novel cell lines from lake trout tissues, which can be used in virology, toxicology or immunology studies to improve the health and maintenance of this treasured species.

MATERIALS AND METHODS

Fish and tissue collection

For this study, three different groups of lake trout were obtained for tissue collection and production of primary cell cultures: 1) a single sexually mature fish (>12 years of age), which

had been spawned and housed its entire life at the University Research Containment Facility (Michigan State University, East Lansing, Michigan); 2) yearling fish collected from the Marquette State Fish Hatchery (MSFH, Marquette, Michigan); and 3) sac fry collected from the Marquette State Fish Hatchery (MSFH, Marquette, Michigan).

The adult lake trout was removed from its holding tank, and euthanized with an overdose of tricaine methansulfonate (MS-222; Argent Chemical Laboratories, Redmond, Washington; 0.25 mg/mL) prior to tissue collection. After dissection each tissue was placed in sterile cell culture media (MEM-0) of Earle's salt-based minimal essential medium (MEM; Invitrogen, Carlsbad, California) and supplemented with 10% tryptose phosphate broth (BD Biosciences, San Jose, California), 29.2 mg/mL L-glutamine (Invitrogen), penicillin (100 IU/mL) (Invitrogen), streptomycin (0.1 mg/mL) (Invitrogen), amphotericin B (250 μg/mL; Invitrogen) and sodium bicarbonate (7.5% w/v) (Sigma) for temporary holding and to prevent trying during transport between facilities.

Blood was drawn directly from the ventricle following a cut down dissection of the heart and diluted 1:2 with MEM-0 containing heparin at a minimum of 30 IU/mL blood. External tissues (i.e., skin and fin) were briefly flame sterilized to remove external pathogens and then dissected and placed into a solution of MEM-3x, which contained triple antibiotic/antifungal (penicillin (300 IU/mL), streptomycin (0.3 mg/mL) amphotericin B (550 μg/mL)), for 30 minutes, after which time tissue were transferred to MEM-0 while remaining tissues were collected. The opercular cavity was briefly dried with sterile gauze after which an entire gill arch was dissected and placed in MEM-3x for 30 minutes and transferred to MEM-0 for transport. Finally, portions of liver, testes, anterior kidney and posterior kidney were aseptically collected and placed directly into MEM-0.

Tissues were collected similarly from yearling lake trout with the exclusion of blood. For lake trout sac fry, fish were dissected in order to remove the yolk sac and the head (cranial to opercular margin) with the remaining body processed as a singular tissue sample. Multiple individual fry were processed together until an adequate amount of tissue was collected.

Isolation and in vitro culture of primary cells

Individual tissues collected from all three groups of fish were transferred to sterile petri dishes where they were manually digested using scissors until they reached a size of approximately 1-2 mm diameter. Next, enzymatic digestion was performed using 0.25% Trypsin-EDTA (Gibco, Life Technologies, Carlsbad, California). Minced tissue was combined with 10 mL trypsin in an Erlenmeyer flask and placed on a stir plate. After three minutes, the initial trypsin was removed and replaced with a fresh 20 mL of trypsin. This solution was left to stir for 20-60 minutes or until tissues visually determined to have reached complete digestion. The tissue-trypsin suspension was then filtered through sterile gauze to remove any remaining tissue pieces, after which, MEM-10 (10% fetal bovine serum; Hyclone Laboratories Inc.) was added to the resulting filtrate at a ratio of 2:1 (medium/trypsin) in order to deactivate the trypsin. The single cell suspension was then centrifuged at 190 x *g* for 5 minutes at 15°C. Following centrifugation the supernatant was discarded and the cell pellet resuspended in 10 mL of MEM-10. This rinsing step was repeated a total of three times. The final cell suspension was seeded into 25cm^2 cell culture flasks (Corning) and incubated at 15°C. This process was repeated for all tissue types with the exception of the blood. Heparinized blood diluted with MEM-0 was centrifuged at 4,700 x *g* for 10 minutes at 4°C. Next, the visible buffy coat plus one half mL red

cells were collected, mixed with sterile water 1:1, then washed with MEM-10 and seeded into culture flasks as above.

Routine subculture

Primary cell flasks were monitored daily for evidence of attachment and replication. Unattached cells and spent media were removed from the flask every 2-3 days, replaced (75%) by fresh MEM-10 and unattached cells checked for viability using Trypan Blue (Sigma-Aldrich, St. Louis, Missouri) exclusion staining. If greater than 20% viable cells were observed in stained sample, remaining cell suspension was pelleted (centrifuged at 190 x *g* for 5 minutes), resuspended in 10 mL of MEM-10, and seeded into a new culture vessel. If no viable cells were detected either attached to the culture surface, or in suspension via exclusion staining, flasks were discarded.

In flasks where primary cell growth occurred, once cultures reached >90% confluence, or if replication rate slowed, they were subcultured per standard laboratory protocols. Growth media was removed from the flask and cells rinsed briefly with 1 mL 0.25% trypsin-EDTA (<10 seconds). After first trypsin rinse was removed, 2 mL fresh trypsin was added to the flask and very gently rocked until cells had released from flask, at which time MEM-10 growth media was added at a ratio of 3:1 (medium/trypsin) in order to deactivate the trypsin. Cell suspension was centrifuged at 190 x *g* for 5 minutes at 15°C after which the supernatant was removed, cell pellet resuspended in MEM-10 and centrifuged a second time. Final cell suspension was reseeded into culture flask with approximately 40-60% of original cells. Subculturing continued in the same manner once flasks reached near 100% confluence with select cultures being cryopreserved in liquid nitrogen (180 µL dimethyl sulfoxide (DMSO) per 1 mL cell suspension).

Optimization of culture conditions

Influence of temperature on cell growth

The effect of incubation temperature on cell growth was evaluated for the two most promising cell lines: yearling gonad tissue (subculture 4) and fry tissue (subculture 11). Gonad and fry cells were seeded into 12.5 cm^2 culture flasks (CELLTREAT Scientific Products, Pepperell, Massachusetts) at a density of $4x10^5$ and $1.5x10^5$ cells per flask respectively. Flasks were incubated at 15, 21, and 25°C while all other culture conditions remained consistent as described above. Flasks were monitored daily for a subjective assessment of percent confluence. Every 2-5 days, cells were detached using trypsin, from $n = 2$ flasks per day, and counted using a hemacytometer in order to assess relative cell growth and density (cells per cm^2).

Influence of serum type and concentration on cell growth

The effect of serum supplementation type and concentration on cell growth was also evaluated for the same two cell cultures, seeded as with the temperature trial. In this experiment, all flasks were incubated at 15°C but contained growth medium supplemented with either 10% fetal bovine serum (FBS), 15% FBS, or 15% FBS plus 1% heat inactivated lake trout serum. All other culture conditions remained identical. Lake trout serum was collected from mature lake trout such as was used to produce primary cultures and heat inactivated at 56°C for 30 minutes. Flasks were monitored and assessed as described above.

Influence of growth medium base on cell growth

The effect of two different growth medium bases was evaluated for the yearling gonad and fry cell cultures as well. All cells were seeded as above in growth medium containing 15% FBS and incubated at 15°C. Growth medium was produced using either Earle's salt-based minimal essential medium as described above, or Leibovitz's L-15 medium. All other culture conditions remained identical. Cells were monitored and counted as above.

Confirmation of species of origin

In order to establish that these novel cell lines were indeed of lake trout origin, a DNA barcoding technique was employed to amplify and sequence the cytochrome *c* oxidase 1 (CO1) gene (Cooper et al. 2007; Ivanova et al. 2007). An early and late passage cell sample from both the fry and yearling gonad cell lines were used, with lake trout skin tissue serving as a positive control and *Epitheliosum papulosum cyprini* (EPC; ATCC) cells as a negative control. DNA extractions were performed using the Mag Bind® Blood and Tissue DNA Kit (OMEGA Bio-tek, Inc, Norcross, Georgia, USA), following the manufacturer's instructions for extractions from cell cultures. All PCR reactions were carried out in a Mastercycler Gradient thermocycler (Eppendorf, Hamburg, Germany). The COI-3 primer cocktail designed by Ivanova et al. (2007) was used to amplify a 631 bp fragment of the COI gene. Each 25 µL reaction mixture was comprised of 12.5 µL 2x Go-Taq Green Master Mix (Promega, Madison, Wisconsin, USA), 0.8 µM of each primer, and 4.5 µL DNA template. Cycling parameters were as described by Ivanova et al. (2007) for the COI-3 primer cocktail. Amplicons and a 1 kb Plus molecular ladder (Roche Applied Science, Penzberg, Germany) were, electrophoresed through a 2% agarose gel with SYBR Safe DNA Gel Stain (Thermo Fisher Scientific) at 100V for 30 minutes, and visualized under ultraviolet light.

Amplicons were prepared for sequencing using ExoSAP-IT (Thermo Fisher Scientific). 1 µL of each PCR product was combined with 3 µL 1x $MgCl_2$ Buffer and 0.25 µL ExoSAP-IT reagent. The ExoSAP-IT mixture was placed in the thermocycler with a program of 37°C for 20 minutes followed by 95°C for 10 minutes. After clean up, amplicons were Sanger sequenced at the Michigan State University Research Technology Support Facility using M13 forward and reverse primers (Ivanova et al. 2007). Sequences and chromatograms provided by the Michigan State University Research Technology Support Facility were visually examined using 4Peaks software (http://nucleobytes.com/4peaks/; Version 1.8) and contigs were assembled and aligned using ClustalW in the Molecular Evolutionary Genetics Analysis software (MEGA; version 6.0) (Tamura et al. 2013). Resulting contigs were then entered into the Barcode of Life Data System (BOLD) (Ratnasingham and Hebert 2007) search function where each sequence was compared against the ID System to identify nearest neighbors using a global alignment of more than 3,000,000 barcode sequences from 180,000 animal species.

