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Title	Development of a loop-mediated isothermal amplification assay for the detection and quantification of epizootic epitheliotropic disease virus (salmonid herpesvirus-3)
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Abstract

Epizootic Epitheliotropic Disease Virus (EEDV; Salmonid Herpesvirus-3) causes a serious disease of lake trout (Salvelinus namaycush) that threatens the restoration efforts of this species in North America. The current inability to replicate EEDV in vitro necessitates the search for a reproducible, sensitive, and specific diagnostic assay that allows for accurate diagnosis that is both time and cost effective. Herein, we describe a loop-mediated isothermal amplification (LAMP) assay that we developed for the rapid and quantifiable detection of EEDV in infected fish tissues. The newly developed LAMP reaction was optimized in the presence of calcein, and the best results were produced using 2 mM MgCl2, 1.8 mM dNTPs and an incubation temperature of 67.1ºC. The analytical sensitivity of the LAMP method was estimated to be as low as 78 pg extracted DNA per reaction from lake trout tissues. The diagnostic sensitivity and specificity of the newly developed LAMP assay compared to the SYBR Green qPCR assay were 84.3% and 93.3%, respectively. The quantitative LAMP for EEDV had a high correlation coefficient (R2 = 0.980), and when compared to the SYBR Green quantitative PCR for validation, no statistical difference found between the two assays ($p > 0.05$). Given its cost- and time-effectiveness, this quantitative LAMP assay is suitable for the surveillance of this herpesvirus in wild fish as well as for the reliable diagnosis of clinical cases.

Keywords	Epizootic epitheliotropic disease virus; loop-mediated isothermal amplification; calcein

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Paul K.S. Chan
Editor-in-Chief, Journal of Virological Methods

April 12, 2018

Dear Mr. Chan,

Please find attached the submission of the manuscript entitled, “Development of a loop-mediated isothermal amplification assay for the detection and quantification of epizootic epitheliotropic disease virus (salmonid herpesvirus-3).” We hope that you find the manuscript to be acceptable for publication in your reputable journal.

Most cordially,

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Highlights

- A quantitative LAMP method for identification of EEDV has been developed.
- Analytical sensitivity of the qLAMP is as low as 78 pg extracted DNA from tissue.
- The method is highly specific for EEDV.
- The EEDV qLAMP method was evaluated against the qPCR method.

Development of a loop-mediated isothermal amplification assay for the detection and quantification of epizootic epitheliotropic disease virus (salmonid herpesvirus-3)

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Abstract

Epizootic Epitheliotropic Disease Virus (EEDV; Salmonid Herpesvirus-3) causes a serious disease of lake trout (*Salvelinus namaycush*) that threatens the restoration efforts of this species in North America. The current inability to replicate EEDV *in vitro* necessitates the search for a reproducible, sensitive, and specific diagnostic assay that allows for accurate diagnosis that is both time and cost effective. Herein, we describe a loop-mediated isothermal amplification (LAMP) assay that we developed for the rapid and quantifiable detection of EEDV in infected fish tissues. The newly developed LAMP reaction was optimized in the presence of calcein, and the best results were produced using 2 mM $MgCl_2$, 1.8 mM dNTPs and an incubation temperature of 67.1ºC. The analytical sensitivity of the LAMP method was estimated to be as low as 78 pg extracted DNA per reaction from lake trout tissues. The diagnostic sensitivity and specificity of the newly developed LAMP assay compared to the SYBR Green qPCR assay were 84.3% and 93.3%, respectively. The quantitative LAMP for EEDV had a high correlation coefficient ($R^2 = 0.980$), and when compared to the SYBR Green quantitative PCR for validation, no statistical difference found between the two assays ($p > 0.05$). Given its cost- and time-effectiveness, this quantitative LAMP assay is suitable for the surveillance of this herpesvirus in wild fish as well as for the reliable diagnosis of clinical cases.

Keywords
Epizootic epitheliotropic disease virus; loop-mediated isothermal amplification; calcein

1. Introduction

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Abbreviations: Epizootic epitheliotropic disease virus (EEDV); loop-mediated isothermal amplification (LAMP); Infectious pancreatic necrosis virus (IPNV); Spring viremia of carp virus (SVCV); Infectious hematopoietic necrosis virus (IHNV); golden shiner reovirus (GSRV); fathead minnow nidovirus (FHMNV); viral hemorrhagic septicemia virus (VHSV); forward inner primer (FIP); backward inner primer (BIP); deoxynucleoside triphosphates (dNTPs)

Viruses in the *Alloherpesviridae* family (order *Herpesvirales*) cause a variety of diseases in amphibians and teleost fish, often with severe economic consequences (Boutier et al., 2015; Hanson et al., 2011). Within the *Alloherpesviridae* family is the genus *Salmonivirus*, which currently contains five viruses: the Salmonid Herpesvirus-1 (Herpesvirus salmonis), Salmonid Herpesvirus-2 (*Oncorhynchus masou* virus), Salmonid Herpesvirus-3 (Epizootic epitheliotropic disease virus; EEDV), Salmonid Herpesvirus-4 (Atlantic salmon papillomatosis virus), and Salmonid Herpesvirus-5 (Namaycush herpesvirus) (Doszpoly et al., 2013; Glenney et al., 2016a; King et al., 2012).

Among the five salmonid herpesviruses, EEDV causes one of the more lethal diseases in its host, leading, for example, to the morality of over 15 million hatchery-reared juvenile lake trout in the early 1980s (Bradley et al., 1989, 1988; McAllister and Herman, 1989). Recently, after 30 years of minimal mortalities associated with EEDV, the virus reappeared in Wisconsin and Michigan hatcheries, resulting in morbidity and mortality in hundreds of thousands of lake trout (Kurobe et al., 2009; Faisal et al., in preparation). In the absence of other available control measures to combat this virus, the implementation of stringent biosecurity measures and use of avoidance strategies remain our only tools to prevent EEDV spread to additional lake trout rearing units or facilities should another outbreak occur. A sensitive and specific diagnostic tool that is rapid and reasonably inexpensive is needed in order to perform testing of wild gamete donor fish as well as periodic testing of hatchery-reared fish throughout their growth. Early detection of EEDV prior to the start of a mortality episode, would allow for more rapid disease control and perhaps prevention of such devastating losses as previously seen.

Endpoint and quantitative PCR-based detection assays for EEDV have been developed that target stretches of the EEDV terminase gene (Glenney et al., 2016b; Kurobe et al., 2009). After the molecular characterization of Salmonid Herpesvirus-4 and -5, it was determined however, that the current EEDV qPCR assay was unable to distinguish between Salmonid Herpesvirus-3, -4, and -5, as the viruses share high sequence identity in the terminase gene. This led Glenney et al. (2016b) to design three primer sets based on the glycoprotein gene. Using these primer sets in a SYBR Green qPCR assay, the authors were able to amplify each virus individually. Herein, we report on the development of a loop-mediated isothermal amplification (LAMP) assay for the detection and quantification of EEDV in infected lake trout tissues, that is faster, more cost effective, and of comparable specificity and sensitivity to the established SYBR Green qPCR.

2. Materials and Methods

2.1 Virus and template DNA

Tissues used in this study for the development and testing of the EEDV LAMP assay were obtained from juvenile naïve lake trout experimentally infected with EEDV-positive tissue homogenate by either intraperitoneal injection or immersion bath (Shavalier, 2017). All research involving live fish adhered to the Michigan State University Institutional Animal Care and Use Committee guidelines (11/14-201-00).

For the purpose of this study, tissues of infected and negative control fish were collected, and enzymatically digested with Proteinase K. Viral DNA extractions were performed manually using the Mag Bind® Blood and Tissue DNA Kit (OMEGA Bio-tek, Inc., Norcross, Georgia, USA), following the manufacturer's instructions and with the addition of a filtering step using the E-Z 96® Lysate Clearance Plate (OMEGA Bio-tek) after tissue digestion (24). Following all nucleic acid extractions, DNA was quantified using a Quant-iT DS DNA Assay Kit and a Qubit

fluorometer (Life Technologies, Grand Island, New York, USA) and diluted to a standard concentration using nuclease free water.

2.2 Primers and LAMP design

A partial sequence of the Salmonid Herpesvirus-3 glycoptorein gene (GenBank accession number JX886027.1) was used as a template to design the EEDV LAMP primer set with the Primer Explorer software, version 4.0 (http://primerexplorer.jp/elamp4.0.0/index.html). The details of the primers are displayed in Table 1. Following alignment of the EEDV primer target sequences on the glycoprotein gene with the same segment of Salmonid Herpesvirus-4 (GenBank accession number JX886028) and Salmonid Herpesvirus-5 (GenBank accession number KP686091), the *in silico* analysis guided the selection of primer sets that are strictly specific to Salmonid Herepesvirus-3 and hence used in this study.

The LAMP reaction was carried out in a 25 μL reaction mixture containing 1.6 μM of each of the forward inner primer (FIP) and backward inner primer (BIP); 0.8 μM of each of the LF and LB primers; 0.2 μM of each of the F3 and B3 primers; 1X isothermal amplification buffer (20 mM Tris-HCl, 10 mM $(NH_4)_2SO_4$, 50 mM KCl, 2 mM $MgSO_4$, 0.1% Tween 20, pH 8.8); 2 mM $MgCl_2$; 1 M betaine; 1.6 mM deoxynucleoside triphosphates (dNTPs); 0.2 mM $MnCl_2$; 20 μM calcein; 8 U *Bst* DNA polymerase (New England Biolabs, Beverly, Massachusetts, USA) and 1 μL template DNA. Calcein was used as a fluorescent indicator which yields strong fluorescence by forming complexes with divalent magnesium ions in LAMP reactions as reported by Tomita et al. (2008).

The mixture was incubated for 50 minutes (one cycle per minute) in an Eppendorf mastercycler realplex ep gradient S PCR machine (Eppendorf, Hauppauge, New York). Changes in fluorescence were monitored every min at 520 nm. Three separate assay factors were optimized: 1) temperature, assay run at 58.3, 60.3, 62.6, 64.9, 67.1, 69.1, and 70.7ºC, (each followed by 80°C for 20 min to terminate the reaction); 2) $MgCl_2$ concentration; and 3) dNTP concentration. $MgCl_2$ and dNTP concentrations were optimized by Taguchi's L16 (2(4)) orthogonal design with two elements (dNTPs and $MgCl_2$) at four concentration levels (Table 2). The reaction optimization of each parameter was performed in triplicate and no-template controls were included in each run.

2.3 Analytical specificity of the EEDV LAMP assay

The specificity of the LAMP primer set was tested by performing the assay under the optimized conditions. Nucleic acids were extracted from a number of DNA and RNA fish pathogenic viruses such as Salmonid Herpesvirus-1, -2, -4, and -5, Infectious pancreatic necrosis virus (family *Birnaviridae*, genus *Aquabirnavirus*; IPNV), Spring Viremia of Carp Virus (order *Mononegavirales*; family *Rhabdoviridae*; genus *Vesiculovirus*; SVCV), Infectious Hematopoietic Necrosis Virus (order *Mononegavirales*; family *Rhabdoviridae*; genus *Novirhabdovirus*; species *Salmonid novirhabdovirus*; IHNV), Golden Shiner Reovirus (family *Reoviridae*; genus *Aquareovirus*; GSRV), Fathead Minnow Nidovirus (order *Nidovirales*; family *Coronaviridae*; subfamily *Torovirinae*; genus *Bafinivirus*; FHMNV), and Viral Hemorrhagic Septicemia Virus (order *Mononegavirales*; family *Rhabdoviridae*; genus *Novirhabdovirus*; species *Salmonid novirhabdovirus*; VHSV) and used as templates in this analysis. Additionally, the 212 bp target sequences of glycoprotein gene of Salmonid Herpesvirus-3, -4, and -5 were aligned and compared using BLAST and the software BioEdit 7.0.

