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Sturgeon nucleo-cytoplasmic large DNA virus phylogeny and PCR tests

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ABSTRACT: Sturgeon epitheliotropic nucleo-cytoplasmic large DNA viruses (NCLDVs) can cause a lethal disease of the integumentary system. These viruses have not been assigned to any currently recognized family or genus. In this study, phylogenetic analyses using the major capsid protein (MCP) showed that the sturgeon NCLDVs formed a cohesive taxonomic group, could be identified to the species or possibly sub-species level and formed a distinct evolutionary lineage within the *Megavirales*. The genetic relatedness of the sturgeon virus MCP allowed design of 3 PCR diagnostic tests with analytical specificity (ASp) inclusive of this group of viruses. The conventional PCR test, C1, had broader ASp than the 2 quantitative PCR tests, Q1 and Q2, and was inclusive of the sturgeon viruses as well as some viruses belonging to the families *Mimi-*, *Phycodna-*, or *Iridoviridae*. Q2 had broader specificity than Q1 but both tests recognized the sturgeon NCLDVs and did not cross-react with co-localizing sturgeon herpesviruses. Analytical test performance characteristics evaluated for Q1 and Q2 revealed sensitive assays with observed 50% limits of detection between 3 and 6.25 plasmid copies and high intra- and inter-assay repeatability. Q1 was used to test for sturgeon viruses in endangered populations of lake sturgeon *Acipenser fulvescens* within the Winnipeg River or Nelson River drainage systems of Manitoba, Canada. Test results indicated that namao virus is endemic in the Nelson River water basin. These tests meet the analytical requirements for diagnostic testing in Canada and are useful tools for disease management in sturgeon conservation stocking programs in North America.

KEY WORDS: Acipenseridae · Nucleo-cytoplasmic large DNA viruses · NCLDVs · Quantitative PCR · Namao virus · *Mimiviridae* · Endangered species

INTRODUCTION

Epitheliotropic nucleo-cytoplasmic large DNA viruses (NCLDVs) can cause a lethal disease of the integumentary system in infected sturgeon (Table 1). Viruses in the group include white sturgeon iridovirus (WSIV) (Hedrick et al. 1990, Raverty et al. 2003), Missouri River sturgeon iridovirus (MRSIV) (Kurobe et al. 2010, 2011), shortnose sturgeon virus (SNSV) (LaPatra et al. 2014), namao virus (NV) (Clouthier et al. 2013), and Russian sturgeon iridovirus (Adkison et al. 1998). They infect epithelial cells (Elliot 2011) of the integumentary system of sturgeon skin and fins, gills, and nasopharynx and olfactory organs (Hedrick et al. 1990, Watson et al. 1998a, Drennan et al. 2007, Kurobe et al. 2011). Damage to epithelial cells of the chemosensory organs leads to a lethal wasting syndrome in infected sturgeon (Watson et al. 1998a).

The viruses belong to an unassigned genus and family (Clouthier et al. 2013) in the proposed order *Megavirales* (Colson et al. 2013), a term we will use here although it has not formally been adopted by the International Committee for the Taxonomy of

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Viruses. The sturgeon NCLDVs are present in hatchery-reared and wild sturgeon found across North America and northern Europe. Outbreaks of virus disease in the hatchery are associated with stress factors such as rearing density, handling, and fluctuations in water temperature, levels, and flow rates (LaPatra et al. 1994, 1996, Watson et al. 1998b, Georgiadis et al. 2000, 2001, Drennan et al. 2005, 2006). The host range of the viruses includes fish in the genera *Acipenser* or *Scaphirhynchus* of the family

Table 1. *Megavirales* included in this study and analytical specificity of the conventional PCR (C1) and qPCR (Q1 and Q2) tests. NCLDV: nucleo-cytoplasmic large DNA virus. +/–: weak positive; blank cells: not tested