Viral susceptibility

The susceptibility of our newly established lake trout cells to three different aquatic viruses was evaluated. Flasks of fry cell cultures were exposed to isolates of viral hemorrhagic septicemia virus (VHSV), and infectious pancreatic necrosis virus (IPNV), while both fry and gonad cells were inoculated with EEDV. Viral stocks of VHSV and IPNV were inoculated into 25cm^2 flasks of fry cells at and incubated at 15°C. After inoculation, cells were monitored via light microscopy for development of cytopathic effect (CPE) at 48 and 72 hours post infection.

As a current *in vitro* model of replication does not exist for EEDV, our lake trout cells were exposed to virus-positive tissue homogenate supernatant (first passage on cells), followed

by a second passage on cells of either first pass supernatant or first pass cells. As optimal incubation temperature for EEDV *in vitro* is unknown, infected cells were incubated at a range of temperatures (i.e., 4, 9, and 15°C).

Quantification of viral DNA

A TaqMan quantitative PCR (qPCR) described by Glenney et al. (2016) was used to compare viral titers in tissue samples to those in cells and supernatant following inoculation of lake trout cells. In this manner, a relative increase in viral loads would suggest replication by active virus rather than merely the presence of viral genetic material. For DNA extractions, the MagMax™ 96 Viral RNA isolation kit (Life Technologies, Grand Island, New York, USA) was used manually, following manufacturer's instructions, after which, extracted DNA was quantified using a Quant-iT DS DNA Assay Kit and a Qubit fluorometer (Life Technologies, Grand Island, New York, USA). All qPCR reactions were carried out in a Mastercycler ep *realplex*^{2} S real-time PCR machine (Eppendorf, Hauppauge, New York, USA) with qPCR protocols as previously described (Glenney et al. 2016).

RESULTS

Primary culture and routine subculture

Out of the eight tissues collected from the lake trout broodstock, within two days of seeding, attached cells were observed from the gills (few, <1% confluence), and the liver (moderate number, ~5% confluence). No attached cells were observed in the skin, testes, anterior kidney, posterior kidney, fin or blood cultures and flasks were discarded on Day 5 post-seeding. The flasks of gill cells improved to contain a few small clusters of attached cells on Day 3,

however by Day 5, almost all of these had detached and on Day 8 the flasks were discarded. The number of attached liver cells decreased between days 3 and 5, at which time a media change was performed. By the following day, small clusters of cells had begun to develop (Figure 1a). The number and size of these small clusters increased through Day 10, however by Day 15 the cells were beginning to detach, and the flask was subcultured. Following subculture, the flask was monitored, with media changes performed once weekly until the flask reached 50% confluence around Day 85 at which time a second subculture was performed. A total of 9 subcultures were performed before cell growth began to significantly decrease, with the flask reaching 100% confluence (Figure 1b) within an average of 3-4 weeks from subcultures 3-8.

Of the tissues collected from yearling lake trout, no cell attachment or growth was observed from either the anterior or posterior kidney. Occasional attached cells were observed over the first few days following seeding of the skin, gill and liver tissues, however only a single liver flask produced replicating cells, and these ceased to grow following the second subculture. Fin cells proceeded to grow (Figure 2) and reached 90% confluence by Day 20, were successfully subcultured and again reached 100% confluence in a second 20 days, however following the second subculture, no growth was recovered. Yearling gonad cells on the other hand had a moderate number of attached cells and a few small clusters by Day 2 after primary seeding (Figure 3a) with large areas of up to 50% confluence by Day 3 (Figure 3b). Subculturing occurred as early as two weeks after primary seeding with subsequent passages occurring approximately every month for the first five months and every 1-2 weeks after that (Figure 3c). To date, gonad cells have reached 35 subcultures and continue to grow (Figure 3d).

Primary cultures established from fry tissues (Figure 4a) reached 50% confluence within the first 3 days after seeding (albeit with patchy growth), and 75-100% confluence by Day 14

(Figure 4b) at which time they were subcultured. The next 3 subcultures occurred 3-4 weeks apart (flasks reaching 75-80% confluence), and after the 4th subculture the flasks were reaching 100% confluence within 2 weeks (Figure 4c). In later passages (e.g., >20), the fry cells could be subcultured weekly. Fry cells have been successfully cultured out to 52 subcultures (Figure 4d).

Optimization of culture conditions

Influence of temperature on cell growth

Both fry and gonad cells grew extremely poorly at 25°C (Figure 5, light grey lines). While some level of growth was achieved in both cell types at 21 and 15°C the trend was for best growth at the coldest temperature (although statistical strength is low due to the size). In fact, in the fry, both the percent confluence as well as the number of cells per cm^2 was higher at all time points beyond 3 days post seeding. As such, all further growth of lake trout cells was performed at 15°C.

Influence of serum type and concentration on cell growth

When comparing the three different serum supplement concentrations, it was clear that a 15% FBS concentration was preferred over 10% FBS (Figure 6). When grown in media containing only 10% FBS, fry cells only reached a final percent confluence level of just under 30%, while the other two trials resulted in more than 80% confluence by the end of two weeks. A similar although less defined trend was observed in the gonad cells. The addition of the lake trout serum did not appear to have a positive affect on cell growth and in fact, for both the fry cells and the gonad cells, the ultimate percent confluence was approximately 20% lower in the flasks

receiving media with the lake trout serum. As such, 15% FBS was used in all future medium preparations.

Influence of growth medium base on cell growth

A comparison of the two main growth medium bases (i.e., MEM vs. L-15) showed mixed results. While 100% confluence was achieved in the fry cells with both media types (Figure 7a), relatively poor growth was observed in the gonad cells grown in MEM (Figure 7b), which was uncharacteristic compared to all previous gonad cell growth. In spite of the inconsistencies in this single trial, all cells continued to be grown in MEM rather than changing to L-15.

Morphologic characteristics

Primary cultures of the gonad cells (Figure 3a, 6.3b) displayed fibroblast-like morphologic characteristics. Cells appeared to be bipolar with a length > 2x cell width. However, by the 6^{th} subculture, a more mixed population of fibroblast-like and epithelioid cell were observed, with the epithelial-like cells appearing more polygonal and in discrete patches between the other cells. This trend toward an epithelial-like cell morphology continued through the later subcultures as pictured in Figure 3d.

Morphologically, the fry cells appeared to be a mixture of fibroblast-like and epithelial-like cells in the primary cultures (Figure 4a, 6.4b). However, through passages, they became consistently more epithelioid with regular dimensions growing in discrete patches.

Confirmation of species of origin

Origin of both fry and gonad cell cultures was verified through DNA barcoding. Resulting barcode sequences for fry cells (early and late subcultures), gonad cells (early and late subcultures), lake trout skin and EPC cells were entered into the BOLD ID System. This analysis returned a 100% probability that all four lake trout cell cultures and the lake trout skin tissue were in fact lake trout (*Salvelinus namaycush*) while the EPC cells were confirmed to be of fathead minnow (*Pimephales promelas*) origin.

Virus susceptibility

Clear changes were observed in the fry cells infected with both VHSV and IPNV within 48 hours post infection (Figure 8). Fry cells began to round, shrink and release from the growing surface, disrupting the monolayer. This lysis and rounding of cells is comparable with the typical CPE seen in EPC cells infected with VHSV.

Both gonad and fry cells were infected with various samples known to be EEDV-positive. While cytological changes were observed following initial inoculation, including cell rounding (Figure 9a, 6.9b), piling of cells, vacuolation (Figure 9c), and mild areas of cell lysis (Figure 9b, 6.9d), upon subsequent passages such changes were no longer observed. In order to account for the potential necessity of cell to cell contact for *in vitro* infection as is seen with Marek's Disease Virus (Schumacher et al. 2000) EEDV infectivity trials were completed using both supernatant and cell suspensions for second passages. However, while mild cytotoxicity was observed from the previous cells being introduced onto new cultures, no overt CPE was observed. Cellular changes were more severe when infected flasks incubated at 9°C than at 4°C or 15°C.

The viral loads from four separate infectivity trials with fry cells are highlighted in Figure 10. This data highlights how the viral load detected from fry cells or supernatant following infection with EEDV tissue homogenate is consistently substantially lower than in the original samples. The exception was in the fourth group where it appeared there might in fact be some viral replication (evidenced by an increase in viral load from sample to P1 supernatant), however upon passage to a P2 using both supernatant and cells, once again viral titers decreased substantially.

DISCUSSION

Cell lines originating from aquatic species are important for a variety of scientific fields, but unfortunately, the number of well established fish cell lines is substantially lacking compared to that of mammals. In this study, we established primary cultures of multiple different lake trout tissues, and subcultured, expanded and characterized two: one from yearling gonads and one from fry. The development of these cell cultures resulted in a much-needed expansion of the available arsenal of fish cell lines. As demonstrated by Hedrick et al. (1991), there are times when situations require the availability of host species specific cell lines, such as was the case when in the 1980s significant mortalities were seen in juvenile white sturgeon (*Acipenser transmontanus*) caused by what was later determined to be three separate viruses (an adenovirus, an iridovirus, and a herpesvirus), but at the time were unculturable in the only two established sturgeon cell lines, originating from different species (Hedrick et al. 1991).

The methods described herein resulted in the creation of primary cell cultures from all three age groups of lake trout tested: liver from the broodstock, fin and gonads from the yearling fish and whole sac fry. While neither the yearling fin nor the adult liver cells survived beyond 10

subcultures, the establishment of primary cultures from both of these tissues indicates that they remain potential options for future studies utilizing primary cultures rather than established cell lines. Both yearling gonad and fry cells on the other hand readily established monolayers and produced stable subcultures. These cells were subcultured with relative ease, suggesting that they will be suitable for use in standard laboratory assays.