2.4 Analytical sensitivity of the EEDV LAMP assay

The detection limit of the EEDV LAMP assay was analyzed with two kinds of templates. One template was a plasmid vector (pCR®2.1-TOPO®) containing the target fragment from the EEDV glycoprotein gene (designated as pCR®2.1-EEDV). The 212 bp PCR product was amplified by using the primer set of F3-III and B3-III and cloned into the plasmid vector following the manufacturer's instructions. Copy number of pCR®2.1-EEDV was calculated using the molecular mass of the vector and amplicon as indicated in the website (http://cels.uri.edu/gsc/cndna.html). A 10-fold serial dilution of plasmid pCR®2.1-EEDV ($1.6x10^7$–10^1 copies/reaction) was used as the template for the LAMP under the predetermined conditions. The other template was gill tissue DNA extracted from infected lake trout and serially diluted ($7.8x10^6$–$7.8x10^0$ pg/reaction).

2.5 Quantitative EEDV LAMP assay

A quantitative LAMP assay was produced by using ten-fold dilutions of purified PCR product as standards (DNA extracted from skin tissue of infected lake trout). The end-point PCR assay for production of quantification standards consisted of a 50 µL reaction containing 25 µL GoTaq Green Mastermix, 0.25 µM each of F3 and B3 primers and 80 ng DNA template. The PCR reaction was 95ºC for 2 minutes followed by 40 cycles of 95ºC for 15 seconds, 50ºC for 15 seconds and 72ºC for 45 seconds and finished with a single cycle of 95ºC for 15 minutes. PCR product was purified using the Wizard SV Gel and PCR Clean-Up System (Promega) and copy number in each 10-fold dilution was calculated as described above for the plasmid.

For real-time monitoring, the qLAMP reactions were incubated at 67.1ºC for 50 cycles (one minute per cycle) with an Eppendorf realplex 2 (Eppendorf). For quantitative detection of samples, a standard curve was generated for EEDV qLAMP ranging from 10^1 to 10^7 copies/reaction.

2.6 Evaluation of the EEDV LAMP assay on clinical samples

In order to validate the quantitative abilities of the EEDV LAMP assay, a group of 100 previously tested lake trout tissue samples with known viral load ranges (i.e., negative, low, medium, or high titers) were chosen in order to test a comprehensive range of virus loads in tissue. All samples came from experimentally infected or negative control group fish (Shavalier, 2017). DNA was extracted from these tissue samples using the kit (OMEGA Bio-tek) described above, after which the qLAMP was run in parallel with the SYBR Green qPCR assay as described by Glenney et al. (2016). Resulting copy numbers from qLAMP and qPCR were analyzed using a paired *t*-test run in SAS software, Version 9.4 of the SAS System (© 2017 SAS Institute Inc.).

The diagnostic sensitivity (DSe) and specificity (DSp), as defined by the World Organization for Animal Health (2011), of the qLAMP compared to the qPCR were calculated according to Zhang et al. (2013) (International Office of Epizootics, 2009).

3. Results

3.1 Optimization of the EEDV LAMP reaction

In order to determine the optimal reaction conditions, the LAMP assay was carried out for 50 minutes at 7 temperatures. As displayed in Table 2, the smallest average *Ct* value (17.35) was achieved when the reaction was incubated at 67.1ºC and resulted in a relatively small standard error of *Ct* value (0.45) compared to other incubation temperatures.

Concerning the optimization of $MgCl_2$ and dNTPs, the results indicated that the smallest average *Ct* value (17.19) was produced when the concentrations of $MgCl_2$ and dNTPs were 2.0 mM and 1.8 mM, respectively (Table 3). The smallest average *Ct* value was accompanied by a standard error of 0.34, indicating negligible fluctuation of amplification efficiency. Meanwhile, the second smallest *Ct* value (17.66) resulted in a higher standard error of 1.05, and was produced when the concentration of $MgCl_2$ and dNTP were 2.0 mM and 1.6 mM, respectively. Therefore, the optimal concentrations of $MgCl_2$ and dNTP were determined to be 2.0 mM and 1.8 mM, respectively. Based on these results, further LAMP assays were incubated for a total of 50 min at 67.1ºC with 2 mM $MgCl_2$ and 1.8 mM dNTPs.

3.2 Analytical specificity of the EEDV LAMP assay

Alignment of the EEDV LAMP target sequence (212 bp) with the corresponding sequences from the closely related Salmonid Herpesvirus-4 and -5 indicated that the eight EEDV LAMP primers covered 35 or more mutation sites in the corresponding sequences of the other two Salmonid Herpesviruses (Figure 1). Positive results were obtained only when the template used contained the DNA from EEDV-infected fish tissue; no amplification was observed for the DNA or RNA extracted from stocks of Salmonid Herpesviruses-1, -2, -4, or -5, IPNV, SVCV, IHNV, GSRV, FHMNV or VHSV samples (Figure 2). Taken together, these results indicate that the LAMP primer set is specific for amplification of EEDV nucleic acid.

3.3 Analytical sensitivity of the EEDV LAMP assay

When the reaction was tested using 1 µL of 10-fold serial dilutions of plasmid pCR®2.1-EEDV DNA (7.2 ng/µL, equivalent to $1.6x10^9$ copies/µL), the analytical sensitivity of the EEDV-LAMP method was estimated to be as low as 16 copies of the plasmid per reaction while becoming more sporadic below 16 copies per reaction. When the reactions were tested using 1 µL of 10-fold serial dilutions of EEDV positive DNA from lake trout, the analytical sensitivities of the LAMP method were determined as 78 pg of DNA extracted from gill tissues (Figure 3).

3.4 Quantitative EEDV LAMP and validation against SYBR Green qPCR

DNA from 100 tissue samples collected from experimentally challenged lake trout were used to compare the newly developed qLAMP assay with the SYBR Green qPCR currently in use (Glenney et al., 2016b). A high correlation coefficient ($r^2 = 0.980$) was obtained by the EEDV qLAMP when the initial template was above 100 copies (Figure 4). The value of correlation coefficient (r^2) would become 0.990 when the initial template was above 1000 copies (Data not shown). Experimental samples were quantified using the standard curve generated from PCR product. Positive qPCR samples ranged from 10.0 to $1.69x10^8$ copies/reaction while positive qLAMP samples ranged from 4.18 to $6.89x10^7$ copies/reaction (Table 4). Statistical analysis comparing the paired samples using a paired *t* test run in SAS software, Version 9.4 of the SAS System (© 2017 SAS Institute Inc.), revealed no significant difference between the quantifications recovered via the two assays ($p > 0.05$).

The qPCR results indicated that 70/100 samples were positive for EEDV. The qLAMP agreed that 59 of those qPCR positives were also positive. Meanwhile, of the 30 qPCR negative samples, the qLAMP agreed that 28 of those were also negative. Therefore, the DSe and DSp values for the qLAMP method compared to the SYBR Green qPCR method were 84.3% and 93.3% respectively.

4. Discussion

In light of the current absence of a cell line that can support the replication of EEDV, diagnostic tools are limited to endpoint PCR (Kurobe et al., 2009), real-time PCR (Glenney et al., 2016b), or electron microscopy (Bradley et al., 1989). In the current study, we developed a time and cost effective LAMP assay for EEDV detection. This method amplifies EEDV DNA in fish tissue with relatively high specificity and sensitivity, and therefore, represents a valuable diagnostic tool for the detection and quantification of this deadly virus.

The optimal reaction temperature was determined to be 67.1°C which is relatively higher than the optimal LAMP reaction temperatures reported for other viruses such as 62°C for the orf virus (family *Poxviridae*; genus *Parapoxvirus*) (Li et al., 2013), 63°C for human papillomavirus (Saetiew et al., 2011), and 64°C for nervous necrosis virus (family *Nodaviridae*; genus *Betanodavirus*)(Hwang et al., 2016). This variation can be explained by the use of different primer sets for different viruses. Actually, the results of the temperature optimization showed that *Bst* DNA polymerase effectively amplified the nucleic acid templates at a relatively wide temperature range from 62.6 to 69.1ºC, which should greatly benefit the possible application of the method under field conditions. The *Ct* value of samples tested using the EEDV LAMP assay showed substantial variation when the concentration of $MgCl_2$ changed from 2 mM to 6 mM, and also when the concentration of dNTPs changed from 1.2 mM to 1.4 mM, both of which are indications that the concentration of $MgCl_2$ and dNTPs are critical parameters in the EEDV LAMP reaction.

Testing the analytical specificity of the EEDV LAMP clearly demonstrated that amplification occurred only when DNA from EEDV was used as a template; no amplification occurred with the other fish pathogenic DNA viruses including the other closely related Salmonid Herpesviruses-4 and -5. The fact that the EEDV LAMP primers designed in this study cover gene stretches with greater than 35 mutation sites compared to the corresponding sequence stretch of Salmonid Herpesvirus-4 and -5, and did not cross react, attests to the high specificity of this newly developed assay for detection of EEDV.

The analytical sensitivity of the EEDV LAMP assay was determined to be 78 pg total DNA extracted from EEDV-positive lake trout gills, which is considerably higher than those reported by Chen et al. (2010) for the swine transmissible gastroenteritis coronavirus, Li and Ling (2014) for the tomato necrotic stunt virus, and Ma et al. (2016) for the Eriocheir sinensis reovirus.

A standard curve was constructed using serial 10-fold dilutions of the pCR®2.1-EEDV plasmid with reference to C*t* value. Based on the standard curve, an equation was calculated using regression analysis comparing C*t* value to the standard copy number. In the range of 10^7 to10^3 plasmid copies, the correlation coefficient was high ($r^2 = 0.990$), which indicates that the LAMP is appropriate as a quantification tool. However, when copy number was less than 1000 copies, the correlation coefficient declines significantly (data not shown). Previous reports also demonstrated that it is difficult to determine the exact correlation of virus quantity and C*t* value at very low concentrations of template (Suzuki et al., 2011; Wei et al., 2013).

When the developed EEDV LAMP assay was compared to the real-time SYBR Green qPCR (Glenney et al., 2016b), the diagnostic specificity was greater than 90%, however the diagnostic sensitivity was only 84.3%. While the qPCR identified 11 samples as positive that the qLAMP did not, all but two of them were less than 1,000 copies and as indicated above, accurate quantification below this level can be difficult.

When the viral loads determined by qLAMP were compared to those of the SYBR Green qPCR, both assays were capable of quantifying viral loads over a wide range (Table 4). While there were some discrepancies with identification of individual positive tissues between the two assays, when all samples were examined together, the paired *t*-test demonstrated no significant difference between the results of the two different assays ($p > 0.05$). The discrepancy was limited to samples with low viral copy numbers. In total, these quantification results lend further support to the use of this qLAMP assay as a diagnostic tool, both in the laboratory and in field conditions.

5. Conclusion

In summary, a specific, sensitive LAMP assay was developed for the detection of EEDV in fish tissues. This novel assay has the advantage of being rapid and is promising for use as a surveillance tool for EEDV diagnosis in clinical samples. Moreover, the qLAMP established in this study provides a low-cost quantification method for EEDV loads in tissue samples, and the use of calcein as a fluorescent indicator, which can also be visualized by the naked eye, or under a UV light, provides a good platform for optimization of an assay that can be used in field conditions, such as at an aquaculture facility.