Virus	Detection by test method			GenBank accession no.	
	C1	Q2	Q1	DNA	Protein
Sturgeon NCLDVs					
Namao virus (NV)	+	+	+	JX155659	AGH17555
Missouri River sturgeon iridovirus (MRSIV)	+	+	+	JX155661	AGH17557
British Columbia white sturgeon virus (BCWSV)	+	+	+	JX155660	AGH17556
Shortnose sturgeon virus (SNSV)	+	+	+	KM606973	
White sturgeon iridovirus (WSIV)	+	+	–	DQ897645	ABK34555.1
Mimiviridae					
Giant mimiviruses					
Clade I - Group A					
Acanthamoeba polyphaga mimivirus (APMV-1)	–	–	–	NC014649	YP003986929
Acanthamoeba polyphaga mimivirus (APMV-2)	–	–	–	JN036606	AEJ34665
Acanthamoeba castellanii mamavirus (ACMV)	–	–	–	JF801956	AEQ60625
Clade I - Group B					
Acanthamoeba polyphaga moumouvirus (APMM)				NC020104	YP007354349
Clade I - Group C					
Megavirus chiliensis (MVChile)	+	–	–	NC016072	YP004894515
Megavirus courdo (MVCour)	–	–	–	JN885991	AEX61606
Clade II					
Cafeteria roenbergensis virus (CroV)	+	–	–	NC014637	YP003969975
Small mimiviruses					
Organic lake phycodnavirus 1 (OLPV–1)				HQ704802	ADX05938
Organic lake phycodnavirus 2 (OLPV–2)	+/–	–	–	HQ704803	ADX06358
Phaeocystis pouchetii virus (PPV)	–			EU006631	ABU23715
Chrysochromulina ercina virus (CEV)	+	–	–	EU006628	A7U6E7
Pyramimonas orientalis virus (POV)	–			EU006630	A7U6EP
Phaeocystis globosa virus (PGV)				HQ634147	AET73005
Phycodnaviridae					
Heterosigma akashiwo virus (HAV)	+	–	–	AB198422	BAE06835
Acanthocystis turfacea chlorella virus (ATCV)	–			NC008724	YP001426761
Paramecium bursaria chlorella virus (PBCV)	–			NC009898	YP001497813
Micromonas pusilla virus (MPV)	–			HQ633072	AET43572
Ostreococcus tauri virus (OTV)	+			NC013288	YP003495004
Feldmannia species virus (FSV)	–			NC011183	YP002154681
Ectocarpus siliculosus virus (ESV)	–		–	FN648730	CBN80416
Iridoviridae					
Chilo iridescent virus (CIV)	+	–	–	NC003038	NP149737
Frog virus 3 (FV3)	–	–	–	FJ459783	ACP19256
Invertebrate iridescent virus 3 (IIV3)	–	–	–	NC008187	YP654586
Infectious spleen & kidney virus (ISKNV)	–	–	–	AF371960	AAL98730
Lymphocystis disease virus (LDV)	–	–	–	EF103188	ABL07488
Marseilleviridae					
Marseillevirus (MV)	–			NC013756	YP003407071
Ascoviridae					
Diadromus pulchellus ascovirus 4a (DPAV)	–			AJ312705	CAC84483
Asfarviridae					
African swine fever virus (ASFV)	–			XM369245.2	XP369245.2

Acipenseridae: white sturgeon *A. transmontanus* (Hedrick et al. 1990), lake sturgeon *A. fulvescens* (Clouthier et al. 2013), pallid sturgeon *S. albus* and shovelnose sturgeon *S. platorynchus* (Kurobe et al. 2011), shortnose sturgeon *A. breviro* (LaPatra et al. 2014), and Russian sturgeon *A. gueldenstaedtii* (Adkison et al. 1998).

Diagnosis of sturgeon NCLDVs can be made by histological observation of pathognomonic cellular changes and visualization of virus particles by electron microscopy. Microscopic signs of infection are hypertrophied, basophilic, or amphophilic- to eosinophilic-staining epithelial cells with eccentric nuclei and cytoplasmic inclusion bodies containing isometric, doubly encapsidated virus particles (242 to 262 nm) with a condensed bar-shaped nucleoid (Hedrick et al. 1990, 1992, Adkison et al. 1998, Kurobe et al. 2011, Clouthier et al. 2013). Some, but not all, of these viruses grow in cell culture (Hedrick et al. 1991, Watson et al. 1998a, Kurobe et al. 2011).

The analytical specificity of current PCR-based tests for detection of the sturgeon viruses is isolate-specific. For example, MRSIV PCR-based tests recognize MRSIV but not the other viruses (Kurobe et al. 2010, Clouthier et al. 2013), and WSIV PCR-based tests do not recognize all sub-types of WSIV (Drennan et al. 2007). Recognition that the epitheliotropic sturgeon NCLDVs are widespread in different sturgeon species found across North America motivated our design and analytical validation of a group-specific quantitative PCR (qPCR) test for their detection.

MATERIALS AND METHODS

Viruses and plasmids

The viruses used in this study, their abbreviations, and GenBank accession numbers for the major capsid protein (MCP) sequences are shown in Table 1. Both genomic viral DNA (Clouthier et al. 2013) and synthetic viral DNA were prepared. Fin tissue samples from shortnose sturgeon were provided by a commercial grower in Atlantic Canada.