We determined that both gonad and fry cells were well adapted for growth in either MEM or L-15 growth media, supplemented with 15% fetal bovine serum and incubated at 15°C. These conditions are comparable to those required by other salmonid cell lines (Fryer and Lannan 1994), however differ slightly from those used in previous attempts at producing lake trout cell cultures which grew best at a higher temperature (18-21°C) (Cheng et al. 1993).

Morphologically, in early passages, all cultures contained mixed populations of cells, both epithelial- and fibroblast-like. This was particularly clear in the fry cells, which was not surprising as these cells originated from whole body tissues as opposed to a single organ. However, as passage number increased, proportions of fibroblast-like and epithelioid cells changed with both cell types becoming more epithelial-like. In many individual flasks, it became clear that with mixed cell populations, the fibroblast-like cells were out competing the epithelioid cells, however through regular subculturing and splitting of flasks, certain cultures of epithelial-like fry cells were able to prevail.

A crucial component of cell line characterization is definitive identification of species origin. Historically, methods such as karyotyping, and isoenzyme analysis have been popular (Freshney 2011), however recent advancements in molecular diagnostics have helped cement a new protocol for cell line species identification, DNA Barcoding, which has been used successfully to determine the species of origin of a wide range of cell lines from all animal

kingdoms (Cooper et al. 2007). By sequencing a stretch of the cytochrome *c* oxidase 1 (COI) gene in early and late passages from both the fry and gonad cell lines, and comparing the sequence to the BOLD database, we established that these cells were indeed of lake trout origin.

Following inoculation of fry cells with VHSV and IPNV we demonstrated the development of cytopathic effect (CPE) including rounding and lysis of cells (Figure 8). This is crucial, as it suggesting the susceptibility of lake trout cells to two key lake trout pathogens in the Great Lakes Basin. If further investigation reveals that these cultures are truly capable of becoming infected with VHSV and IPNV, the have the potential to serve as a diagnostic tool in the detection and identification of these important aquatic pathogens. Inoculation of both fry and gonad cells with EEDV resulted in the development of mild, inconsistent CPE that included some lysis and rounding of cells, as well as vacuolation of cells exposed to the virus, indicating a decreased health of the cells. However, qPCR data indicates a substantial difference in viral titers in tissue samples compared to that recovered from cell cultures. With the exception of one inoculation on fry cells (trial number 4 in Figure 10), the identification of EEDV in these cell cultures is consistent with the detection of genetic material rather than active and replicating virus. While these results with EEDV are inconclusive, and do no fully support our hypothesis, we have demonstrated the ability to produce lake trout cell cultures and further attempts can be made with additional cell types and culture conditions to improve changes at supporting EEDV replication *in vitro*.

The lake trout is a commercially, recreationally and ecologically important fish species throughout the Great Lakes Basin (GLB). Unfortunately, lake trout populations in the Midwestern United Stated have faced continued threats due to invasive species, overfishing and infectious diseases throughout the past half century (Redick 1967; Bronte et al. 2008). In order to

combat these threats to species rehabilitation, we have established two novel cell lines of lake trout origin that have the potential to be used in future diagnostic assays and research studies on viral diseases of lake trout as well as other fields such as genetics, toxicology and medicine.

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Figure Legends

Figure 1. Liver cells cultured from an adult lake trout (*Salvelinus namaycush*). A) primary culture, 6 days after seeding (original magnification 200x); B) 6^{th} subculture, confluent (original magnification 100x).

Figure 2. Fin cells cultured from yearling lake trout (*Salvelinus namaycush*), primary culture (original magnification 200x).

Figure 3. Gonad cells cultured from yearling lake trout (*Salvelinus namaycush*). A) primary culture, 3 days after seeding (original magnification 200x); B) primary culture, 5 days after seeding, ready for subculture (original magnification 200x); C) 6^{th} subculture (original magnification 100x); D) 32^{nd} subculture (original magnification 200x).

Figure 4. Cell cultures established from body tissue of lake trout (*Salvelinus namaycush*) sac fry. A) primary culture, 2 days after seeding (original magnification 200x); B) primary culture, ready for subculture (original magnification 200x); C) 14^{th} subculture (original magnification 100x); D) 47^{th} subculture (original magnification 100x).

Figure 5. Influence of incubation temperature on growth of novel lake trout cell cultures. A) fry cells; B) gonad cells. Solid lines indicate cell density (cells per cm^2); segmented lines indicate subjective percent confluence. 2 flasks examined for confluence and cell density per sampling day.

Figure 6. Influence of serum supplement concentration and type on growth of novel lake trout cell cultures. A) fry cells; B) gonad cells. Solid lines indicate cell density (cells per cm^2); segmented lines indicate subjective percent confluence. 2 flasks examined for confluence and cell density per sampling day.

Figure 7. Influence of growth medium base on growth of novel lake trout cell cultures. A) fry cells; B) gonad cells. Solid lines indicate cell density (cells per cm^2); segmented lines indicate subjective percent confluence. 2 flasks examined for confluence and cell density per sampling day.

Figure 8. Lake trout (*Salvelinus namaycush*) cell cultures infected with VHSV or IPNV. A) fry cell negative control (original magnification 200x); B) fry cells infected with VHSV, cell lysis (original magnification 200x); C) fry cell negative control (original magnification 100x); D) fry cells infected with IPNV, cell lysis (original magnification 100x).

Figure 9. Lake trout cells inoculated with EEDV. A) fry cells (47^{th} subculture), cell rounding (original magnification 100x); B) fry cells (47^{th} subculture), cell rounding, some lysis (original magnification 200x); C) gonad cells (32^{nd} subculture), vacuolation, some rounding (original magnification 200x); D) gonad cells (32^{nd} subculture), vacuolation, early lysis (original magnification 200x).

Figure 10. Relative viral loads following inoculation of lake trout fry cells with EEDV positive tissue sample homogenate; represented as number of viral copies per qPCR reaction. P1 = 1^{st}

pass infection, P2 = 2nd pass infection, Cells = cell pellet tested for presence of EEDV, Sup = flask supernatant tested for presence of EEDV. Number in parentheses indicates temperature of incubation. Numbers at bottom indicate separate infection trials, but have no sequential significance.

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ARTICLE

A Quantitative Polymerase Chain Reaction Assay for the Detection and Quantification of Epizootic Epitheliotropic Disease Virus (EEDV; Salmonid Herpesvirus 3)

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Abstract

Epizootic epitheliotropic disease virus (EEDV; salmonid herpesvirus [SalHV3]; family Alloherpesviridae) causes a systemic disease of juvenile and yearling Lake Trout *Salvelinus namaycush*. No cell lines are currently available for the culture and propagation of EEDV, so primary diagnosis is limited to PCR and electron microscopy. To better understand the pervasiveness of EEDV (carrier or latent state of infection) in domesticated and wild Lake Trout populations, we developed a sensitive TaqMan quantitative PCR (qPCR) assay to detect the presence of the EEDV terminase gene in Lake Trout tissues. This assay was able to detect a linear standard curve over nine logs of plasmid dilution and was sensitive enough to detect single-digit copies of EEDV. The efficiency of the PCR assay was 99.4 ± 0.06% (mean ± SD), with a 95% confidence limit of 0.0296 (R^2 = 0.994). Methods were successfully applied to collect preliminary data from a number of species and water bodies in the states of Pennsylvania, New York, and Vermont, indicating that EEDV is more common in wild fish than previously known. In addition, through the development of this qPCR assay, we detected EEDV in a new salmonid species, the Cisco *Coregonus artedi*. The qPCR assay was unexpectedly able to detect two additional herpesviruses, the Atlantic Salmon papillomatosis virus (ASPV; SalHV4) and the Namaycush herpesvirus (NamHV; SalHV5), which both share high sequence identity with the EEDV terminase gene. With these unexpected findings, we subsequently designed three primer sets to confirm initial TaqMan qPCR assay positives and to differentiate among EEDV, ASPV, and NamHV by detecting the glycoprotein genes via SYBR Green qPCR.

Epizootic epitheliotropic disease virus (EEDV; salmonid herpesvirus [SalHV3]; family Alloherpesviridae) causes a systemic disease that is often associated with epidermal lesions in juvenile and yearling Lake Trout *Salvelinus namaycush*. Since the mid-1980s, epizootic epitheliotropic disease has periodically been a serious problem for federal and state Lake Trout hatcheries in the Great Lakes basin (Bradley et al. 1988, 1989; McAllister and Herman 1989). At federal and state hatcheries in Michigan and Wisconsin, a total of over 15 million Lake Trout died or were destroyed in the 1980s due to EEDV (Bradley et al. 1989). During fall 2012, our laboratory confirmed the diagnosis of EEDV among juvenile Lake Trout (Lake Superior inshore lean and Seneca Lake strains) from a Michigan state fish hatchery experiencing chronic low-level mortality among production fish being raised in surface water. These fish experienced peak mortality at around 9°C, whereas mortality decreased as temperature declined to 5°C (Gary Whelan, Michigan Department of Natural Resources, personal communication). Although natural infections have only been observed in Lake Trout, Lake Trout × Brook Trout *Salvelinus fontinalis* crosses have been experimentally infected. Species known to be refractory to EEDV are Chinook Salmon *Oncorhynchus tshawytscha*, Atlantic Salmon *Salmo salar*, Brook Trout, Rainbow Trout *O. mykiss*, and Brown Trout *Salmo trutta* (McAllister 1991).

Clinical signs observed with EEDV-infected fish include inappetence, spiraling, and lethargy, with periods of hyperexcitability. Lesions often associated with EEDV-infected fish

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include proliferative epithelial lesions as well as hemorrhages of the eyes, mouth, and fins. Soon after initial presentation of clinical signs, affected fish tend to show rapid increases in mortality, which can approach 100% among the youngest fish (Bradley et al. 1989; McAllister and Herman 1989). Secondary fungal infections are commonly observed in survivors, which can become long-term carriers of the virus (McAllister 1991).