5. Acknowledgements

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6. Data Statement

Data used in this study can be made available upon reasonable request.

7. Declarations of interest: none

8. References

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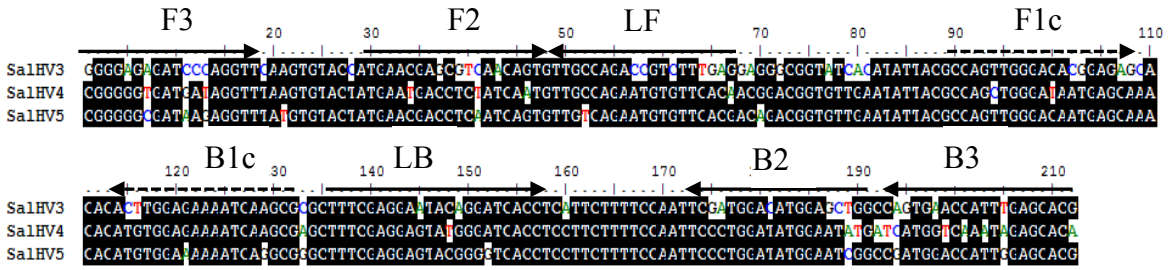

Figure 1. Target gene sequence alignments. Alignments of the epizootic epitheliotropic disease virus (EEDV; Salmonid Herpesvirus-3) target gene region (GenBank JX886027) with the most related sequences of viruses available in GenBank including Atlantic salmon papillomatosis virus (Salmonid Herpesvirus-4; JX886028) and Namaycush herpesvirus (Salmonid Herpesvirus-5; KP686091). Notice that the eight EEDV loop-mediated isothermal amplification (LAMP) primers cover 35 or more mutation sites in the corresponding sequences of the other two SalHV strains. F: forward primer, B: backward primer, LF: loop-forward primer, LB: loop-backward primer.

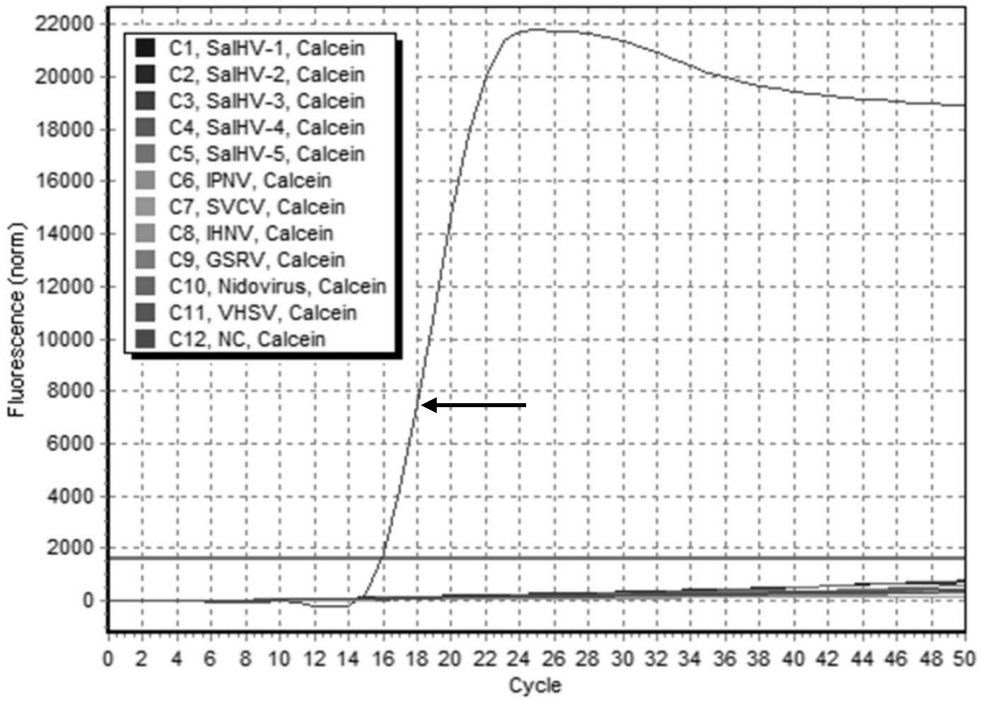

Figure 2. EEDV amplification plot. Ability of the epizootic epitheliotropic disease virus (EEDV) loop-mediated isothermal amplification (LAMP) assay to discriminate EEDV from other viruses (i.e., analytical specificity). The amplification plot of EEDV is indicated by the arrow and appears as expected.

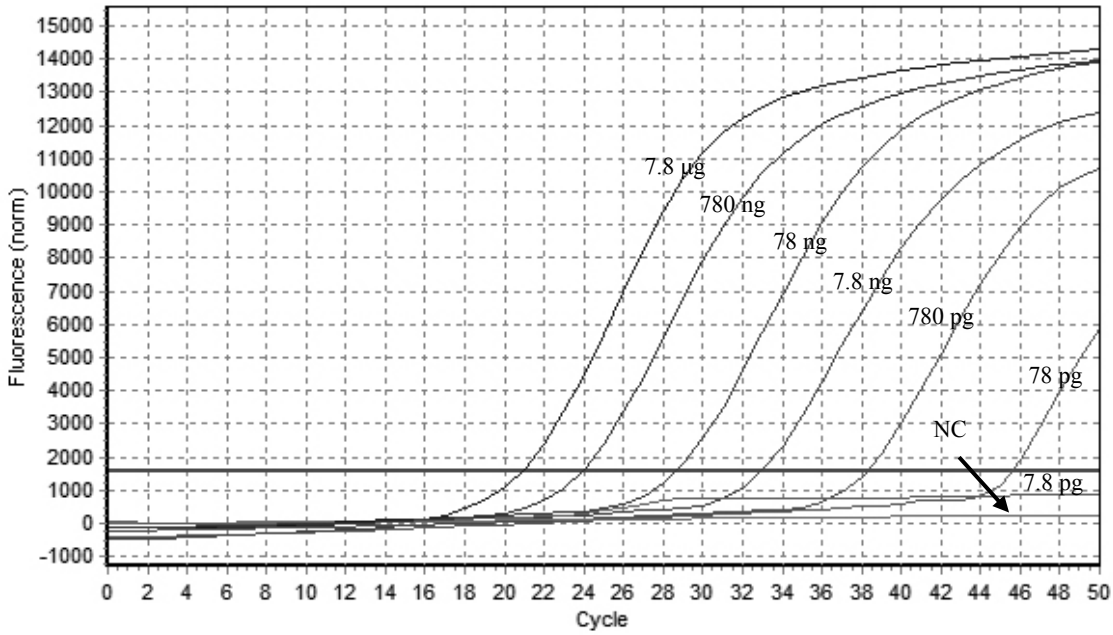

Figure 3. EEDV qLAMP sensitivity. Analytical sensitivity or limits of detection of epizootic epitheliotropic disease virus (EEDV)-positive lake trout gill DNA by the diagnostic loop-mediated isothermal amplification (LAMP) assay for EEDV. Amplification plots 1–7 (from left to right): reaction conducted using 10-fold serial dilutions of DNA from lake trout: $7.8x10^6$, $7.8x10^5$, $7.8x10^4$, $7.8x10^3$, $7.8x10^2$, $7.8x10^1$, and 7.8 pg, respectively. Amplification plot 8 was the negative control (NC).

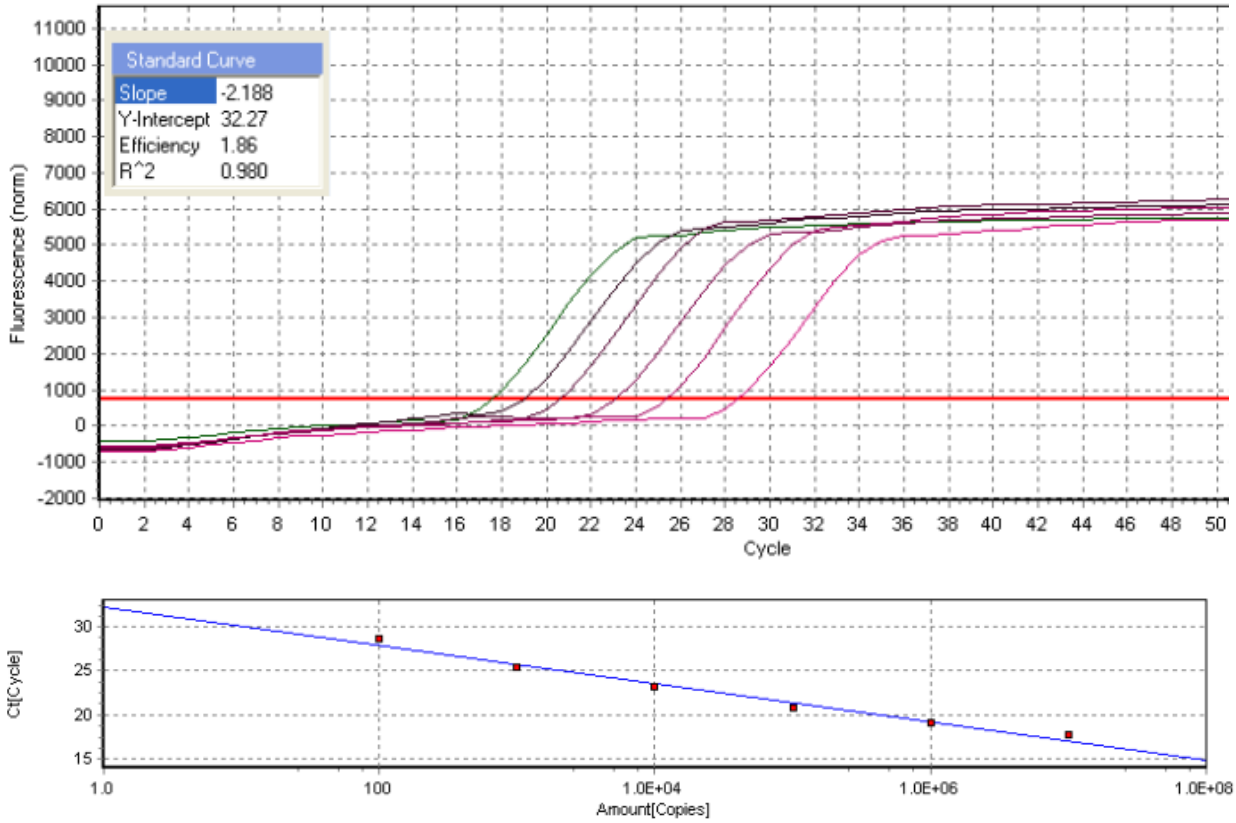

Figure 4. EEDV standard curve. Standard curve and standard curve equation for the EEDV-specific qLAMP assay generated from the amplification plots between the serial 10-fold diluted pCR®2.1-EEDV plasmid and *Ct* value. Plasmid was serially diluted 10-fold from $1.0x10^7$ to $1.0x10^2$ copies /reaction over three replicates.

Primer	*Sequence*
F3	GGGGAGAGATCCCAGGTTC
B3	CGTGCTCAAATGGTTCACTG
FIP (F1c+TTTT+F2)	GCTCTCCGTGTCCCAACTGGTTTTTGAACGAGCGTCAACAGTG
BIP (B1c+TTTT+B2)	ACTTGGAGAAAATCAAGCGCGCTTTTCCAGCTCCATGTCCATCGA
LF	CCTCAAAGACGGTCTGGCAA
LB	TTTCGAGGAATACAGGATCACCT

Table 1 Primers used for epizootic epitheliotropic disease virus (EEDV) loop-mediated isothermal amplification (LAMP).