WSIV and type 2 acipenserid herpesviruses (AciHV-2) were grown in cell culture on white sturgeon skin (WSSK-1) or white sturgeon spleen (WSS-2) cell lines (Hedrick et al. 1991). Each cell line was propagated at 16°C in minimum essential medium with Hanks salts (MEM-H) supplemented with 10% fetal bovine serum (FBS) and 2 mM L-glutamine (Life Technologies). Cell monolayers were inoculated with a suspension of virus, and after an absorption period of 1 h, MEM-H supplemented with 2% FBS (MEM-H2), 2 mM L-glutamine, and 1× antibiotic/antimycotic (Life Technologies) was added to each flask, the flasks were incubated at 16°C, and the monolayers were observed daily for evidence of cytopathic effect (CPE). At the time of harvest, each inoculated monolayer was scraped from the bottom of the flask into the culture fluid and the cell suspension was transferred to a storage vial and placed at −80°C.

Genomic viral DNA was obtained from virus-infected tissue. This was used to infer partial MCP nucleotide and protein sequence of NV, MRSIV, BCWSV, and SNSV as described by Clouthier et al. (2013). This information was used to design synthetic DNA encoding the sturgeon virus MCP which was then inserted into cloning vector pMA-RQ (Life Technologies) or pJ204 (DNA2.0). These constructs were named pNVmcp, pWSIVmcp, pMRSIVmcp, pBCWSVmcp, or pSNSVmcp.

Plasmids containing synthetic viral MCP DNA (DNA2.0) were made for 25 of the 33 viruses included in Table 1. Each synthetic construct consisted of the target sequence for the conventional (C1) or qPCR tests (Q1 or Q2; Fig. 1) inserted in cloning vector pJ204 (DNA2.0). Three constructs were made for 14 of the viruses. For example, the virus APMV-1 was used to create plasmids pAPMV-1 C1, pAPMV-1 Q1, and pAPMV-1 Q2. The abbreviations C1, Q1, or Q2 refer to the test for which they were designed.

Artificial positive control (APC) plasmids were designed for the Q1 and Q2 tests using the strategy outlined by Snow et al. (2009) (Fig. 2). For these constructs, the common vector backbone was pJ204 (DNA2.0) into which a segment of synthetic NV MCP DNA was inserted to create plasmids pNV-APC Q1 and pNV-APC Q2 (DNA2.0). Both of these contain a non-viral segment of DNA complementary to the pAPC probe (Fig. 2).

Plasmids were transformed into *Escherichia coli* K12 or DH5α (Life Technologies), and DNA was purified from the host using a QIAprep Spin Miniprep Kit (Qiagen).

Primer/probe design

Sequence alignments of full or partial MCP nucleotide or protein sequence for NV, WSIV, BCWSV, MRSIV, and SNSV were performed using T-Coffee (Notredame et al. 2000, Di Tommaso et al. 2011) (Fig. 1). Q1 and Q2 primers and hydrolysis probes were identified using Beacon Designer and Allele ID software (Premier Biosoft International).