Epizootic epitheliotropic disease virus continues to cause mortality events in domesticated Lake Trout at rearing facilities, thus necessitating the development of a sensitive and specific diagnostic assay. No cell lines are available for the isolation of EEDV, so diagnosis is limited to clinical signs, lesions, and PCR. Kurobe et al. (2009) described a conventional PCR assay that was capable of detecting the presence of EEDV DNA in Lake Trout skin during active viral outbreaks. Those authors were also able to detect EEDV in healthy appearing juveniles and in the ovarian fluid of spawning adults (Kurobe et al. 2009). However, we found that conventional PCR was inadequate to detect EEDV-positive fish in the carrier or latent state of infection, and thus we needed a higher level of sensitivity that only quantitative PCR (qPCR) offers. To obtain greater sensitivity and to better understand the pervasiveness of EEDV in hatchery and wild Lake Trout populations, we developed a TaqMan qPCR assay that was able to consistently detect EEDV down to double-digit copies. During assay development, we unexpectedly found that the TaqMan qPCR assay described here was able to detect two additional herpesviruses, the Atlantic Salmon papillomatosis virus (ASPV; SalHV4) and the Namaycush herpesvirus (NamHV; SalHV5), which both share high sequence identity with the EEDV terminase gene. Prompted by these unexpected findings, we subsequently designed three primer sets to independently confirm the initial TaqMan qPCR assay detection by targeting an alternative, more variable gene. These confirmatory assays differentially detected the glycoprotein genes of EEDV, ASPV, and NamHV via SYBR Green qPCR. We feel that the initial testing with the TaqMan qPCR assay provides a sensitive tool to identify carrier or latent infections of EEDV, ASPV, and/or NamHV. Subsequent testing with the SYBR Green assays allows for specific differentiation and confirmation of viral species.

We modeled our qPCR validation after the Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) guidelines (Bustin et al. 2009) by conducting a variety of quantitative assays to best demonstrate their limits and capabilities.

METHODS

Tissue Samples and Virus Isolation

Lake Trout tested in the present study were collected via gillnetting and were submitted by state and federal agencies for fish health testing as part of the National Wild Fish Health Survey (U.S. Fish and Wildlife Service [USFWS]; www.fws.gov/wildfishsurvey). Kidney and skin (ventral surface) tissue samples were aseptically removed from individual fish at the sample site, or the fish were transported on ice to the Lamar Fish Health Center (USFWS, Lamar, Pennsylvania) for tissue sampling. All fish were necropsied within 24 h of capture. Kidney tissue homogenates were immediately diluted 1:10 in Hank's balanced salt solution containing gentamicin at 200 µg/mL (HBSS-G; Mediatech, Manassas, Virginia) and were centrifuged for 20 min at 3,000 × gravity. A sample of the supernatant was diluted to a final concentration of 1:100 in Eagle's minimal essential medium containing gentamicin at 1,000 µg/mL and was inoculated on confluent monolayers of two cell lines: Chinook Salmon embryo (CHSE-214) and epithelioma papulosum cyprini (EPC). These cultures were incubated at 20°C for a total of 28 d (USFWS and AFS-FHS 2007). Kidney and/or skin samples were frozen at –20°C until DNA extractions were conducted.

Extraction of DNA

All nucleic acid extractions were conducted by using a commercial kit according to the manufacturer's protocol for tissue DNA (Mag Bind Tissue DNA Kit M6329-02; OMEGA Bio-tek, Inc.). A filtering step was added during nucleic acid extraction to reduce PCR inhibition from melanin (E-Z 96 Lysate Clearance Plate FL96-01; OMEGA Bio-tek). The DNA extraction process was performed with the aid of an automated extraction machine and software (KingFisher Instruments, BindIt Software version 3.1; Thermo Scientific). All extractions were performed with 10 mg of tissue in a 96-well format, eluted with elution buffer, and measured by spectrophotometry (NanoDrop 2000; Thermo Scientific, Waltham, Massachusetts).

Quantitative Polymerase Chain Reaction

Primer and probe selection.—Polymerase chain reaction primers and hydrolysis probe (TaqMan; Applied Biosystems, Inc. [ABI]) were designed by using the terminase gene sequence for EEDV (GenBank accession number EU349284; Table 1). Primer and probe design was conducted with ABI Primer Express version 3.0 (Figure 1). To streamline the confirmation process, we designed primers that were specific to the glycoprotein gene to confirm and differentiate between EEDV (GenBank JX886027), ASPV (JX886026), and NamHV (KP686091) via separate SYBR Green qPCR assays (Table 1; Figure 2).

Quantitative PCR.—The qPCR reactions for EEDV were prepared in 30-µL volumes consisting of 15 µL of 2× TaqMan Universal PCR Master Mix, 2.7 µL of 10-µM forward and reverse primers, 0.75 µL of 10-µM FAM-labeled probe, 3.85 µL of distilled H_2O, and 5.0 µL of DNA template. The PCR was carried out on a qPCR machine (ABI 7300 Real-Time PCR System; Life Technologies, Grand Island, New York) by using the manufacturer's software (ABI 7300 System

TABLE 1. Primers and probe used for TaqMan and SYBR Green quantitative PCR (qPCR) assays listed by gene and purpose (MGB = minor groove binder; % GC = guanine–cytosine content). Conventional PCR primers used for nested confirmation, plasmids, and sequencing are listed for each gene. Standard vector primers, M13 forward and reverse, were used for cloned terminase sequencing reactions (SalHV = salmonid herpesvirus; SalHV2 = *Oncorhynchus masou* virus; SalHV3 = epizootic epitheliotropic disease virus; SalHV4 = Atlantic Salmon papillomatosis virus; SalHV5 = Namaycush herpesvirus).

Primer name	Gene	PCR assay	Use	Nucleotide sequence (5′–3′)	Length (bp)	% GC	Melting temperature (°C)
SalHV_F1	Terminase	qPCR–MGB probe	Initial detection	CCT TTG TGA ACC TCA CCT CCA T	22	50	59
SalHV_R1	Terminase	qPCR–MGB probe	Initial detection	CCC GGC GAC CAG CAT	15	73	59
SalHV_Probe1	Terminase	qPCR–MGB probe	Initial detection	6FAM ACT AGT CTG ATC CCC C MGBNFQ	16	56	69
194F	Terminase	Conventional	Nested confirm	TAG TCT GAT CCC CCT CAT GC	20	55	56
214F	Terminase	Conventional	Nested confirm	TAA CAA CGT GGG CGA CAT TA	20	45	56
249R	Terminase	Conventional	Nested confirm	GTC GAG TCC GAC ACC AGA TT	20	55	56
224_R[a]	Terminase	Conventional	Nested confirm	GTA GAA AGC CGA AAC TTC G	19	47	52
SalHV3_23F	Glycoprotein	SYBR Green	Differentiation	TGG GAG TCC GTC GTC GAA	18	61	58
SalHV3_23R	Glycoprotein	SYBR Green	Differentiation	TCC ACA CAG GAG CTC ACG AA	20	55	58
SalHV4_1F	Glycoprotein	SYBR Green	Differentiation	CGG GCG CGT TTG CA	14	71	58
SalHV4_1R	Glycoprotein	SYBR Green	Differentiation	TGA AGA TCA ACC GTC CCA TTG	21	48	55
SalHV5_16F	Glycoprotein	SYBR Green	Differentiation	GGA GGA CCA TCA TCG GAC TTT	21	52	57
SalHV5_16R	Glycoprotein	SYBR Green	Differentiation	CCC CAT CTA TCG CCT GCT T	19	58	57
3-Glyco_F1	Glycoprotein	Conventional	Plasmid	TTT CTC TGA GTG GCA CGA CC	20	55	58
4-Glyco_F3	Glycoprotein	Conventional	Plasmid	CTC TCA TGT TGG TGA GCG GT	20	55	57
4-Glyco_R5	Glycoprotein	Conventional	Plasmid	TGT GGT GGA CTT TGG TTC CC	20	55	59

[a]Kurobe et al. (2009).

```
            A   F   V   N   L   T   S   I   T   S   L   I   P   L   M   L   V   A   G
SalHV3    GCC TTT GTG AAC CTC ACC TCC ATC ACT AGT CTG ATC CCC CTC ATG CTG GTC GCC GGG
SalHV1    GCC TTT GTC AAC CTC ACA TCC ATC ACT AGC CTC ATT CCG CTG ATG CTC GTC GCT GGC
SalHV2    GCC TTT GTC AAT CTC ACC TCG ATT ACC AGT CTC ATC CCT TTG ATG CTC GTC GCT GGT
SalHV4     CC TTT GTC AAC CTC ACC TCC ATC ACC AGT CTG ATT CCC CTC ATG CTG GTC GCC GGG
SalHV5                                 C ACA AGT CTG ATC CCC CTC ATG CTG GTC GCC GGG
```

FIGURE 1. Primers and TaqMan minor groove binder probe locations are underlined within the terminase gene sequence of epizootic epitheliotropic disease virus (salmonid herpesvirus [SalHV] 3; GenBank EU349284). Homologous gene sequences from SalHV1 (GenBank EU349281), *Oncorhynchus masou* virus (SalHV2; EU349282), Atlantic Salmon papillomatosis virus (SalHV4; JX886026), and Namaycush herpesvirus (SalHV5; KP686092) are provided for comparison. The predicted amino acid sequence is conserved and listed above the nucleotide sequence. Known nucleotide differences from SalHV3 are shaded.