Temperature	**Primer set III**	
	Mean* of *Ct* value	SD* of *Ct* value
58.3	27.07	0.28
60.3	24.13	0.01
62.6	18.99	0.01
64.9	18.41	0.09
67.1	17.35	0.45
69.1	18.22	1.22
70.7	24.40	1.15

Table 2 Results of epizootic epitheliotropic disease virus (EEDV) loop-mediated isothermal amplification (LAMP) temperature optimization. Mean and standard deviation produced from duplicate repeats of LAMP assay.

$MgCl_2$ concentration	**dNTP** concentration	**Primer set**	
		Mean* of *Ct* value	SD* of *Ct* value
2mM	1.2 mM	21.39	0.74
2mM	1.4 mM	18.50	0.39
2mM	1.6 mM	17.66	1.05
2mM	1.8 mM	**17.19**	**0.34**
4mM	1.2 mM	36.93	0.68
4mM	1.4 mM	32.52	1.13
4mM	1.6 mM	28.24	1.00
4mM	1.8 mM	27.03	0.93
6mM	1.2 mM	-	-
6mM	1.4 mM	47.51	0.60
6mM	1.6 mM	45.02	1.15
6mM	1.8 mM	40.63	1.32
8mM	1.2 mM	-	-
8mM	1.4 mM	-	-
8mM	1.6 mM	-	-
8mM	1.8 mM	-	-

Table 3 Results of $MgCl_2$ and dNTP concentration optimization for epizootic epitheliotropic disease virus (EEDV) loop-mediated isothermal amplification (LAMP). Mean and standard deviation produced from duplicate repeats of LAMP assay.

#	qPCR	qLAMP	#	qPCR	qLAMP	#	qPCR	qLAMP
1	-	-	35	$1.79x10^4$	$7.54x10^4$	69	$3.47x10^5$	$2.16x10^5$
2	-	-	36	960	820	70	$2.07x10^5$	$1.71x10^5$
3	-	-	37	122	-	71	$6.38x10^4$	$5.48x10^4$
4	-	-	38	-	-	72	$1.05x10^4$	$4.94x10^3$
5	-	-	39	159	-	73	$9.24x10^3$	635
6	-	-	40	$1.86x10^3$	$9.66x10^3$	74	$1.86x10^4$	$8.25x10^3$
7	-	-	41	347	-	75	$2.49x10^5$	$2.52x10^5$
8	-	-	42	$1.40x10^4$	$8.60x10^4$	76	$2.30x10^3$	144
9	-	-	43	$3.00x10^5$	$1.03x10^6$	77	$3.30x10^4$	$1.04x10^4$
10	-	-	44	$1.60x10^4$	$4.96x10^4$	78	$3.09x10^6$	$6.45x10^6$
11	-	-	45	$3.63x10^5$	$6.96x10^5$	79	$7.71x10^6$	$1.27x10^7$
12	-	-	46	$1.80x10^3$	$3.42x10^3$	80	$6.62x10^6$	$2.97x10^6$
13	-	-	47	220	-	81	$9.44x10^7$	$6.11x10^7$
14	-	-	48	495	$3.04x10^3$	82	$2.47x10^7$	$2.01x10^7$
15	-	-	49	527	95.3	83	$1.83x10^7$	$2.59x10^7$
16	-	-	50	$1.40x10^3$	$5.40x10^3$	84	$1.23x10^7$	$1.45x10^7$
17	-	-	51	$4.50x10^3$	579	85	$7.12x10^7$	$4.31x10^7$
18	-	-	52	937	4.18	86	$6.74x10^7$	$5.13x10^7$
19	-	-	53	$3.13x10^3$	267	87	$2.60x10^7$	$1.53x10^7$
20	-	-	54	-	566	88	$1.69x10^8$	$6.89x10^7$
21	-	-	55	$1.95x10^3$	119	89	$3.14x10^7$	$3.49x10^7$
22	-	$2.54x10^3$	56	825	-	90	$1.47x10^7$	$4.18x10^7$
23	202	-	57	$1.34x10^3$	283	91	$1.84x10^7$	$1.62x10^7$
24	256	-	58	$4.02x10^3$	205	92	$1.73x10^7$	$1.37x10^7$
25	166	-	59	$2.18x10^3$	-	93	$1.47x10^7$	$1.23x10^7$
26	-	-	60	$1.38x10^3$	-	94	$2.71x10^7$	$2.87x10^7$
27	-	-	61	$1.62x10^6$	$1.86x10^6$	95	$2.15x10^7$	$6.48x10^6$
28	-	-	62	$1.20x10^6$	$1.41x10^6$	96	$7.40x10^6$	$4.42x10^6$
29	-	-	63	$2.22x10^5$	$1.53x10^5$	97	$5.55x10^6$	$4.11x10^6$
30	84.9	-	64	$1.83x10^6$	$3.17x10^6$	98	$1.58x10^7$	$4.34x10^6$
31	102	18.3	65	$1.69x10^6$	$1.78x10^6$	99	$1.12x10^7$	$4.99x10^6$
32	-	-	66	$1.64x10^6$	$2.80x10^6$	100	$7.02x10^6$	$1.44x10^6$
33	-	-	67	$5.93x10^5$	$8.43x10^5$			
34	$1.41x10^3$	$1.14x10^4$	68	$3.44x10^4$	$1.53x10^3$			

Table 4 Comparison of SYBR Green qPCR assay (*14*) results and newly developed quantitative loop-mediated isothermal amplification (qLAMP) assay results performed on 100 experimental samples of lake trout skin tissue. Data is presented as viral copies per reaction (50 ng template DNA added to each reaction, qPCR and qLAMP) for the epizootic epitheliotropic disease virus (EEDV). There was no statistical difference between qPCR and qLAMP quantification ($p > 0.05$) using a paired t test run in SAS software, Version 9.4 of the SAS System (© 2017 SAS Institute, Inc.).

Progression of epizootic epitheliotropic disease virus (*Salmonid herpesvirus 3*) in target tissues and cells of its host, the lake trout (*Salvelinus namaycush*)

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Subject category: Animal, DNA Viruses

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Abbreviations:

Abstract

Salmonid herpesvirus 3 (epizootic epitheliotropic disease virus; EEDV) is an Alloherpesvirus (Order *Herpesvirales*) responsible for the deaths of millions of hatchery-raised lake trout (*Salvelinus namaycush*) in the Laurentian Great Lakes Basin over the past three decades. There is yet little known about the tissue and cellular tropism of EEDV. In this study, we investigated the presence of EEDV in experimentally challenged lake trout over the course of six weeks. Individual tissue samples were collected from 6 infected and 3 control fish on days 0, 1, 3, 6, 9, 12, 15, 21, 28, 35, and 42 post-infection. EEDV viral load was determined using a quantitative real-time PCR targeting the EEDV glycoprotein gene, and virus tropism was visualized using oligoprobes designed to target the same gene in an *in situ* hybridization (ISH) assay. Skin, fin, and ocular tissues were the first viral targets, and yielded the highest viral loads throughout the course of infection. During early stages of disease manifestation, intense labeling for EEDV DNA was identified in epithelial cells of the epidermis, with subsequent labeling detected in the epithelial lining of primary and secondary gill lamellae. During advanced disease, EEDV-positive staining was observed in endothelial and dendritic cells as well as blood monocytes. This study characterized EEDV tissue tropism and associated pathology for the first time. Our results will serve to guide future research aimed at understanding EEDV disease ecology, as well as helping to improve strategies for diagnostic sampling and disease control.

Introduction

The family *Alloherpesviridae* is comprised of a group of highly pathogenic viruses that often result in devastating mortality events in their fish hosts, such as is the case with the OIE-reportable Koi Herpes Virus (KHV; *Cyprinid herpesvirus 3*) [1] in common carp (*Cyprinus*

carpio), *Ictalurid herpesvirus 1* and *2* in the channel catfish (*Ictalurus punctatus*) [2, 3], and *Salmonid herpesvirus 2* and 3 in salmonids [4, 5]. Despite the losses caused by each of these viruses, little is known about their cellular targets, information that is essential to improving our understanding of the pathogenesis of this group of viruses.

In a previous study using quantitative PCR (qPCR) and *in situ* hybridization (ISH) assays, Miwa et al. [6] demonstrated that in immersion challenged Koi and Common Carp (*Cyprinus carpio*), skin is the major entry point of KHV, followed by the gills within an additional 1-4 days and internal organs after that. ISH positive labeling was particularly intense in the epithelial cells of both skin and gills of these fish [6]. Similarly, a fluorescence ISH assay, paired with conventional PCR targeting the polymerase gene, was used to identify the gill, kidney and spleen as the target tissues of *Cyprinid herpesvirus 2* (the causative agent of Goldfish Hematopoietic Necrosis Virus) in Prussian carp (*Carassius auratus gibelio*) [7]. The use of ISH assays has also allowed for the elucidation of viral tissue targets of herpesviruses outside the Alloherpesvirus family, including the localization of *Ostreid herpesvirus 2* (Family *Malacoherpesviridae*) DNA, RNA and viral proteins in a wide variety of tissues, including the gills, mantle, heart, adductor muscle, and labial palps of Pacific oyster (*Crassostrea gigas*) [8].

Of particular concern to fishery conservation efforts in the United States, is the Alloherpesvirus *Salmonid herpesvirus 3*, (epizootic epitheliotropic disease virus; EEDV), which causes devastating losses in lake trout (*Salvelinus namaycush*) [5, 9, 10]. A highly prized, indigenous species in North America, the lake trout is of high economic and recreational importance in addition to being a key apex predator [11]. To date, EEDV has not been successfully replicated *in vitro*, making the study of its pathogenesis especially difficult. Recently, we developed an *in vivo*, reproducible model for EEDV infection under controlled

laboratory conditions. Using this protocol, the current study was designed in order to follow EEDV within it host and to identify its cellular target throughout the course of disease. This was achieved by quantification of viral load using real time quantitative PCR (qPCR), and visualization of viral DNA by *in situ* hybridization (ISH).

RESULTS

EEDV infection, and assessment of viral load in tissues of experimentally infected fish, using qPCR

Gross disease signs in experimentally challenged fish were consistent with those seen during natural EEDV outbreaks [12]. Clinical signs were observed as early as three days post-infection (p.i.) in the form of petechiae to ecchymoses in the lower quadrant of the eyes as well as congestion of visceral blood vessels. By Day 15 p.i., skin of infected fish exhibited multifocal to coalescing erosions and ulcerations, along with congestion (at the base) and erosion (at the tip) of all fins. Abnormalities in visceral organs were observed by Day 6 p.i., and ranged from mild pallor to congestion, particularly of both hepatic and enteric vessels. Fish density within the tank continuously decreased due to a predetermined, periodic sampling schedule, and the death of four fish on Day 28 p.i. No clinical signs were observed and no mortalities occurred in the negative control group.

The earliest detection of EEDV DNA was from a single fish on Day 9 p.i. By Day 18 p.i., viral DNA was present in half of the fish sampled (3/6). As the infection advanced further, EEDV DNA was detected in multiple tissues from all fish sampled on Day 21 p.i., through the end of the observation period, i.e. Day 42 (Table 1). No EEDV DNA was detected in any of the tissues sampled from the negative control fish.