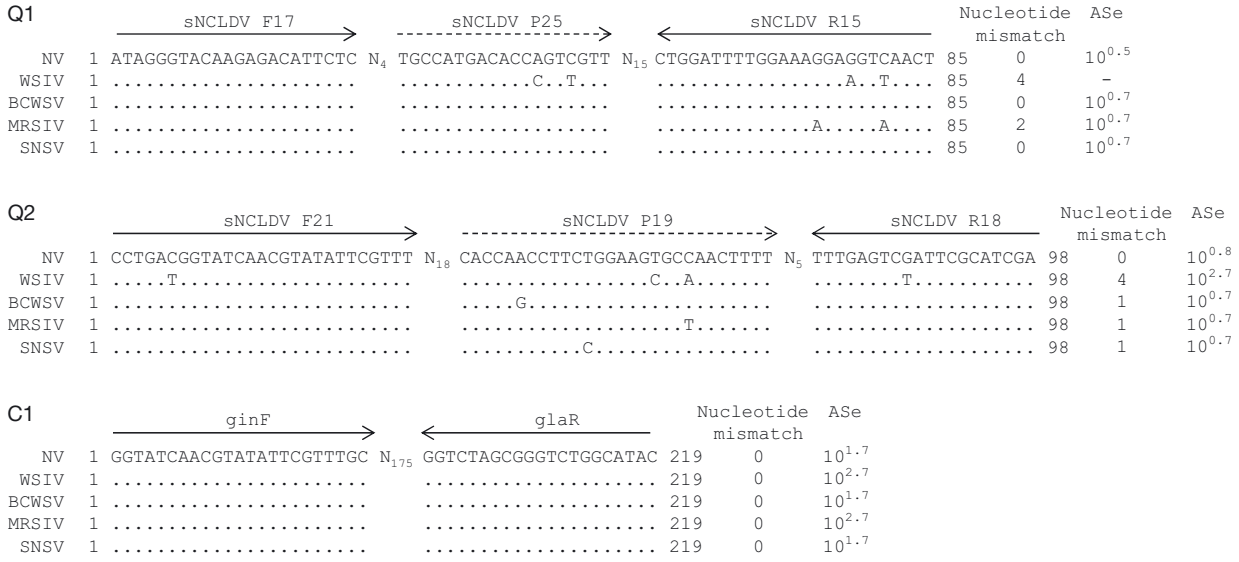


Fig. 1. qPCR (Q1 and Q2) and conventional PCR (C1) tests for the major capsid protein gene of sturgeon viruses. Namao virus (NV) is the referent sequence for nucleotide mismatches in primer/probe binding regions of Q1 (85 nucleotides), Q2 (98 nucleotides), and C1 amplicons (219 nucleotides). The symbols for primers (solid arrows) and probes (dashed arrows) indicate polarity (5′ to 3′). The sequence for sNCLDV R15, sNCLDV R18, and glaR are the reverse complement of the primer sequence shown above. The number of intervening nucleotides between primer and probe (Q1, Q2) or primers (C1) is N_x. Analytical sensitivity (ASe) of the tests is given for targets pNVmcp, pWSIVmcp, pBCWSVmcp, pMRSIVmcp, and pSNSVmcp. Virus abbreviations are described in Table 1

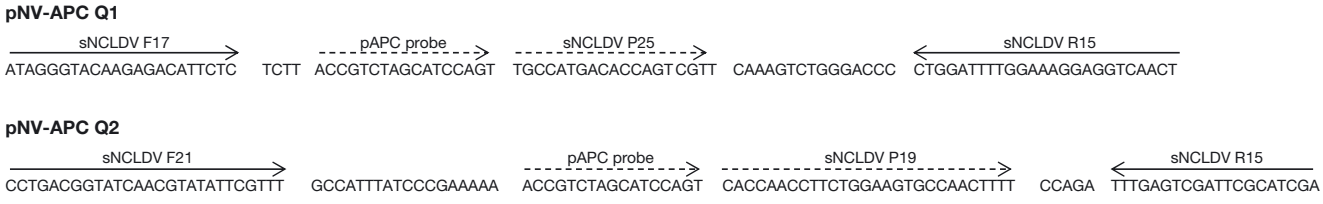


Fig. 2. Artificial positive control (APC) plasmids pNV-APC Q1 and pNV-APC Q2. Namao virus (NV) is the referent for synthetic DNA sequences which also include a non-viral sequence (i.e. pAPC probe). Vector sequence (pJ204) is not shown. The symbols for primers (solid arrows) and probes (dashed arrows) indicate polarity (5′ to 3′)

PCR tests

Conventional PCR test (C1)

The primers for the conventional PCR test C1 were forward primer ginF (5′-GGT ATC AAC GTA TAT TCG TTT GC-3′) and reverse primer glaR (5′-GCA AAC GAA TAT ACG TTG ATA CC-3′) (Sigma-Aldrich; Clouthier et al. 2013). The C1 test involved 25 µl containing 100 nM ginF, 100 nM glaR, 3 mM $MgCl_2$, 100 µM deoxynucleotide triphosphates (dNTPs), 1.25 U AmpliTaq Gold (Applied Biosystems), and 500 to 1500 ng genomic DNA. It was performed using an Applied Biosystems Veriti thermocycler. The thermocycling profile was 1 cycle of 5 min at 95°C; 40 cycles of 30 s at 95°C, 30 s at 54°C, and 30 s at 72°C; followed by 1 cycle of 10 min at 72°C. Amplicons were separated by electrophoresis on 2% agarose gels (Life Technologies) containing 1× fluorescent nucleic acid stain GelRed (Biotium). Data were recorded using the Kodak Gel Logic 200 imaging system.

qPCR tests (Q1 and Q2)

The primers for qPCR test Q1 were forward primer sNCLDV F17 (5′-ATA GGG TAC AAG AGA CAT TCT C-3′), reverse primer sNCLDV R15 (5′-AGT TGA CCT CCT TTC CAA AAT CCA G-3′) (Sigma-Aldrich), Q1 assay probe sNCLDV P25 (5′-6FAM-TGC CAT GAC ACC AGT CGT T-MGBNFQ-3′), and positive control pAPC probe (5′-VIC-ACC GTC TAG CAT CCA GT-MGBNFQ-3′) (Life Technologies). The

Q1 test involved 25 µl containing 1000 nM sNCLDV F17, 800 nM sNCLDV R15, 250 nM sNCLDV P25, 250 nM pAPC probe, 1× Taqman Universal PCR Master Mix (Applied Biosystems), and 1500 ng genomic DNA.