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SalHV3  430-TGGGAGTCCGTCGTCGAAAGTCCACGGAAGACCGAGGTGTTCGTGAGCTCCTGTGTGGAT-489
SalHV4       TGGAGCGCAGTGACCGAAAGCACCAAGAAGACCGAGGCCTTCGTCGACTCCTGTGTGGAG
SalHV5       TGGAGCGCAGTGACCGAAAGTGCCAAGAAGACCGAGGCGTTCGTCGGCTCCTGTGTGGGG
SalHV3  556-GGGGGTCCATCACCGTCGTTCACCAAGATAGCGGAGGCGTTCAGTAAAGTAATGGGGGAGA-616
SalHV4       GGGGGACCCTCATTGGACTTTACCAAGATAGCCGAGGCCTTCAAGCGGGCAATTGGGGGGG
SalHV5       GGAGGACCATCATCGGACTTTACTAAGATAGCCGAGGCCTTCAAGCAGGCGATAGATGGGG
SalHV3 1310-GGACGGGACCGTTTACAGTATGTCCCGGGATTATAGCCACGATGGGCAAACTGGTCTTCA-1369
SalHV4       GCACGGGCGCGTTTGCAGTGTGTCCAAATATCATTGCCACAATGGGACGGTTGATCTTCA
SalHV5       GCACGGGTGCGTTTACAGTGTGTCCAAATATCATTGCCACAATGGGACGCTTGATCTTCA
```

FIGURE 2. Differentiation primer locations are underlined along respective aligned glycoprotein genes of salmonid herpesvirus (SalHV) members (epizootic epitheliotropic disease virus [SalHV3; GenBank JX886027]; Atlantic Salmon papillomatosis virus [SalHV4; JX886028]; and Namaycush herpesvirus [SalHV5; KP686091]). Sections of sequence that did not contain primer sites have been excluded. Nucleotide differences between genes are shaded.

Sequence Detection Software, version 1.3). The target sequence was amplified using absolute quantification with the machine's default thermocycling profile of 50°C for 2 min and initial denaturation at 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. The TaqMan qPCR assay amplicon has a total length of 56 base pairs (bp). To detect the presence of inhibitory factors, an internal positive control (IPC) was used (TaqMan Exogenous IPC, VIC probe).

The SYBR Green qPCR assays were performed in 30-μL reactions containing 4.6 μL of PCR-grade water, 15 μL of SYBR Select Master Mix (2×; Life Technologies), 2.7 μL of forward and reverse primers (10 μM), and 5 μL of sample DNA. To avoid competitive inhibition, multiplexing was not conducted. Real-time amplification reactions consisted of 50° C for 2 min; 95°C for 10 min; and 40 cycles of 95°C for 15 s, 60°C for 60 s, followed by a dissociation stage (95°C for 15 s, 60°C for 60 s, and 95°C for 15 s) on an ABI 7300.

Positive-control plasmid.—To establish positive controls, PCR products were amplified from DNA originating from (1) EEDV-positive Lake Trout skin; (2) ASPV-positive Atlantic Salmon papilloma tissue; and (3) NamHV-positive Lake Trout skin (Lake Ontario, New York, samples). Nested PCRs were used when low levels of target DNA warranted (for the EEDV terminase gene [GenBank EU349284]: SalHV_F1 and 224_R [438 bp] first, SalHV_F1 and 249_R [349 bp] second; for the glycoprotein gene of EEDV [JX886027], ASPV [JX886028], and NamHV [KP686091]: 3-Glyco_F1 and 4-Glyco_R5 [1,146 bp] first, 4-Glyco_F3 and 4-Glyco_R5 [1,087 bp] second; Table 1). Amplifications for both rounds were performed in 50-μL reactions containing 21 μL of PCR-grade water, 5 μL of PCR reaction buffer (10×), 2.5 μL of $MgCl_2$ (50 mM), 1 μL of 10.0-mM deoxynucleotide triphosphates, 5 μL of forward and reverse primers (10 pM/μL), 5 μL of Rediload (10×), 0.5 μL of *Taq* polymerase (5 units/μL), and 5 μL of template in the first round or 1 μL of template and make-up water in the second round. Amplification for both rounds consisted of 95°C for 15 min; 35 cycles of 94°C for 30 s, 58°C for 30 s, and 72°C for 60 s; and a final extension at 72°C for 10 min. The PCR products were cloned as described below and were sequenced on an ABI 3100 Sequencer. Plasmid copy numbers were calculated by volume from the concentration of extracted DNA as measured by spectrophotometry.

Sequence analysis.—Due to the low levels of virus present in the samples that were found positive by the TaqMan qPCR assay, we conducted nested PCR to obtain terminase gene products for confirmatory sequencing. The amplification parameters were the same as listed above for positive-control plasmids. The primers used were 194_F and 249_R (324 bp) in the first round and 214_F and 249_R (249 bp) in the second round. Purified plasmid DNA obtained from the nested PCR products were cloned into pCR 2.1-TOPO (Invitrogen, Carlsbad, California), and the resulting plasmids were used to transform Top 10 Competent Cells (Invitrogen). Competent *Escherichia coli* cells were plated onto Luria-Bertani media

containing kanamycin at 50 µg/mL (Mediatech). Selected colonies were grown overnight in 5.0 mL of LB broth containing kanamycin at 50 µg/mL. Plasmid DNA was extracted with the QIAprep Spin Miniprep Kit (QIAGEN, Valencia, California) and was sequenced using an ABI 3100 Sequencer.

Quantitative Polymerase Chain Reaction and Hydrolysis Probe Assay Analysis

We followed the MIQE guideline of establishing assay efficiency and testing intraassay and interassay variances by determining the SD and CV between copy numbers instead of using quantification cycle (Cq) values. To perform these validation assays, we used EEDV-positive clinical material that was obtained from a natural outbreak in Michigan. The EEDV-positive tissues were kindly provided by Thomas Loch and Mohamed Faisal (Aquatic Animal Health Laboratory, Michigan State University, East Lansing).

Standard curve and dynamic range.—A standard curve was produced by conducting tenfold serial dilutions of purified EEDV control plasmid DNA in molecular-grade water to determine qPCR assay linearity and the limit of detection (LOD). This initial dilution was frozen in batches for future use. This assay was run with tenfold dilutions ranging from 2.41×10^{10} to 2.41 copies/reaction in triplicate. A standard curve was also produced with fish tissue by conducting a tenfold serial dilution of DNA from naturally infected Lake Trout skin to determine linearity and assay efficiency. In addition, a total of 14 independent, overlapping standard curve assays were conducted over the course of 1 year to determine the dynamic range and reaction efficiency of the TaqMan qPCR assay. A more narrow dilution range of six logs (ranging from 2.41×10^5 to 2.41 copies/reaction) was used.

Analytical specificity.—To measure specificity of the TaqMan qPCR forward and reverse primers against Lake Trout DNA, skin samples from eight naturally EEDV-infected Lake Trout were run without the minor groove binder probe via a SYBR Green assay. Five tenfold dilutions were carried out, and the dissociation curves were analyzed for a total of 40 samples. The PCR products from fish that were EEDV positive via the TaqMan qPCR assay were initially sequenced for verification (data not shown). Confirmations were then conducted with the SYBR Green assay upon development. The uniqueness of the TaqMan qPCR assay (terminase) and SYBR Green (glycoprotein) target sequences were verified through standard Basic Local Alignment Search Tool (BLAST) analysis (National Library of Medicine; blast.ncbi.nlm.nih.gov/Blast.cgi). To verify specificity, closely related herpesvirus samples and bacterial, parasitic, and viral fish pathogen DNA samples were tested with the TaqMan qPCR assay and the SYBR Green differentiation assays for cross-reactivity.

Repeatability (intraassay variance).—To measure short-term precision or robustness of the TaqMan qPCR assay, a single assay was conducted using samples from a total of eight naturally EEDV-infected Lake Trout. The DNA extractions were conducted on three skin samples (10 mg) from each fish; each skin sample was run in triplicate for a total of 9 samples/fish. A standard curve was generated with plasmid dilutions ranging from 2.41×10^6 down to 2.41 copies/reaction to estimate sample copy numbers for all variance testing. Intraassay variance was found by determining the SD and CV for a total of 24 tissue samples.

Reproducibility (interassay variance).—To measure long-term precision, the interassay variance assessment was conducted as described above for intraassay variance except that we calculated the SD and CV for mean EEDV copy number from individual fish over five assays conducted on separate days by alternating operators.