As displayed in Table 1, ocular tissues were the first to harbor EEDV DNA. As infection progressed, detection extended to the skin and fins by Day 18 p.i. From Day 21 through the end of the observation period, EEDV DNA was most prevalent in these tissues (i.e., eye, skin, fin) with the addition of gill tissue. Detection of viral DNA in internal organs did not occur until Day 21 p.i. when brain, kidney, liver, and spleen tissues from multiple fish were EEDV-positive. The infection seems to have peaked by Day 28 p.i., at which point the virus was present in all tissues of all fish and four mortalities occurred. As infection subsided across the following two sampling events, EEDV was detected in some but not all internal organs of sampled fish. In contrast, EEDV remained detectible in all external tissues of the eyes, skin, fins, and gills through the end of the observation period.

Comparisons of the number of EEDV-positive samples per tissue type, by day p.i. were performed. On Day 18 p.i., the number of positive skin and fin tissues ($n = 2$ each) was statistically significantly different from the number of positive gill, kidney, spleen, heart, liver, intestine, or brain tissues ($n = 0$ each; $p < 0.01$). On Day 21 p.i., in pairwise comparisons, the number of positive eye, skin, fin, or gill tissues ($n = 5$ each) was statistically significantly different from the number of positive kidney ($n = 3$), spleen ($n = 2$), heart ($n = 2$), intestine ($n = 3$), or brain ($n = 2$) tissues ($p < 0.01$). On Day 35 p.i., in pairwise comparisons, the number of positive eye, skin, fin, or gill tissues ($n = 6$ each) was statistically significantly higher than the number of positive spleen ($n = 3$) or heart tissues ($n = 4$) ($p < 0.01$). Finally, on Day 42 p.i., the number of positive eye, skin, fin, or gill tissues ($n = 6$ each) was statistically significantly higher than the number of positive kidney ($n = 2$), heart ($n = 1$), liver ($n = 4$) or intestine ($n = 3$) tissues.

When examining differences between tissue types across the entire study, eyes, skin, fins and gills were EEDV-positive most frequently, however, the only statistically significant pairings were between heart (n = 13) and eye, skin or fin (n = 25 each; $p < 0.05$).

In addition to having a higher number of positive samples throughout the study, eye, skin and fin also consistently contained the highest EEDV DNA loads, often 100 to 1,000 fold higher than that of internal organs (Table 1, Figure 1). When first detected in the eye, fin and skin (Days 9 and 18 p.i.), EEDV DNA loads ranged from 10^3 to 10^5 viral copies per mg host tissue (Table 1). A similar range was observed on Day 21 p.i., when EEDV was first detected in the gills and remaining internal tissues, while the viral loads in the eye, skin and fin reached 10^7 to 10^8 copies per mg host tissue.

At the apparent peak of infection, on Day 28 p.i., viral loads in external tissues ranged from 10^6 to 10^9 copies per mg host tissue while the viral loads in internal tissues ranged from 10^4 to 10^7 copies per mg host tissue (Table 1). While certain gill tissues contained a viral load equivalent to those of the eye, skin and fin, as a whole, viral loads in gill tissues were more similar to viral loads of internal tissues, as is evidenced in Figure 2.

While infection appeared to wane throughout the final two sampling events (Days 35 and 42 p.i.), and the number of positive tissues decreased, the viral loads in internal tissues decreased as well. In contrast, the viral loads in the eye, skin and fin remained high (up to 10^9 viral copies per mg host tissue).

When analyzing pairwise comparisons of viral loads, the eye, skin, and fin tissues had statistically significantly higher viral loads than internal organs on Day 21 (kidney, liver, and brain; $p < 0.05$), Day 28 (kidney, spleen, heart, liver, intestine, and brain; $p < 0.05$), Day 35 (all other tissue types; $p < 0.01$), and Day 42 p.i. (liver, intestine, and brain; $p < 0.05$).

An additional statistical analysis was performed comparing the number of positive samples, as well as the average viral load, by sampling day, from all external tissues combined versus all internal organs combined on Days 21-42. On each of these four sampling days, the external tissues had a statistically significantly higher viral load compared to internal organs ($p < 0.01$) and except for Day 28 when EEDV was detected in all 60 tissues tested, the external tissues also harbored the virus in a higher number of tissue samples than the internal organs ($p < 0.01$).

ISH assay designed and verified for identification of EEDV DNA

As a positive control, we used skin and gill tissues collected from fish exhibiting classical EEDV clinical signs and with high EEDV loads (based on qPCR, ct values of < 20) from previous experimental infections [12]. Negative control tissues were collected from adult lake trout population, obtained as gametes and that have been held in a biosecure quarantine facility since 2003. These tissues were used to standardize the ISH procedure and confirmed a lack of non-specific reaction. Using this standardized procedure, no signal was detected in any negative control tissues, while specific intranuclear labeling was detected in positive tissues.

Visualization of EEDV-infected tissues and cells using _ISH_ assay

Due to the inherent lower sensitivity of ISH compared to qPCR, the assay was run on samples with relatively high viral copies from all tissues sampled to determine cellular targets of EEDV. Positive labeling was observed in the skin, gills, and spleen as well as endothelial cells and monocytes of vessels in different organs, from fish sampled on Days 28, 35, and 42 p.i. The number of positive cells varied between days with the largest number of positive cells correlating

to the most advanced stages of disease and the highest viral load based on qPCR. Positive labeling was not evident in any of the negative control tissues tested.

In skin tissue collected from fish in early stages of disease course, positive ISH labeling confirmed the presence of EEDV in the nuclei of degenerating epithelial cells as well as in infiltrating lymphocytes and dendritic cells (Figure 3). In early skin lesions (Figure 3a) viral nucleic acid was detected in nuclei of individual necrotic epithelial cells. In advanced cutaneous lesions (Figure 3b), viral nucleic acid was readily detected in large numbers of nuclei of degenerate and necrotic epithelial cells that commonly sloughed off. In the most severe skin lesions (Figure 3c), viral nucleic acid was detected in the nuclei of the vast majority of epithelial cells throughout all layers prior to epithelial loss.

In the gills, viral nucleic acid was detected in the nuclei of morphologically unremarkable epithelial cells (Figure 4a) during early stages of infection. More advanced gill disease (Figure 4b) saw viral nucleic acid the in nuclei of attenuated epithelial cells and nuclei of infiltrating mononuclear cells (Figure 4c).

During the later disease stages, lesions in internal organs most likely developed secondary to viremia, as is supported by the sudden detection of large amounts of virus in internal organs by qPCR. Viral nucleic acid was detected in nuclei of large numbers of mononuclear cells in the spleen (Figure 5a). A severe lymphohistiocytic perivasculitis was observed [12], most likely secondary to viral infection of endothelial cells (Figure 5b). Significant nuclear labeling of mononuclear cells (Figure 5b) in the vessels of different organs indicates likely development of viremia in later stages of disease.

DISCUSSION

The present study is the first to unravel which lake trout tissues and cell types support the replication of *Salmonid herpesvirus 3* (epizootic epitheliotropic disease virus; EEDV). The quality of EEDV DNA visualization in tissues collected from infected lake trout attests to the soundness of this ISH protocol on formalin-fixed, paraffin-embedded tissues. The coupling of qPCR detection with ISH visualization sheds light on the spread of this virus within the tissues of its host, thereby improving our understanding of EEDV pathogenesis.

Our data demonstrated that the EEDV eclipse period is between 9 and 18 days following a water-borne infection. Many factors may affect the length of this initial incubation period including virus exposure dose, fish density and stress, water temperature or pre-existing health conditions [12–14]. qPCR findings clearly point to the early involvement of the integument and ocular tissues in EEDV infection, underscoring the potential of the integument as a portal for EEDV to enter its host. While the experimental infection in this study took place via exposure to virus-laden water, it is plausible to consider skin-to-skin contact with infected fish as another potential source of infection. Skin-to-skin contact is known as a primary portal of infection in a number of herpesvirus infections of terrestrial animals such as with the equine and feline herpesviruses [15, 16].

In the early stages of infection, EEDV seems to target squamous and cuboidal cells of the epidermis, and as the infection progresses, the virus becomes ubiquitously distributed throughout the layers of the skin epithelium, often leading to necrotic changes in infected cells, resulting in erosions and ulcerations. The fact that the integument and eye tissues maintained relatively high copy levels of EEDV DNA along with intense intranuclear ISH staining, attests to these organs being a major site of virus replication throughout the disease course. As the epithelium erodes and ulcerates in advanced stages of disease, these sloughing epithelial cells, full of virus, may

183 serve as a primary source of infection for cohabitating fish. In addition to the skin epithelial cells,
184 intense staining was noticed in the nuclei of infiltrating mononuclear cells, whose numbers also
185 increased as the infection progressed. The intranuclear ISH staining of the mononuclear cells
186 indicates that EEDV replicates in these infiltrating cells as well, a matter that may aid in the virus
187 dissemination.

188 The embryonic origin (ectoderm vs. endoderm) of the gills in bony fish is currently
189 debated. However, recent evidence using cell lineage tracers demonstrated that pharyngeal gills
190 of gnathostomes (jawed vertebrates – cartilaginous and bony fishes) are endodermally derived
191 [17]. Data acquired in the current study demonstrated that gills actually harbored significantly
192 less EEDV copies than the integument, despite their external location. This is in contrast to
193 trends seen with *Ictalurid herpesvirus 1*, *Cyprinid herpesvirus 2*, and *Anguillid herpesvirus 1*,
194 where host gills are preferentially and persistently infected [18–20]. Similar to the skin however,
195 ISH staining was noticed in the branchial epithelium. The virus was present in the mononuclear
196 cells not only infiltrating gill tissues but also those inside the blood vessels, representing a likely
197 cause of viremia. Even the endothelial cells lining blood vessels seem to support EEDV
198 replication.

199 The identification of these EEDV-positive intravascular mononuclear cells is important,
200 not only as a vehicle for virus spread within the fish during active infection, but also as a
201 potential source for reinfection in surviving fish. Previous work with the hepatitis c virus
202 identified viral RNA in peripheral blood mononuclear cells in serum negative patients
203 undergoing chemotherapy [21]. Viral DNA has also been detected in the peripheral blood
204 mononuclear cells of a clinically normal horse infected with *equine herpesvirus 5* [22] and
205 multiple humans infected with *human herpesvirus 6* [23]. Therefore, it is possible that these

peripheral mononuclear cells might serve as a reservoir for recrudescence of EEDV in surviving fish, albeit at low levels.

Positive staining of mononuclear cells was also observed in extremely large numbers in the spleen of fish at advanced stages of the disease, suggesting that viral replication also occurs within the splenic hematopoietic cells. As with the epithelial and mononuclear cells of the skin and gills, this staining is intranuclear, lending support to active viral replication. These infected splenic cells represent a large source from which virus can spread to other organs and tissues.

Following the initial detection of EEDV DNA in external tissues, it was approximately two weeks before any virus was detected in internal tissues. This apparent delayed or prolonged spread of virus to visceral organs (e.g., kidney, spleen, and liver) after initial detection is evidence of EEDV first targeting and establishing an infection in external tissues, followed later by spread and development of systemic disease. This pattern of an initial infection site in external tissues followed by systemic spread has also been observed in the herpesviruses of cyprinids, catfish, and eels [24].