The primers for qPCR test Q2 were forward primer sNCLDV F21 (5′-CCT GAC GGT ATC AAC GTA TAT TCG TTT-3′), reverse primer sNCLDV R18 (5′-TCG ATG CGA ATC GAC TCA AA-3′) (Sigma-Aldrich), Q2 assay probe sNCLDV P19 (5′-6FAM-CAC CAA CCT TCT GGA AGT GCC AAC TTT T-TAMRA-3′), and positive control pAPC probe (5′-VIC-ACC GTC TAG CAT CCA GT-TAMRA-3′) (Life Technologies). For the Q2 test, we used 25 µl containing 400 nM sNCLDV F21, 800 nM sNCLDV R18, 200 nM sNCLDV P19, 250 nM pAPC probe, 1× Taqman Universal PCR Master Mix (Applied Biosystems), and 1500 ng genomic DNA.

The sample set for each test run included control samples of a known composition. This control material was 50 mg lake sturgeon tissue (N1), 50 mg lake sturgeon tissue + $10^{7.8}$ pNV-APC Q1 or Q2 (P1), pNV-APC Q1 or Q2 diluted from $10^{7.7}$ to 5 copies per reaction (P2), and water (N2). The N1 and P1 samples were included at the nucleic acid extraction step, whereas N2 and P2 were added at the qPCR step of the Q1 or Q2 tests.

Q1 and Q2 tests were run using the Stratagene Mx3000P system in a 96-well format. The thermocycling profile was 1 cycle of 2 min at 50°C, 1 cycle of 10 min at 95°C; followed by 40 cycles of 15 s at 95°C and 60 s at 60°C. Tests were run with 3 to 5 replicates of each sample. Data were analyzed using MxPro software (Stratagene) using the adaptive baseline and amplification-based threshold algorithm enhancements. The PCR cycles used for quantification in Q1 and Q2 will be referred to as the quantification cycle (Cq) (Bustin et al. 2009).

The copy number of pNV-APC Q1 or Q2 was calibrated using the standard curve method provided in the MxPro software and was used to infer the copy number equivalent in test samples. These constructs are 2812 and 2853 bp in length, respectively, corresponding to 1.83×10^6 and 1.85×10^6 g mol^{-1}. The copy number per µg DNA was calculated as 3.29×10^{11} and 3.26×10^{11}, respectively.

Conventional and qPCR assays for MRSIV serpin gene or WSIV MCP gene

The MRSIV serpin conventional or qPCR assays were performed as described by Kurobe et al. (2010). The WSIV PCR assays were performed as outlined by Kwak (2006) and Kwak et al. (2006).

Primer/probe screening and optimization

qPCR primer pair combinations (N = 69) were analyzed with SYBR Green (Life Technologies) using 300 nM of each primer. Templates for the qPCR tests were DNA extracted from NV-infected lake sturgeon (1 µg) or plasmid DNA encoding a portion of the MCP gene from WSIV ($10^{6.7}$ copies). Amplicons were evaluated using dissociation curves and gel electrophoresis. Primer pair combinations that failed to amplify both NV and WSIV DNA templates were removed from the study.

Hydrolysis probe assays were performed using NV-infected lake sturgeon DNA or WSIV MCP plasmid DNA. The primer and probe combinations were 400 nM (equimolar) and 80 nM, respectively. Primer/probe combinations that produced a detectable Cq value with the NV template alone or with both templates were selected for further analysis.

Tests were optimized by selecting for their ability to detect NV, WSIV, BCWSV, and MRSIV. The template was NV-infected lake sturgeon tissue or plasmid DNA encoding the full or partial MCP DNA sequence (i.e. pNVmcp, pWSIVmcp, pMRSIVmcp, pBCWSVmcp, or pSNSVmcp). Tests were selected for their ability to amplify plasmid DNA from $10^{7.7}$ to $10^{0.7}$ copies per reaction with concentrations of primers, in increments of 100 nM, from 100 to 1000 nM. In all cases, the hydrolysis probe concentration was 250 nM. Primer concentrations for each test giving the lowest Cq value were selected for further analysis. Probe concentrations, in increments of 50 nM from 100 to 250 nM, were tested and those giving the highest final fluorescence value in the baseline-corrected, ROX-normalized view (i.e. dRn) were selected for further analysis.

Primer and probe concentrations and cycling conditions for the Q1 test were optimized for detection of NV in infected tissue while those for Q2 were optimized for detection of NV, WSIV, MRSIV, and BCWSV.

The conventional PCR reaction was optimized with the same templates, primer concentrations from 100 to 800 nM, 1 to 3 mM $MgCl_2$, and 0.1 to 0.3 mM dNTPs. Primer concentrations and cycling conditions were selected from those tests giving the highest relative fluorescence of amplified products with all 4 sturgeon NCLDVs.