Sample variance.—To measure sample collection precision, three skin samples were collected as described above from eight EEDV-positive Lake Trout, thus totaling 24 samples. The sampling variance was determined by calculating the SD and CV for average copy number between tissues from each fish over five assays.

RESULTS

Virus Detection

Among the 23 Lake Trout sample sites from a total of nine water bodies (total number of fish sampled = 548), six sample sites were positive for EEDV based on the developed TaqMan qPCR assay (terminase gene) and sequencing or were positive based on SYBR Green qPCR (glycoprotein gene; Table 2). Initial EEDV detection was in Lake Trout sampled from Lake Ontario, New York, during 2011. In that same year, EEDV was detected in the ovarian fluid of Lake Trout from Lake Champlain, Vermont; EEDV was detected again from Lake Champlain in 2012 via analysis of skin and kidney samples from adult Lake Trout. In 2014, we tested skin samples from 15 Lake Trout fry collected from the same Lake Champlain location where the positive adults were sampled in 2011 and 2012; however, the samples from fry were negative for EEDV. While we understand that these sample numbers are low, this finding may indicate that vertical transmission is limited. In 2013, the skin of Lake Trout from two additional water bodies, Seneca Lake and Otsego Lake, New York, were found to be positive for EEDV. Namaycush herpesvirus was also found in this same group of Otsego Lake fish, although not in the same individuals that were positive for EEDV. During 2014, similar results were obtained with Lake Trout from Otsego Lake: six fish were positive for NamHV, and one of those six was co-infected with EEDV (Table 2). The co-infected individual was initially found positive by the TaqMan qPCR assay (terminase gene) and then tested positive by both the EEDV and NamHV primer sets (glycoprotein gene) in separate SYBR Green qPCR assays.

TABLE 2. Lake Trout samples that tested positive for epizootic epitheliotropic disease virus (salmonid herpesvirus [SalHV] 3) and Namaycush herpesvirus (SalHV5) listed by sample date, water body and state of sample collection, and tissue. Positives were either confirmed by nested PCR and sequencing (terminase gene) or via SYBR Green quantitative PCR (qPCR; glycoprotein; Glenney et al. 2016, this issue). See Table 1 for primer sequences (A/U = attempted but unsuccessful; N/A= not attempted).

Sample date	Lake	Tissue	*n*	Percent qPCR positive	Percent consistently positive via real-time PCR assay[b]	Exact binomial CI[c]	Amplicons cloned and confirmed by sequencing	Isolates positive by SYBR Green glycoprotein PCR	Latitude (°N), longitude (°W)
Sep 13, 2011	Ontario (New York)	Kidney	20	40	0	$0.000 \leq p \leq 0.168$	N/A	N/A	43.32, –77.54
		Skin	60	32	15	$0.071 \leq p \leq 0.266$	(2) SalHV3	(6) SalHV5	
Nov 8, 2011	Champlain (Vermont)	Ovarian fluid	4 (5 fp)[a]	50	50	$0.068 \leq p \leq 0.932$	(2) 5fp-SalHV3	N/A	44.63, –73.24
Nov 14, 2012	Champlain	Kidney	18	33	22	$0.064 \leq p \leq 0.476$	A/U	N/A	44.63, –73.24
		Skin	18	83	61	$0.357 \leq p \leq 0.827$	(1) SalHV3	(6) SalHV3	
		Ovarian fluid	4	25	0	$0.000 \leq p \leq 0.602$	N/A	N/A	
Jul 17, 2013	Seneca (New York)	Skin	42	9.5	7	$0.015 \leq p \leq 0.195$	N/A	(3) SalHV3	42.85, –76.98
Nov 14, 2013	Otsego (New York)	Skin	10	30	30	$0.067 \leq p \leq 0.652$	N/A	(2) SalHV3 (1) SalHV5	42.76, –74.89
Sep 19, 2014	Otsego	Skin	16	50	4	$0.152 \leq p \leq 0.646$	(1) SalHV3	(6) SalHV5 (1) SalHV3	42.76, –74.89

[a]4 (5 fp) = a total of 20 fish, pooled into four pools of 5 fish each.

[b]Samples that were positive by two repetitions in a single assay and that were positive in two or more separate real-time assays upon re-extraction of the original sample tissue.

[c]95% binomial probability confidence interval (Clopper–Pearson exact model).

Seven additional species from various water bodies in New York and Pennsylvania and from one research facility in New York were tested for the presence of EEDV. Within these samples, all but one alternative species was EEDV negative; EEDV was found in juvenile Ciscoes *Coregonus artedi*, which were the offspring of Lake Ontario, New York, wild brood (Table 3).

Virus Isolation

None of the corresponding EEDV-positive qPCR samples was observed to exhibit cytopathic effect on either the CHSE-214 cell line or the EPC cell line.

TaqMan Assay Analysis

Standard curve, dynamic range, and efficiency.—In a single experiment, the TaqMan qPCR assay standard curve was linear over nine logs of plasmid dilution and consistently detected EEDV plasmid at a copy number of 24.1 copies/reaction, whereas it only detected two of the three samples at 2.41 copies/reaction (Figure 3). Standard curves of diluted plasmid containing EEDV terminase gene template ranging from 2.41×10^5 down to 2.41 copies per 5.0-µL reaction sample were analyzed over the course of 14 experiments (Figure 4). The standard curve for diluted DNA from EEDV-positive Lake Trout skin ranged from 2.23×10^8 down to 79.22 copies/reaction. The standard curve for the naturally infected tissue was linear over the entire eight logs of dilution, with an efficiency of 101.8% (data not shown).

Analytical specificity.—Primers used for the TaqMan qPCR assay presented one dissociation curve for each sample during SYBR Green melt curve analysis of serially diluted EEDV-positive Lake Trout tissue, with a melting temperature of 78.0°C. The nucleic acids of nine viruses, two bacteria, and two parasites were tested for potential cross-reactions with the TaqMan qPCR assay by using the minor groove binder probe. All but two were negative with the TaqMan qPCR assay (Table 4). The ASPV and NamHV both cross-reacted with the hydrolysis probe assay, prompting us to design additional SYBR Green confirmatory qPCR assays to detect the more variable glycoprotein gene and thus differentiate among the three Alloherpesviridae species.

Repeatability (intraassay variance).—In one experiment, the same skin samples from EEDV-infected fish were repeatedly analyzed in triplicate to measure the robustness or precision of the TaqMan qPCR assay. The mean copy number detected from a fish undergoing a natural EEDV infection was 30,124.0 copies/reaction (Table 5).

Reproducibility (interassay variance).—The mean SD for Lake Trout skin samples was 2,586.2 copies/reaction, while the CV ranged from 4.9% to 13.8% and averaged 8.5% (Table 6).

Sample variance.—The mean SD for skin samples collected from the same individual Lake Trout was 17,908.2 copies/reaction, with a mean CV of 54.1% (Table 7).

SYBR Green Quantitative Polymerase Chain Reaction Assay

All three SYBR Green qPCR assay standard curves were linear over seven logs of plasmid dilution (Figure 5). Each assay detected its respective plasmid down to single-digit copies per reaction. Cross-reaction testing showed that each primer set failed to detect nontarget plasmid and genomic DNA (gDNA) sequences from similar salmonid herpesvirus members. Melt curve analysis of SYBR Green primer sets indicated melting temperatures of 81.5°C for EEDV, 78.6°C for ASPV, and 78.3°C for NamHV.

DISCUSSION

We described the validation and implementation of a TaqMan qPCR assay that was designed to detect the terminase gene of EEDV. With data from a number of species and water bodies in Pennsylvania, New York, and Vermont, the described TaqMan and SYBR Green qPCR assays in combination were found to be sensitive, precise, and specific in detecting EEDV from wild populations of nonclinically affected Lake Trout and, in one case, hatchery Lake Trout exhibiting clinical signs and lesions associated with EEDV. From the data collected, it appears that EEDV is present in wild Lake Trout but not to the same extent as NamHV (Glenney et al. 2016). To our knowledge, this work is first of its kind to present data describing the presence of EEDV in wild Lake Trout and other salmonids.