The viral copy levels in the brain tissue were comparable to those of other internal organs, meaning EEDV can potentially overcome the blood-brain barrier, however EEDV could not be visualized in the brain tissue despite the high number of virus DNA copies. One explanation is that high brain DNA titers originated from the blood or other tissue fluids with brain cells in fact not supporting EEDV viral replication. Similar observations were noted in the liver, kidney, heart, and intestine where major parenchymal cells of these organs were negative for ISH despite the high levels of EEDV DNA copies via qPCR. We believe that cells in these organs are not supportive of the virus replication and the virus detected with the qPCR originated from the tissue fluids and circulating blood. The ability of detecting intense ISH staining in

mononuclear cells but not in other cells types within the visceral organs enforces our notion that EEDV replication occurs in integument epithelial cells, endothelial cells, and mononuclear cells (in skin, gills, circulating blood and spleen) only.

Identification of viral targets throughout a course of disease is important for many reasons, one of which is pathogen identification. Kidney and spleen are commonly used for diagnosis of pathogenic aquatic viruses, however, as is also the case with some of the other aquatic herpesviruses such as koi herpesvirus [6], these are not appropriate tissues for diagnosis of EEDV. When compared to external tissues (e.g., eye, skin, fin), kidney and spleen carry consistently lower viral loads, which are also not detectible as early in, or as long throughout, the course of infection. While internal tissues collected and tested from a highly infected individual may have readily detectible levels of EEDV, in order to maximize chances at detecting low-level carriers of the virus, external tissues should be used for screening instead. Because of high virus copy number in both skin and fin samples from the current study, fin clips emerge as a promising, cost-effective, non-lethal method of sampling both captive and wild fish, which will allow for continuous monitoring of the EEDV in a population.

The data provided herein establishes that EEDV replication is supported by a myriad of cells of different embryonic origins such as skin epithelium, gill epithelium, endothelial cells, and circulating mononuclear cells underscoring its wide range of susceptible cell types. This information can be used to alter screening efforts of Great Lakes Basin lake trout populations as well as to focus future research into the location and establishment of latency as an explanation for the long periods of undetection in this virus' history.

METHODS

Fish and maintenance

Juvenile, Lake Superior strain lake trout (6 months post-hatch), obtained from Marquette State Fish Hatchery (Marquette, Michigan), were used for experimental infections with EEDV. The lot from which these fish originated was determined to be free of reportable pathogens of interest at a 95% confidence level based on recommendations by the American Fisheries Society Fish Health Section blue book [25] and the Model program for fish health management by the Great Lakes Fishery Commission [26]. Additionally, the presence of EEDV was excluded from these fish with the use of qPCR on a subset of the fish prior to performing the experiments of this study.

All experiments were performed at the Michigan State University – Research Containment Facility (East Lansing, Michigan) in accordance with the Institutional Animal Care and Use Committee. Fish were allowed to acclimate to laboratory conditions for a minimum of one month prior to the start of experimental challenges while being held in a 680-liter fiberglass aquarium with continuous, oxygenated well water (12.0 ± 1.0°C), and fed *ad lib* with 1.0 mm sinking trout feed (BioOregon, Westbrook, Maine, USA).

All experimental challenges were performed in fiberglass aquaria receiving flow-through, chilled, oxygenated well water. Studies were performed at a water temperature of 9.0 ± 0.5°C, and fish were allowed to acclimate to colder water temperatures for a minimum of 48 hours prior to the start of experimental challenges.

Infectious virus stock

As EEDV has not been successfully replicated *in vitro*, a stock of infectious virus for use in experimental challenges was produced from the skin of lake trout collected during a natural

outbreak and stored at -80ºC. Skin was homogenized in a sterile phosphate buffered saline solution, (pH 7.5±0.5; Sigma-Aldrich, St Louis, Missouri, USA) at a ratio of 1:3 (w/v), and clarified via low speed centrifugation (1,400 x *g*) for 20 minutes at 4°C. This supernatant was then used to infect naïve juvenile lake trout via an intraperitoneal injection. Fish were anesthetized using tricaine methansulfonate (MS-222; Argent Chemical Laboratories, Redmond, Washington; 0.1 mg ml^{-1}) then injected with 300 µl of virus stock and allowed to recover from sedation prior to return to flow-through aquaria for the duration of the study. Following virus exposure, fish were monitored daily for development of morbidity or mortality, and upon death or development of severe clinical disease, the fish were collected or euthanized with an overdose of MS-222 (0.25 mg ml^{-1}), and their skin sampled and processed as described above to create a new batch of EEDV stock. After the initial stock production, skin samples were homogenized with an Earle's salt-based minimal essential medium (MEM; Invitrogen, Thermo Fisher Scientific, Waltham, Massachusetts, USA), supplemented with 12 mM Tris buffer (Sigma-Aldrich, St Louis, Missouri, USA), penicillin (100 IU ml^{-1}; Invitrogen), streptomycin (100 µg ml^{-1}; Invitrogen), and amphotericin B (250 µg ml^{-1}; Invitrogen) rather than PBS. This process of infection and stock production was repeated with new groups of naïve fish until an adequate volume of 7^{th} passage virus stock was produced for use in the current study [12].

Experimental challenge

For this study, 84 lake trout were immersion challenged with a previously determined moderately lethal dose of EEDV [12] while 48 lake trout were exposed to a sham suspension of MEM as a negative control group. Immersion exposure was achieved by transferring experimental fish to aerated glass aquaria where the infectious or control dose was added. Fish

were maintained and monitored for 1 hour during which time the water was held at a constant temperature (9 ± 0.5°C). After one hour, fish were transferred back to their flow-through aquaria and monitored daily for mortalities or development of clinical disease for the duration of the study. Experimentally challenged fish were maintained at a water temperature of 9 ± 0.5°C for the 42 day study period.

Sample collection

Seven infected fish and four negative control fish were collected in parallel and euthanized on Days 0, 1, 3, 6, 9, 12, 15, 18, 21, 28, 35, and 42 post-infection (p.i.), focusing on minimizing stress for both sampled and remaining fish throughout the sampling event. On these days, one fish from each group was preserved whole in 10% neutral buffered formalin (v/v) following creation of a ventral midline incision to allow for improved fixation. External and internal examinations were performed on the remaining 6 infected and 3 control fish at which time individual portions of skin, fin, gill, eye, brain, spleen, heart, liver, intestine, and kidney were collected from each fish. Each tissue was divided, one portion to be frozen at -20°C for quantification of EEDV DNA while the other portion was fixed in 10% neutral buffered formalin (v/v) for viral DNA visualization. Eyes were collected whole, utilizing both right and left rather than attempting to split, portions of both anterior and posterior kidney were collected for "kidney" samples, and "intestine" tissues consisted of a portion of the intestine approximately 1 cm oral to the vent. Fixed tissues were processed for paraffin embedding, sectioned and applied to glass slides.

Quantification of EEDV DNA in tissues

Tissues collected for viral DNA quantification were individually digested and DNA extractions performed using the Mag Bind® Blood and Tissue DNA Kit (OMEGA Bio-tek, Inc, Norcross, Georgia, USA), following the manufacturer's instructions and with the addition of a filtering step using the E-Z 96® Lysate Clearance Plate (OMEGA Bio-tek, Inc, Norcross, Georgia, USA) based on the protocol outlined by Glenney et al. (2016) [27]. After individual digestion, negative control tissues were extracted from in pools of 3, by tissue type. Eluted DNA was quantified using a Quant-iT DS DNA Assay Kit and a Qubit fluorometer (Life Technologies, Grand Island, New York, USA). All PCR reactions were carried out in a Mastercycler ep *realplex*^{2}S real-time PCR machine (Eppendorf, Hauppauge, New York, USA) with a total reaction volume of 20 µL. Each reaction contained 10 µL SYBR Select Master Mix (2x; Life Technologies, Grand Island, New York, USA), 1.0 µM of forward and reverse primers [27] and 50 nmol total DNA template. Positive control standards were produced using known positive skin samples following the method outlined in Glenney et al. (2016) [27]. Viral loads (copies per mg) were then calculated using resulting reaction copy number following qPCR and original tissue weights (mg) prior to digestion.

Statistical analysis

Statistical analyses were performed in order to evaluate the relationships between the number of positive samples or the viral DNA load with respect to organ, days post viral exposure, and external vs. internal tissue groups. These comparison analyses were generated using a generalized linear mixed model in SAS software, Version 9.4 of the SAS System (Copyright © 2017 SAS Institute Inc.). For viral loads, analyses were performed on log-

transformed copies per mg tissue in order to increase normality of distribution. Statistical significance was determined based on a probability level of 1% or 5% as indicated below.

Design and preparation of ISH probes

An EEDV specific oligonucleotide probe was designed following a previously described algorithm [28], using the computer program Oligo 6 and based on the glycoprotein gene sequence published in GenBank (JX886027.1). This oligonucleotide probe (5'-GCT CAA TTT ATC GTG CTC AAA TGG TTC ACT GGC CAG CTC CAT GTC CAT CG-3') is labeled with digoxigenin at the 5' end (IDT). This specific probe was developed to differentiate EEDV from the other four salmonid herpesviruses, and use of the Basic Local Alignment Search Tool (www.ncbi.nlm.nih.gov/blast.cgi) demonstrated no cross-reactivity with *Salmonid herpesvirus 1*, *2*, *4*, or *5*. The probe was purified by high performance liquid chromatography (HPLC) (IDT).

Performance of ISH on fixed tissue sections

In order to maximize the sensitivity and specificity of this ISH assay, preliminary tests were performed in order to identify the optimal protocol and reagent concentrations as previously described [29]. Briefly, 5 μm thick sections were cut from paraffin-embedded tissues previously collected and placed onto positively charged slides, which were then deparaffinized and fixed using the Discovery XT automated slide-processing system (Ventana Medical Systems, Inc., Tucson, Arizona) as programed in the protocol for the RiboMap *in situ* hybridization reagent system (Ventana Medical Systems). Protease 3 (0.02 units ml^{-1} alkaline protease; Ventana Medical Systems) was used for 12 minutes at 37°C for a proteolytic treatment followed by a mild cell conditioning step using the citrate buffer-based RiboCC reagent

(Ventana Medical Systems) for 4 minutes at 95°C. The slides were then denatured for 4 minutes at 37°C, followed by hybridization for 1 hour at 37°C with the antisense oligonucleotide probe for EEDV suspended in hybridization buffer (RiboHybe; Ventana Medical Systems). The concentration used for the EEDV probe was 1.59 ng ml^{-1} (1:10,000 dilution). Four stringency washing steps were performed at 42°C using 0.1× RiboWash (equivalent to 0.1× saline sodium citrate; Ventana Medical Systems) for 4 minutes for the first three and for 8 minutes for the fourth washing step. After the stringency washes, the slides were incubated with a rabbit monoclonal antidigoxigenin antibody (Invitrogen Corporation, Frederick, MD) at a dilution of 1:10,000 for 32 minutes at 37°C. Slides were then incubated in streptavidin-alkaline phosphatase conjugate (UMap anti-Rb AP; Ventana Medical Systems) for 16 minutes at 37°C and the signal was detected automatically using the BlueMap nitroblue tetrazolium-BCIP (5-bromo-4-chloro-3-indolyl phosphate) substrate kit (Ventana Medical Systems) for 2 hours at 37°C. The final step involved conterstaining the slides with nuclear fast red-equivalent reagent Red Counterstain II (Ventana Medical Systems) for 4 minutes before adding a coverslip. Skin and gill tissues collected from naïve lake trout raised in a bio-secure containment facility were used as negative controls while experimentally infected lake trout with qPCR confirmed EEDV-positive tissues were used as positive controls.

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Conflicts of interest

The authors declare that there are no conflicts of interest.

Ethical statement

Fish were housed and all experiments carried out under the oversight of the Institutional Animal Care and Use Committee (IACUC) of Michigan State University (XXXXX).