The effect of tissue type, storage, and quantity of DNA template on C1, Q1, and Q2 was examined with 100, 500, 1000, 1500, 2000, 3000, and 4000 ng of gill, fin, snout, and abdominal skin tissue.

Analytical validation

The analytical sensitivity (ASe) of C1, Q1, and Q2 was evaluated using standard curves generated with (1) plasmid DNA encoding 96% of the MCP gene from NV, MRSIV, BCWSV, WSIV, or SNSV; (2) APC plasmid DNA; or (3) DNA extracted from NV-infected tissue. Standard curves generated with plasmids containing 1 of the 5 sturgeon NCLDVs were used to compare qPCR reaction efficiencies across virus isolates, whereas DNA derived from plasmid versus NV-infected tissue was used to determine whether plasmid DNA could be used as a proxy for absolute quantification of NV in infected tissue. The effect of plasmid DNA conformation (i.e. linear versus circular), host DNA (1500 ng), and/or the APC probe on reaction efficiency and ASe was investigated. Standard curves were constructed from 2-, 5-, or 10-fold serial dilutions, and each dilution was assayed by qPCR in triplicate or in replicates of 5 or 6. Each qPCR assay was performed using reaction conditions described above. The 50 and 100% limit of detection for each assay was expressed as copies of plasmid DNA and was derived from the measured concentrations of the last dilution with at least 50% or 100% detection. The 95% limit of detection was estimated using a logistic regression with the positive/negative status of the sample as the outcome and the concentration ($\log_{10}$) as the predictor. A linear regression was used to predict the Cq value at the 95% probability of detection.

The analytical specificity (ASp) of C1, Q1, and Q2 was tested for exclusivity with synthetic constructs consisting of target sequence from a panel of NCLDVs listed in Table 1. Each construct was tested at $10^{9.7}$, $10^{6.7}$, and $10^{3.7}$ plasmid copies. Exclusivity of the 3 tests was also investigated with AciHV-1 and AciHV-2 isolates from white sturgeon or shortnose sturgeon using DNA (at least 50 ng) extracted from infected tissue or virus amplified in cell culture. Inclusivity of these assays was established using pNVmcp, pWSIVmcp, pMRSIVmcp, pBCWSVmcp, and pSNSVmcp as well as genomic DNA isolated from NV-, MRSIV-, BCWSV-, or SNSV-infected tissue and from WSIV amplified in cell culture. Tests were performed using 3 replicates per sample and the reaction conditions outlined in section 'qPCR tests (Q1 and Q2).' The level of agreement between the observed and expected results was calculated using 2-way tables (Table 2) and expressed as inclusivity or exclusivity (i.e. degree to which the assay detects all intended viruses and does not detect other viruses, respectively). The 95% confidence intervals for these estimates were also calculated for each assay.

Table 2. Determining analytical specificity of the conventional (C1) and qPCR (Q1 and Q2) tests. Inclusivity = A/(A + B); exclusivity = D/(C + D)

		Observed		
		Positive	Negative	Total
Expected	Positive	A	B	A + B
	Negative	C	D	C + D
	Total	A + C	B + D	A + B + C + D

The analytical repeatability of Q1 and Q2 was tested with pNVmcp serially diluted 10-fold, from $10^{7.7}$ to $10^{0.7}$ copies per reaction. The intra- and inter-assay repeatability was determined by analyzing each dilution in replicates of 5 in 5 independent runs. The repeatability of Q1 was also evaluated with positive control samples P1 and P2. Samples from 3 batches of P1 and 8 batches of P2 were assessed in 88 independent runs performed by 1 analyst over a 9 mo period. Q1 and Q2 were performed using reaction conditions outlined in section 'qPCR tests (Q1 and Q2).' The results were evaluated in a scatter plot of the mean of the replicates plotted against the standard deviation. A linear regression was used to determine whether the inter-run or inter-batch variability was statistically significant (i.e. $p < 0.5$). The coefficient of variation (CV) was calculated as SD/mean × 100 using the continuous outcome data (i.e. Cq values). All statistical analyses were performed in StataI/C (12.1).