In wild Lake Trout showing no clinical signs of disease, EEDV detection tended to be on the lower end of viral detection with the qPCR assay, ranging between 4.0 and 827.8 copies/mg of tissue. Seasonality and natural stressors may play a role in detectable levels of EEDV from wild fish. The majority of sampling in the present study was conducted during August and September, so we were unable to recognize any seasonal trends. Gilad et al. (2004) observed similar results of nearly undetectable viral copy numbers with koi herpesvirus (CyHV-3) in koi (a variant of the Common Carp *Cyprinus carpio*) at 62 d postinfection. Gilad et al. (2004) stated that the koi appeared healthy and that the detection of viral DNA alone was inadequate to determine whether these fish were undergoing latent infections or low-level, persistent infections.

In the present study, samples with low viral copy numbers commonly presented inconsistencies due to the Poisson distribution. Repeated extraction of initial tissue samples along with increasing the number of repetitions increased our confidence in designating a sample as positive or negative. With these low-copy-number samples, nested conventional PCR was needed to obtain amplicons for initial confirmation and sequencing. During primer and probe optimization, we also observed that doubling the probe concentration from 50 to 100 nM and 200 nM increased the copy numbers detected by 50% in the detection of template in double-digit and single-digit copy number reactions. Increasing primer concentrations from

TABLE 3. Various fish species that were tested for the presence of epizootic epitheliotropic disease virus (salmonid herpesvirus [SalHV] 3) via TaqMan quantitative PCR (qPCR). Positives were confirmed by semi-nested PCR and sequencing of the terminase gene (USGS = U.S. Geological Survey; STT = steelhead [anadromous Rainbow Trout]; BNT = Brown Trout; LWF = Lake Whitefish *Coregonus clupeaformis*; CIS = Cisco; RWS = Rainbow Smelt *Osmerus mordax*; ALW = Alewife *Alosa pseudoharengus*; SPL = Splake [Brook Trout × Lake Trout]).

Sample date	Water body	Species	Tissue	*n*	Percent qPCR positive	Percent consistently qPCR positive[b]	Exact binomial CI[c]	PCR amplicons cloned and confirmed by sequencing	Latitude (°N), longitude (°W)
Apr 6, 2011	Salmon River, New York	STT	Kidney	60	0	N/A		N/A	43.54, –75.86
			Gill	60	0	N/A		N/A	43.54, –75.86
			Ovarian fluid	30	0	N/A		N/A	43.54, –75.86
Apr 18, 2011	Cattaraugus Creek, New York	STT	Kidney	30	0	N/A		N/A	42.57, –79.10
Jul 25, 2011	Roeliff Jansen Kill, New York	BNT	Kidney	3 (5 fp)[a]	0	N/A		N/A	42.18, –73.86
Jul 28, 2011	Lake Ontario, New York	LWF	Kidney	25	0	N/A		N/A	43.53, –76.43
Jan 29, 2013	USGS Tunison Laboratory of Aquatic Science, New York	CIS	Skin	60	5	3.3	$0.004 \leq p \leq 0.115$	(2) SalHV3[d]	42.56, –76.25
			Kidney	60	8.3	5	$0.010 \leq p \leq 0.139$	(1) SalHV3[d]	
Feb 13, 2013	Lake Erie, Pennsylvania	STT	Kidney	60	0	N/A		N/A	42.08, –80.24
Apr 25, 2013	Lake Ontario, New York	RWS	Kidney	4 (5 fp)	0	N/A		N/A	43.32, –77.54
		ALW	Kidney	6 (5 fp)	0	N/A		N/A	43.32, –77.54
Jun 4, 2013	Lake Ontario	RWS	Skin	20	0	N/A		N/A	43.32, –77.54
Jul 16, 2013	Lake Ontario	ALW	Skin	15	0	N/A		N/A	43.32, –77.54
Jul 16, 2013	Lake Ozonia, New York	SPL	Skin	4	0	N/A		N/A	44.60, –74.62
Aug 14, 2014	USGS Tunison Laboratory of Aquatic Science	BTR	Skin	30	0	N/A		N/A	42.56, –76.25

[a]Number of pools (outside the parentheses) and the number of fish per pool (inside the parentheses). For example, 4 (5 fp) = a total of 20 fish, pooled into four pools of 5 fish each.

[b]Samples that were positive by two repetitions in a single assay and that were positive in two or more separate real-time assays upon re-extraction.

[c]95% binomial probability confidence interval (Clopper–Pearson exact model).

[d]Nested PCR primers for the first round (194_F and 249_R) and second round (214_F and 249_R).

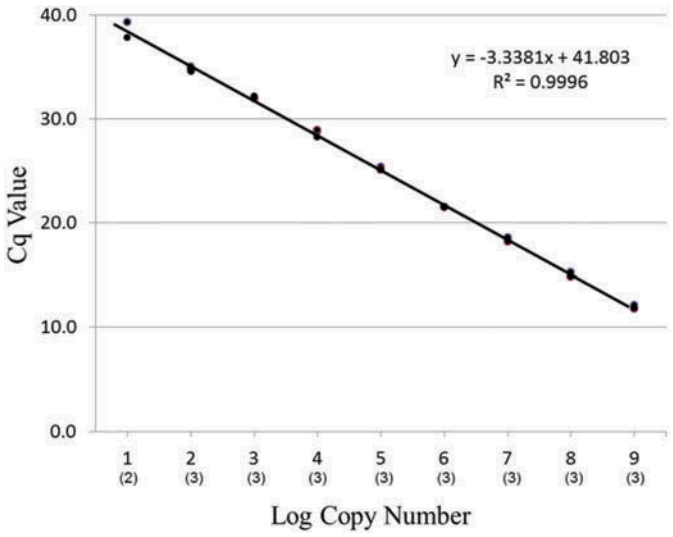

FIGURE 3. Standard curve showing linearity of the quantitative PCR (qPCR) assay for known concentrations of epizootic epitheliotropic disease virus terminase gene plasmid (Cq = quantification cycle). Plasmid was serially diluted tenfold from 2.41×10^8 to 2.41 copies/reaction (n = 1). Values in parentheses indicate the number of positive samples out of three replicates. The qPCR efficiency was 99.3%.

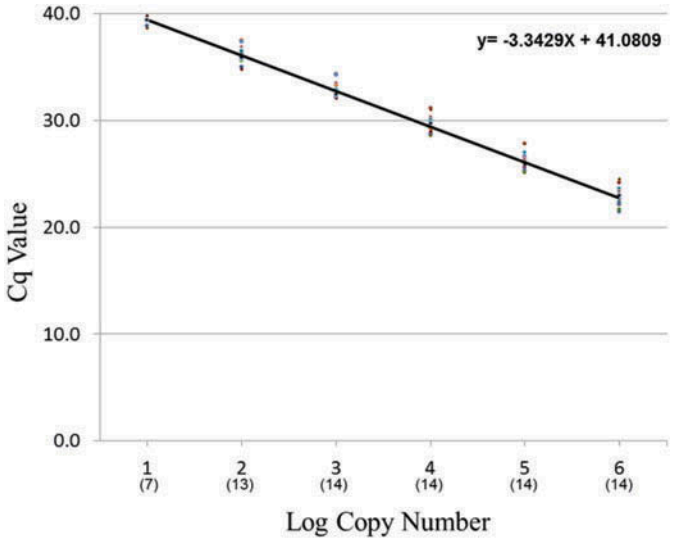

FIGURE 4. Quantitative PCR (qPCR) standard curve for known concentrations of diluted plasmid containing epizootic epitheliotropic disease virus terminase gene template (Cq = quantification cycle). Values in parentheses indicate the number of data points that were recorded at each dilution for 14 overlapping, independent experiments. The mean (±SD) qPCR efficiency was 99.4 ± 0.06%, with a 95% confidence limit of 0.0296 (R^2 = 0.994).

300 to 900 nM also increased detection by 30% at these lower levels of detection.

Although we did not detect EEDV in any wild species other than Lake Trout, we did unexpectedly detect EEDV in an additional salmonid species: domesticated Ciscoes that were

TABLE 4. Cross-reactivity with designed TaqMan quantitative PCR assay, listed by fish pathogen, source, and reaction (SalHV = salmonid herpesvirus; SalHV2 = *Oncorhynchus masou* virus; SalHV4 = Atlantic Salmon papillomatosis virus; SalHV5 = Namaycush herpesvirus; CCV = Channel Catfish virus; ISAV = infectious salmon anemia virus; IPNV = infectious pancreatic necrosis virus; LMBV = Largemouth Bass virus; ATCC = American Type Culture Collection; USFWS = U.S. Fish and Wildlife Service; FHC = Fish Health Center).

Pathogen	Source	Real-time PCR
SalHV1 (plasmid)	Scott Weber, University of California, Davis	–
SalHV1	ATCC, cell culture	–
SalHV2 (plasmid)	Scott Weber, University of California, Davis	–
SalHV4 (DNA, plasmid)	Andor Doszpoly, Hungarian Academy of Sciences, Budapest, Hungary	+
SalHV5 (DNA, plasmid)	USFWS Lamar FHC, Lamar, Pennsylvania	+
CCV isolate 1	USFWS La Crosse FHC, La Crosse, Wisconsin	–
CCV isolate 2	USFWS La Crosse FHC	–
ISAV complementary DNA	USFWS Lamar FHC	–
IPNV (seg-A plasmid)	USFWS Lamar FHC	–
LMBV	USFWS Lamar FHC	–
Renibacterium salmoninarum	USFWS Lamar FHC	–
Flavobacterium psychrophilum	USFWS Lamar FHC	–
Myxobolus cerebralis	USFWS Lamar FHC	–
Nucleospora salmonis	USFWS Lamar FHC	–

the progeny of wild brood from Chaumont Bay, Lake Ontario, New York. The EEDV copy numbers were also low in the Cisco samples, ranging from 10 to 76 copies/mg of kidney tissue and from 10 to 150 copies/mg of skin tissue. This finding may not be all that surprising, as coregonids have historically been an important endemic prey species for Lake Trout in the Great Lakes (Van Oosten and Deason 1938; Christie 1974). Whether Ciscoes are asymptomatic carriers of EEDV or can exhibit clinical signs is yet to be determined.

Cell culture was conducted on all samples tested via qPCR, and cytopathic effect was not observed on either of the cell lines used for PCR-positive samples. This lack of replication in cell lines is common for salmonid herpesviruses, such as EEDV and ASPV (Carlisle 1977; Bradley et al. 1988; Wolf 1988; McAllister and Herman 1989; Shchelkunov et al. 1992).

TABLE 5. Repeatability (intraassay variance) or short-term precision of TaqMan quantitative PCR in detecting the presence of epizootic epitheliotropic disease virus (salmonid herpesvirus 3). The mean SD for all Lake Trout skin samples was 2,551.2 copies/reaction; the CV ranged from 3.7% to 16.9%, with a mean of 8.6%.

Fish number	Sample number	Mean copy number (copies/reaction)	SD (copies/reaction)	CV (%)
1	1	32,660.5	2,535.8	7.7
	2	17,189.7	2,247.9	13.2
	3	20,299.4	2,053.2	10.2
2	1	5,947.2	452.0	7.6
	2	4,342.2	249.7	5.4
	3	22,315.6	1,312.3	6.1
3	1	110,879.3	5,342.1	4.8
	2	11,726.2	955.5	8.3
	3	10,309.9	909.1	8.8
4	1	10,670.8	1,005.6	9.4
	2	9,840.5	750.7	8.0
	3	13,090.4	467.2	3.7
5	1	28,042.3	2,064.1	7.7
	2	37,087.0	3,446.3	9.3
	3	57,439.5	3,707.1	7.0
6	1	55,826.1	5,181.1	9.2
	2	24,919.5	2,269.1	9.2
	3	46,211.3	3,453.7	7.8
7	1	77,252.2	8,761.5	11.3
	2	18,761.3	1,642.9	9.0
	3	30,675.1	3,930.4	9.6
8	1	21,588.3	1,894.4	8.7
	2	29,005.3	2,051.9	7.0
	3	26,893.3	4,546.3	16.9