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Tables

Table 1. EEDV glycoprotein gene copies per mg tissue by day post-infection following experimental exposure of lake trout to the virus via immersion bath as calculated using SYBR qPCR. Data points marked with a "--" indicate no virus detected. ‡All tissues from all fish tested prior to day 18 showed no detectible levels of EEDV except the eye of a single fish on day 9 (3.81x104 copies/mg).

Day‡	Fish	Brain	Eye	Fin	Gill	Heart	Intestine	Kidney	Liver	Skin	Spleen
18	1	--	--	$6.59x10^{3}$	--	--	--	--	--	--	--
	2	--	--	$8.56x10^{3}$	--	--	--	--	--	--	--
	3	--	$1.37x10^{3}$	--	--	--	--	--	--	--	--
	4	--	--	--	--	--	--	--	--	--	--
	5	--	--	--	--	--	--	--	--	$2.93x10^{5}$	--
	6	--	--	--	--	--	--	--	--	$3.45x10^{4}$	--
21	1	$4.25x10^{2}$	$2.50x10^{4}$	$2.65x10^{7}$	$3.32x10^{4}$	--	$4.81x10^{4}$	$1.82x10^{4}$	$3.25x10^{5}$	$2.23x10^{7}$	$7.48x10^{4}$
	2	$5.37x10^{4}$	$1.41x10^{6}$	$6.95x10^{7}$	$1.27x10^{5}$	--	$1.89x10^{3}$	--	$4.26x10^{3}$	$1.23x10^{8}$	--
	3	--	$2.69x10^{6}$	--	--	--	$3.94x10^{5}$	$1.83x10^{4}$	$3.43x10^{4}$	$1.54x10^{5}$	$1.51x10^{5}$
	4	--	$2.98x10^{6}$	$5.27x10^{5}$	$1.67x10^{4}$	$1.07x10^{4}$	--	--	--	$1.87x10^{3}$	--
	5	--	$2.32x10^{7}$	$1.29x10^{7}$	$2.80x10^{5}$	--	--	--	--	--	--
	6	--	--	$1.79x10^{4}$	$2.52x10^{5}$	$1.33x10^{5}$	--	$1.30x10^{4}$	$2.46x10^{3}$	$8.07x10^{4}$	--
28	1	$1.44x10^{5}$	$4.11x10^{7}$	$9.27x10^{7}$	$1.01x10^{7}$	$1.39x10^{6}$	$1.33x10^{7}$	$9.26x10^{5}$	$1.57x10^{5}$	$1.29x10^{8}$	$1.02x10^{6}$
	2	$2.70x10^{5}$	$8.09x10^{8}$	$1.82x10^{9}$	$1.65x10^{7}$	$9.99x10^{5}$	$6.88x10^{6}$	$4.67x10^{6}$	$1.68x10^{6}$	$1.64x10^{8}$	$6.68x10^{5}$
	3	$1.98x10^{5}$	$1.14x10^{8}$	$5.70x10^{8}$	$1.29x10^{6}$	$1.46x10^{7}$	$1.78x10^{6}$	$2.83x10^{6}$	$3.45x10^{6}$	$4.96x10^{8}$	$4.18x10^{6}$
	4	$1.43x10^{4}$	$1.62x10^{8}$	$4.30x10^{8}$	$2.12x10^{6}$	$8.61x10^{7}$	$1.51x10^{5}$	$1.32x10^{5}$	$3.58x10^{4}$	$1.40x10^{8}$	$9.01x10^{4}$
	5	$9.62x10^{4}$	$2.20x10^{6}$	$9.56x10^{7}$	$3.03x10^{6}$	$1.15x10^{5}$	$1.38x10^{5}$	$5.88x10^{5}$	$5.21x10^{5}$	$3.91x10^{8}$	$3.01x10^{6}$
	6	$6.48x10^{4}$	$1.08x10^{8}$	$1.83x10^{9}$	$1.62x10^{7}$	$1.91x10^{6}$	$5.26x10^{6}$	$2.13x10^{6}$	$4.62x10^{6}$	$2.07x10^{9}$	$3.42x10^{7}$
35	1	--	$1.78x10^{8}$	$8.97x10^{8}$	$2.63x10^{6}$	$1.25x10^{6}$	$8.50x10^{5}$	$3.80x10^{5}$	$2.49x10^{5}$	$4.74x10^{8}$	--
	2	$7.97x10^{6}$	$1.72x10^{8}$	$7.58x10^{8}$	$1.67x10^{4}$	$6.08x10^{4}$	$2.22x10^{5}$	$2.30x10^{5}$	$3.86x10^{4}$	$5.57x10^{8}$	$8.85x10^{4}$
	3	$3.20x10^{5}$	$3.82x10^{6}$	$4.06x10^{8}$	$2.91x10^{4}$	--	$2.96x10^{4}$	$1.35x10^{4}$	--	$8.53x10^{7}$	$1.85x10^{4}$
	4	$1.21x10^{5}$	$9.76x10^{7}$	$5.27x10^{6}$	$2.00x10^{4}$	$3.42x10^{3}$	$9.84x10^{4}$	$1.91x10^{4}$	$1.83x10^{4}$	$6.00x10^{8}$	--
	5	$3.43x10^{5}$	$1.01x10^{8}$	$6.45x10^{7}$	$2.57x10^{4}$	--	$1.69x10^{5}$	--	$6.95x10^{4}$	$1.68x10^{9}$	--
	6	$4.74x10^{3}$	$1.81x10^{8}$	$9.33x10^{8}$	$4.22x10^{6}$	$2.11x10^{5}$	$7.91x10^{5}$	$1.10x10^{5}$	$7.18x10^{3}$	$5.97x10^{6}$	$6.32x10^{4}$
42	1	$1.34x10^{6}$	$2.03x10^{8}$	$5.82x10^{8}$	$9.68x10^{7}$	--	$1.70x10^{4}$	$3.30x10^{5}$	$2.36x10^{4}$	$3.13x10^{8}$	$2.09x10^{4}$
	2	$2.36x10^{6}$	$8.61x10^{7}$	$6.53x10^{7}$	$1.95x10^{4}$	--	--	--	$5.20x10^{4}$	$3.91x10^{7}$	$5.06x10^{5}$
	3	$6.29x10^{4}$	$1.75x10^{8}$	$3.54x10^{8}$	$1.92x10^{9}$	$2.39x10^{6}$	$9.26x10^{5}$	$1.22x10^{5}$	$1.05x10^{6}$	$1.75x10^{9}$	$1.56x10^{5}$
	4	$9.90x10^{3}$	$1.49x10^{8}$	$3.45x10^{6}$	$9.50x10^{4}$	--	$5.03x10^{4}$	--	$1.57x10^{5}$	$3.29x10^{7}$	$2.33x10^{6}$
	5	--	$1.19x10^{5}$	$2.93x10^{4}$	$5.74x10^{4}$	--	--	--	--	$4.87x10^{7}$	$1.79x10^{7}$
	6	$2.05x10^{6}$	$4.13x10^{7}$	$3.09x10^{4}$	$1.15x10^{4}$	--	--	--	--	$1.25x10^{7}$	--

Figures

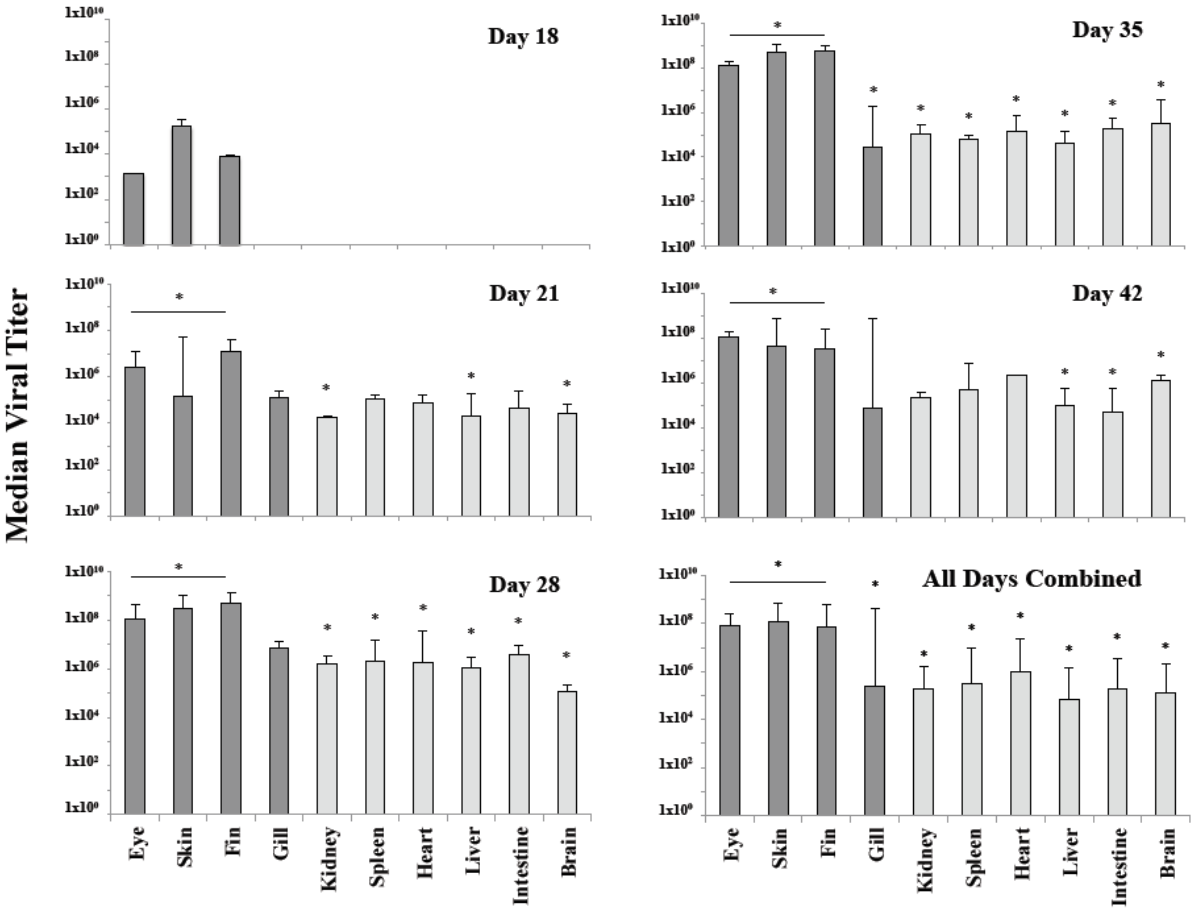

Figure 1. Median EEDV viral titer by tissue type and sampling day. "*" indicates statistical significance compared to tissues below the horizontal bar; Day 18: no statistical difference; Day 21: $p < 0.01$ for brain vs. eye and fin, liver vs. eye and fin and kidney vs. fin; $p < 0.05$ for all remaining combinations. Day 28: $p < 0.01$ for all pictured comparisons with the exception of eye vs. heart ($p < 0.05$); Additional significant pairings include brain vs. heart, intestine and spleen ($p < 0.05$). Day 35: $p < 0.05$. Day 42: $p < 0.01$ for all skin and eye comparisons, $p < 0.05$ for all fin comparisons. Error bars signify one standard deviation.