BLASTP, sequence alignment, and phylogenetic analysis

Sequences displaying similarity to the deduced amino acid sequence of the NV, WSIV, BCWSV, MRSIV, or SNSV MCP were identified from protein databases searched using the BLASTP program (Altschul et al. 1990, 1997; Table 1). Protein sequence alignments were performed using T-Coffee (Notredame et al. 2000, Di Tommaso et al. 2011) with sequences that were trimmed to the first amino acid aligning with the N-terminal end of the NV MCP

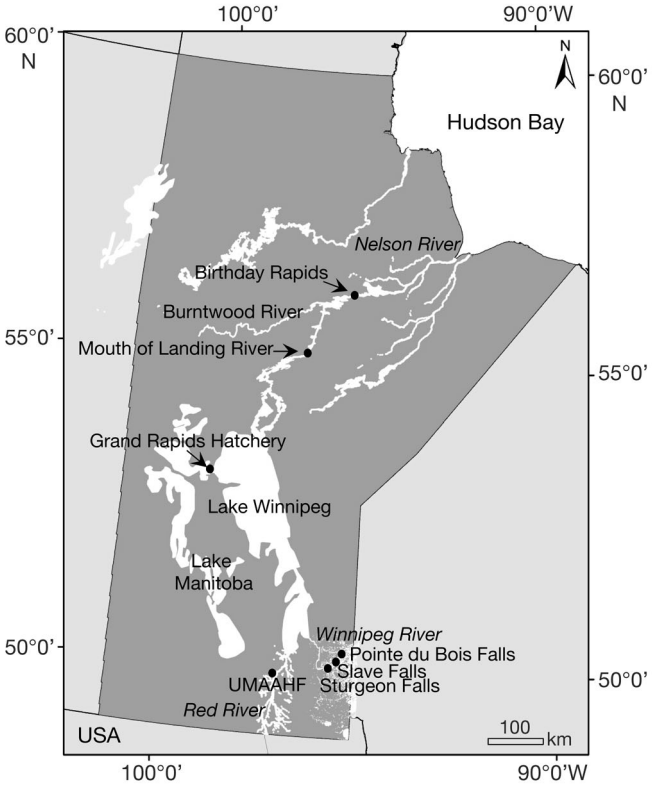


Fig. 3. Study area in Manitoba, Canada. Broodstock collection sites and hatchery rearing facilities for juvenile lake sturgeon *Acipenser fulvescens* are shown

fragment and the last amino acid at the C-terminal end.

Phylogenetic tree reconstructions were estimated by 2 methods: Bayesian inference analysis as implemented by MRBAYES v3.2.1 (Huelsenbeck & Ronquist 2001, Ronquist & Huelsenbeck 2003) and maximum-likelihood as implemented by PHYML 3.69 (Guindon et al. 2010). The Bayesian trees were estimated using multiple independent runs of aamodelpr = mixed and default settings for 1 000 000 generations when the average deviation of the split frequencies was < 0.002. For maximum-likelihood trees, clade confidences were estimated from 1000 bootstrap replicates. The output tree produced from each method was visualized using FigTree v1.3.1 software (http://tree.bio.ed.ac.uk/software/figtree).

Field samples

Samples were collected for histology and/or qPCR from lake sturgeon in the Nelson River and Winnipeg River drainage systems in Manitoba (Fig. 3). Broodstock sample collection sites in the Nelson River drainage were the Burntwood River, the mouth of the Landing River, and Birthday Rapids. Pointe du Bois Falls was the site of broodstock sample collection on the Winnipeg River. Fish were reared at 2 different hatcheries: Grand Rapids Hatchery (GRH; Grand Rapids, MB) or University of Manitoba Aquatic Animal Holding Facility (UMAAHF; Winnipeg, MB). Samples were collected from hatchery-reared fish originating from broodstock captured at Pointe du Bois Falls, Sturgeon Falls, or Slave Falls on the Winnipeg River or all 3 sites in the Nelson River drainage (Fig. 3).

Tissue samples for genomic DNA extraction or histological examination were processed as described by Clouthier et al. (2013). Samples consisted of gametes, whole larvae, and/or pectoral fin clips. Reproductive products were placed in liquid nitrogen, whereas larvae were paired and fin clip samples were separated into 2 parts; the first was placed in RNA*later*® (Life Technologies), and the second was put in Safe-Fix® (Fisher Scientific).

RESULTS

Test development

Phylogenetic analyses of the MCP from sturgeon viruses NV, WSIV, MRSIV, BCWSV, and SNSV show that they form a distinct group separate from other known viruses (Fig. 4; Clouthier et al. 2013). The results indicate that they do not form part of any currently recognized virus genera or family but do belong to the order *Megavirales*.

This information was captured in the intended purpose of the C1, Q1, and Q2 tests, specifically their ASp and ASe. C1 has broader ASp than Q1 and Q2 and is inclusive of the sturgeon viruses as well as some viruses belonging to the families *Mimi-*, *Phycodna-*, or *Iridoviridae* (Table 1). Q2 has broader specificity than Q1, but both tests recognize NV, WSIV, MRSIV, BCWSV, and/or SNSV. Q1 was optimized for detection of NV but also recognizes MRSIV, BCWSV, and SNSV. The WSIV PCR tests targeting the MCP gene and the MRSIV PCR tests targeting the serpin gene did not detect NV, BCWSV, or SNSV.