Although exceptions exist, the replication of many herpesviruses requires cell lines from specific host species and specific cell types, even during active infections to culture (Waltzek et al. 2009; Hanson et al. 2011).

We were able to detect EEDV in the kidney, skin, and ovarian fluid of wild Lake Trout. Although no formal studies were conducted to determine which tissues would be most sensitive for EEDV detection, anecdotal evidence from Lake Ontario, New York, and Lake Champlain, Vermont, suggested that skin would be a more sensitive tissue than kidney for detecting EEDV in asymptomatic wild Lake Trout (Table 2). It was determined that kidney sample elutions with abundant melanin granules or residual melanin pigmentation had periodic PCR inhibition, as evidenced in the delayed amplification of the IPC. An added filtering step during nucleic acid extraction addressed the melanin inhibitory effects.

As measured with plasmid dilutions, the LOD in wild Lake Trout samples was consistently above 10 copies/reaction (5.0-μL sample) while becoming more sporadic below 10 copies/reaction.

The TaqMan qPCR assay's reaction efficiency of 99.4 ± 0.06% (mean ± SD) for detecting diluted EEDV plasmid was well within accepted limits of 90–110% for assay validation (*n*

TABLE 7. Variance measured by TaqMan quantitative PCR assay between epizootic epitheliotropic disease virus (salmonid herpesvirus 3) positive skin samples collected from the same individual Lake Trout (three tissue samples per fish) over a total of five experiments performed on different days by different operators.

Fish number	Mean copy number (copies/reaction)	SD (copies/reaction)	CV (%)
1	23,383.2	8,198.2	35.1
2	10,868.4	9,949.9	91.5
3	44,305.1	57,659.8	130.1
4	11,000.6	1,407.3	12.8
5	40,856.3	15,389.0	37.7
6	42,318.9	15,836.9	37.4
7	42,229.5	30,923.1	73.2
8	25,829.0	3,901.2	15.1

TABLE 6. Reproducibility (interassay variance, copies/reaction) or long-term precision of the TaqMan quantitative PCR assay in detecting the presence of epizootic epitheliotropic disease virus (salmonid herpesvirus 3). Variance was measured (three tissue samples, each in triplicate per Lake Trout) over five experiments performed on different days (1–5) by different operators.

	Day							
Fish number	1	2	3	4	5	Mean copy number (copies/reaction)	SD (copies/reaction)	CV (%)
1	22,579.4	25,731.2	20,861.5	23,994.1	23,749.8	23,383.2	1,804.6	7.7
2	9,595.3	11,439.8	10,519.4	11,492.3	11,295.0	10,868.4	812.4	7.5
3	40,740.6	45,635.0	44,380.2	44,368.7	46,401.1	44,305.1	2,172.4	4.9
4	9,821.7	11,451.0	10,818.7	11,637.8	11,273.8	11,000.6	725.7	6.6
5	39,306.0	47,502.4	42,451.7	35,151.7	39,869.5	40,856.3	4,545.2	11.1
6	39,476.5	45,693.9	38,630.2	43,209.0	44,585.1	42,318.9	3,122.7	7.4
7	40,976.9	46,421.7	36,989.7	40,800.6	45,958.9	42,229.5	3,954.4	9.4
8	21,510.3	25,687.3	23,934.8	27,002.3	31,010.2	25,829.0	3,552.5	13.8

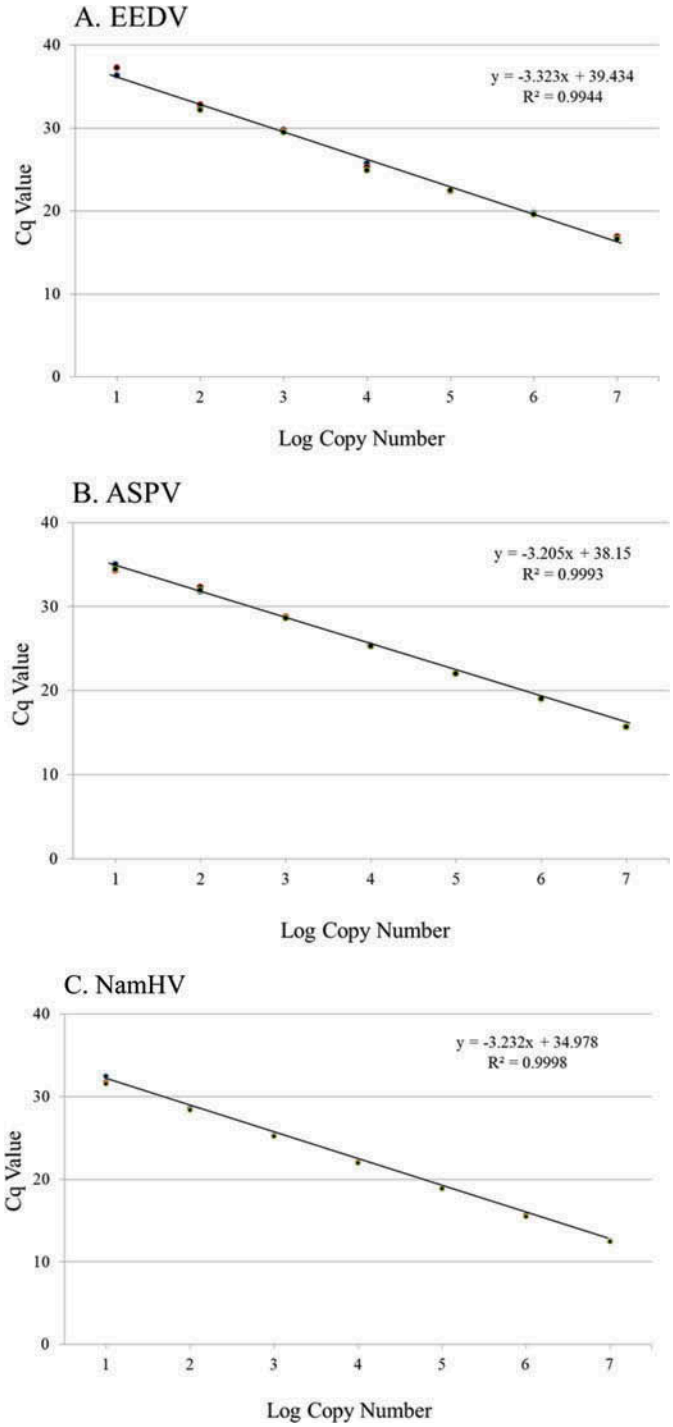

FIGURE 5. Standard curves showing linearity of SYBR Green quantitative PCR assays for known concentrations of glycoprotein gene control plasmid (Cq = quantification cycle): **(A)** epizootic epitheliotropic disease virus (EEDV; reaction efficiency = 99.9%), **(B)** Atlantic Salmon papillomatosis virus (ASPV; reaction efficiency = 105.1%), and **(C)** Namaycush herpesvirus (NamHV; reaction efficiency = 103.8%). Each dilution was run in triplicate, and all assays were linear over seven logs of dilution. Plasmid was serially diluted tenfold as follows: from 4.0×10^6 to 4.0 copies/reaction for EEDV; from 3.82×10^6 to 3.82 copies/reaction for ASPV; and from 3.19×10^6 to 3.19 copies/reaction for NamHV (n = 1).

= 14). The assay's efficiency varied only slightly to 101.8% when we tested diluted, naturally EEDV-infected tissues (n = 1). Both the short- and long-term precision of the TaqMan qPCR assay presented mean CVs below 10% (Tables 5, 6). These results are comparable with Getchell and Bowser's (2011) review of 33 published studies detailing fish pathogen qPCR assays; those authors reported that a majority of the assays had CVs under 15% for intraassay variation (short-term precision/repeatability). Interestingly, the highest observed CVs or mean SDs in our study were calculated for skin samples taken from the same individual Lake Trout. The SDs for skin samples collected from the same fish ranged greatly—from 1,407.3 to 57,659.8 copies/reaction, with a mean CV of 54% (Table 7). The higher variation observed in certain fish could have been due to a variety of factors ranging from error in tissue collection to inconsistencies in DNA extraction to localization of virus "hot spots" in skin samples.

The detection of EEDV in hatchery Lake Trout exhibiting clinical signs of disease ranged from 20.8 to 1.9×10^6 copies/mg of tissue. In hatchery fish that exhibited signs of disease, virus was detected in the skin, gills, and kidney tissue, with the highest copy numbers present in the skin and gills. This is consistent with EEDV histopathological descriptions primarily involving alterations of the epithelium (Bradley et al. 1989; McAllister 1991). In addition to the hatchery fish described above, our laboratory blindly tested two groups of hatchery Lake Trout fingerlings for the presence of EEDV: 60 fish (kidney samples) from Bath State Fish Hatchery, New York; and 60 fish from Chateaugay State Fish Hatchery, New York. Both samples were negative for EEDV.

During initial primer and probe design *in silico*, steps were taken to avoid having the assay detect two well-known pathogenic salmonid herpesviruses: SalHV1 and *Oncorhynchus masou* (Cherry Salmon) virus (SalHV2; Wolf et al. 1978; Kimura et al. 1981; Waltzek et al. 2009). We then determined ASPV gDNA (GenBank JX886026) to cross-react with our TaqMan qPCR assay. Although ASPV as molecularly classified by Doszpoly et al. (2013) has not been described in the United States or isolated from any other species besides Atlantic Salmon, we felt the need to develop a confirmatory assay that would enable us to rule out a potential ASPV cross-reaction. According to Wolf (1988), a papilloma condition has been infrequently observed in young salmon in North America, so there is the potential for an ASPV or ASPV-like salmonid herpesvirus to exist in the United States. In addition, sequence analysis revealed that certain qPCR-positive isolates in the current study consistently varied by more than 5% across 307 nucleotides from the EEDV terminase gene (GenBank EU349284). These results prompted us to sequence the terminase, polymerase, and glycoprotein genes from these isolates, leading us to discover that the qPCR assay was detecting a new alloherpesvirus member in wild Lake Trout, which we named Namaycush herpesvirus (Glenney et al. 2016). Thus, with the inability to culture EEDV on available

cell lines and with the two unexpected cross-reaction findings, we decided to design three primer sets to the more variable glycoprotein gene, thus allowing us to confirm initial detections with an alternative gene and then be able to differentiate among EEDV (GenBank JX886027), ASPV (JX886026), and NamHV (KP686091) via the described SYBR Green qPCR assay. This methodology has worked well with wild Lake Trout sampling. We have been able to detect and differentiate between EEDV and NamHV, which we have found in the same water bodies and, in one case, within the same fish. Currently, it is unknown whether NamHV is pathogenic to Lake Trout, but we feel that it is beneficial to gather more information about which viral agents are present, especially if some form of synergistic co-infection is occurring. Without the ability to culture EEDV, additional targeting of alternative genes through PCR and/or sequencing was necessary in this case for assay validation and to ensure dependable detection and reporting of this relatively new, emerging pathogen. We hope that the assays described here will be useful tools for researchers and diagnosticians working with these salmonid herpesviruses.

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