Figure 2

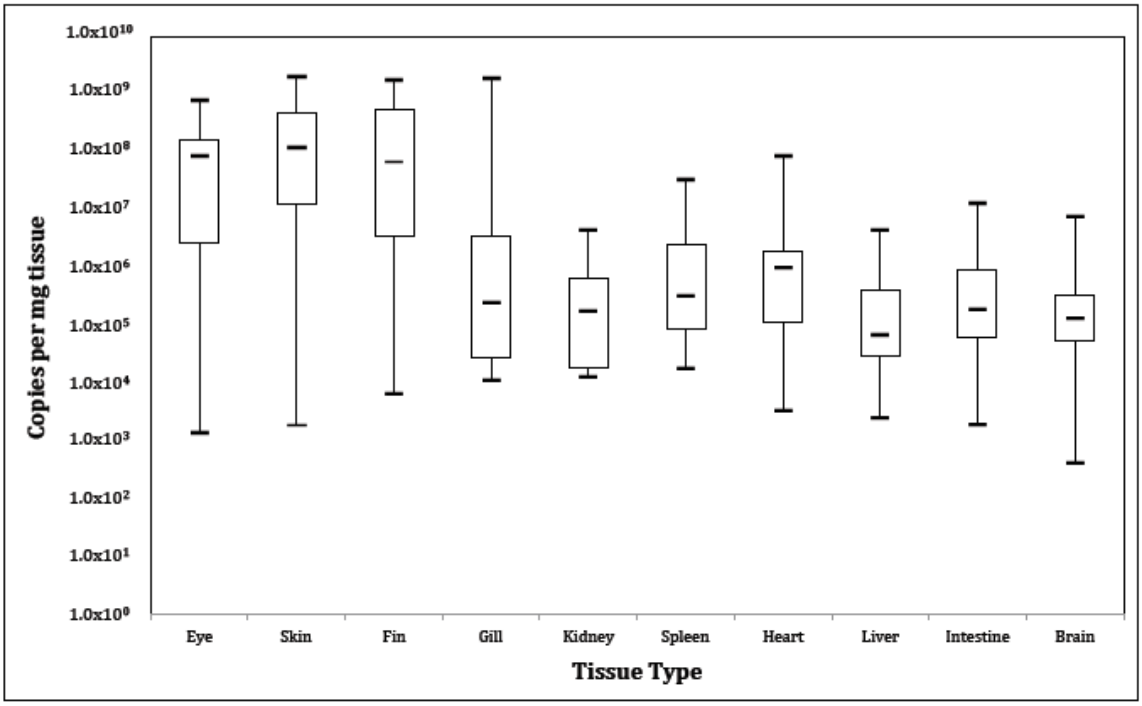

Figure 2. Box plots showing minimum, 1st quartile, median, 3rd quartile and maximum viral glycoprotein gene copies per mg of tissue by tissue type across all EEDV-positive samples on all sampling days.

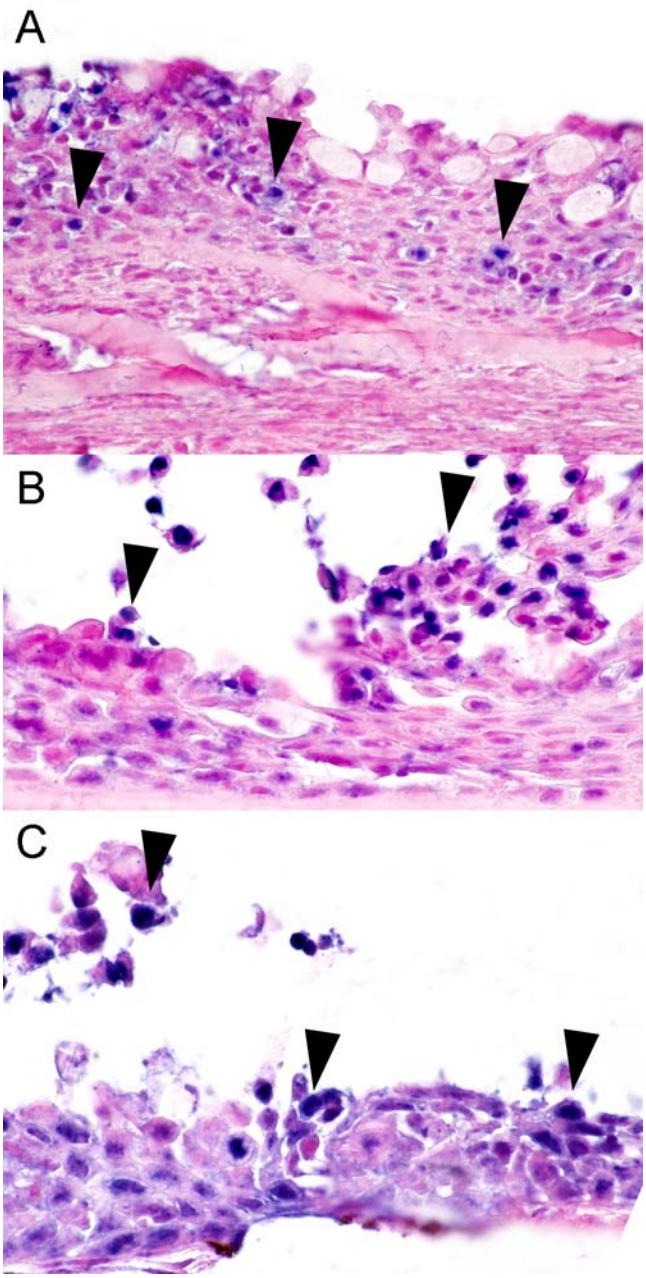

Figure 3. Distribution of EEDV within skin lesions of lake trout during early and late stages of experimental infection. Magnification 400X, In-situ hybridization for EEDV with NBT labeling (blue) and nuclear fast red counterstaining. In early skin lesions viral nucleic acid is detected in individual necrotic epithelial cells (A: arrowhead). At advanced stages (B), viral nucleic acid is readily detected in large numbers of degenerate and necrotic cells that commonly slough off (B: arrowheads). The most severe lesions (C) are characterized by viral nucleic acid detected in the vast majority of epithelial cells throughout all layers (C: arrowheads) prior to epithelial loss.

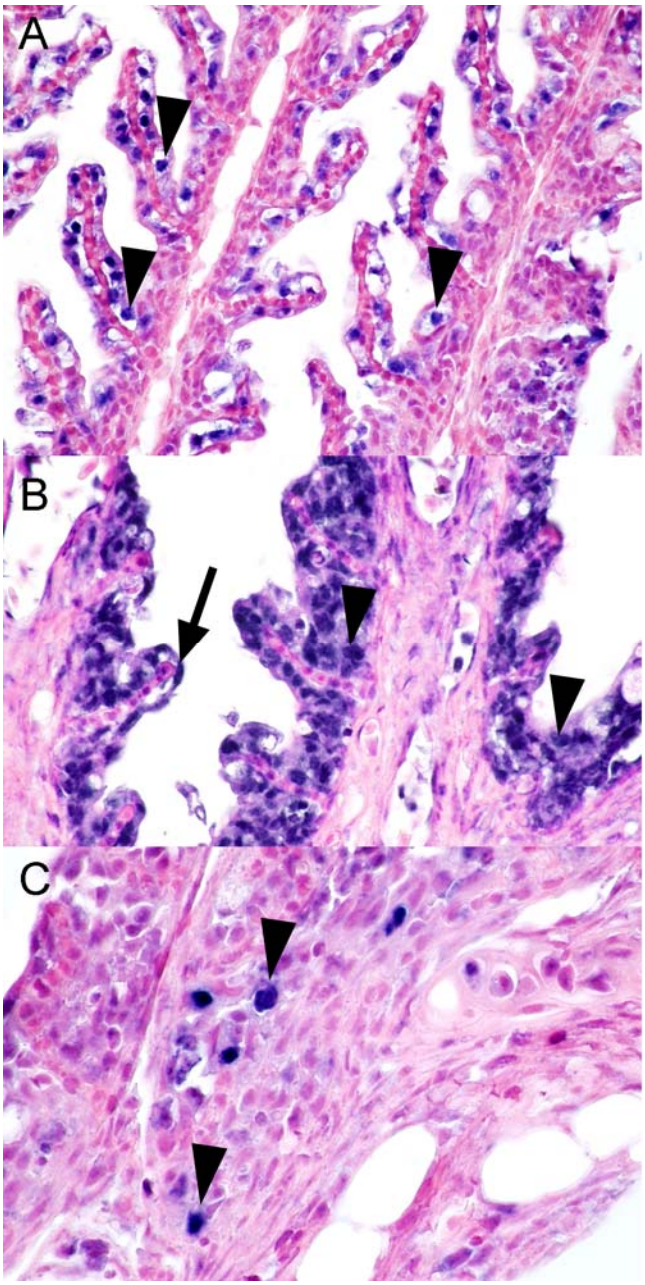

Figure 4. Distribution of EEDV within gill lesions of lake trout during early and late stages of experimental infection. Magnification 400X, In-situ hybridization for EEDV with NBT labeling (blue) and nuclear fast red counterstaining. In early gill lesions, viral nucleic acid is detected in nuclei of morphologically unremarkable epithelial cells (A: arrowheads). More advanced gill disease (B, C) expose viral nucleic acid in attenuated epithelial cells (B: arrow) and nuclei of infiltrating mononuclear cells (B and C: arrowheads).

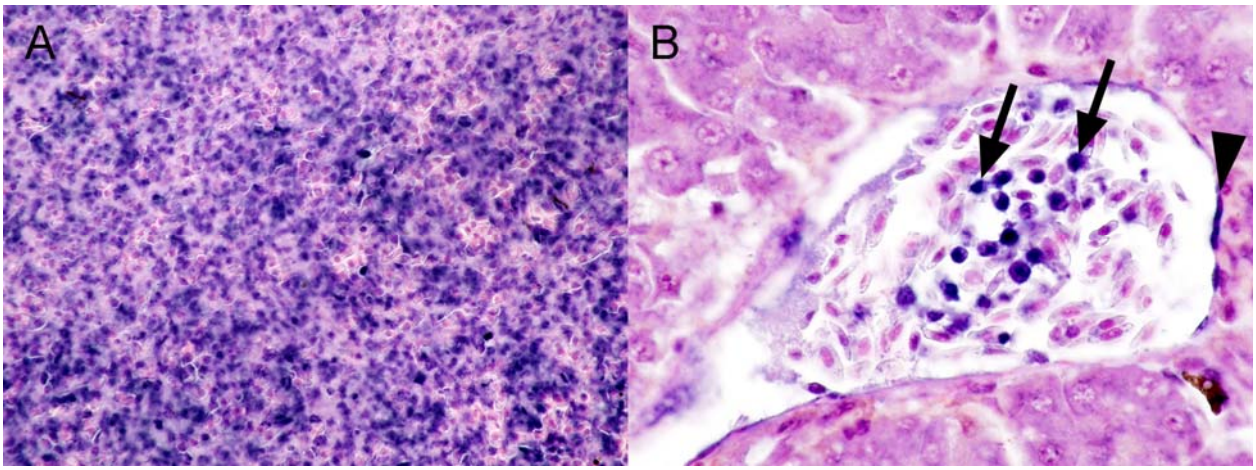

Figure 5. Distribution of EEDV within lesions of internal organs in lake trout during late stages of experimental infection. Magnification 400X, In-situ hybridization for EEDV with NBT labeling (blue) and nuclear fast red counterstaining. Viral nucleic acid can be detected in large numbers of mononuclear cells in the spleen (A). A severe lymphhistiocytic perivasculitis can be found in multiple organs. This perivasculitis is most likely secondary to viral infection of endothelial cells (B: arrowhead). Viremia is caused by large numbers of monocytes being infected as evidenced by significant nuclear labeling of mononuclear cells (B: arrows) in this hepatic vessel within.