The qPCR tests Q1 and Q2 target 2 different, nonoverlapping parts of sturgeon virus MCP nucleic acid sequences (Fig. 1). Using the WSIV MCP (DQ897645.1) sequence as the referent, Q1 targets nucleotides 85 to 169, and Q2 targets nucleotides

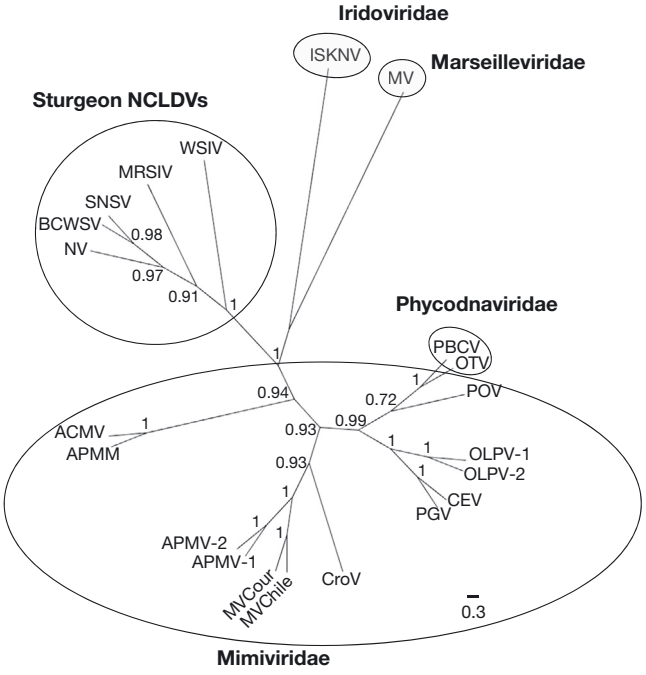


Fig. 4. Bayesian inference of phylogeny for sturgeon viruses using the major capsid protein. Sequence from other nucleocytoplasmic large DNA viruses was included for comparison. Bayesian inference posterior probabilities are given at the nodes. Virus abbreviations and accession numbers are provided in Table 1

1363 to 1460. The conventional PCR test C1 (Clouthier et al. 2013) targets nucleotides 1369 to 1587, which overlap with the target region of Q2 (Fig. 1).

The quantity of template DNA added to each C1 test reaction was 500 to 1500 ng, and for Q1 or Q2 test reactions 1500 ng were used regardless of tissue type. Interference in test performance was observed when more than 2000 ng of DNA was added.

Positive control samples P1 and P2 were used to monitor the efficiency of nucleic acid isolation and qPCR test processes. These samples were also used to monitor for cross-contamination that can occur at either step. To do this, 2 fluorescently labeled probes were present in each qPCR reaction, with each probe recognizing a different target. The FAM-labeled probes P25 and P19 can duplex with viral sequences or artificial plasmid sequences found in pNV-APC Q1 or Q2 (Figs. 1 & 2). The VIC-labeled probe pAPC only recognizes sequences found in pNV-APC Q1 or Q2 (Fig. 2). The pAPC probe was used to detect false positive results arising from cross-contamination of a test sample with P1 or P2. Mixing of the pAPC probe together with P25 or P19 in a single qPCR reaction did not affect Q1 or Q2 test sensitivity. Results of linear regression analysis of Cq produced from dilutions of pNV-APC Q1 or Q2 showed that the presence of the corresponding pAPC probe did not have any effect on Q1 or Q2 test efficiency or sensitivity (data not shown). As linearization of plasmid DNA had a minimal or negative effect on Q1 or Q2 test efficiency, circular plasmid DNA was used throughout the study.

Analytical validation

Q1, Q2, and the internal positive control VIC-based test for these 2 assays had similar reaction and detection efficiencies. The relative slopes of the qPCR standard curves for Q1 or Q2 and the internal APC test varied by less than 0.1 (see Fig. S1 in the Supplement, available at www.int-res.com/articles/suppl/d117p093_supp.pdf). The assays were linear across 8 orders of magnitude with a strong correlation between cycle number and dilution factor (R^2 = 1.0 in both cases; Fig. S1). Amplification efficiencies of Q1 and Q2 qPCR reactions containing plasmid or NV-infected tissue DNA serially diluted 5- or 10-fold ranged from −3.4 to −3.5 (Fig. S2 in the Supplement). The similarity in reaction efficiencies independent of template type showed that plasmid DNA could be used to define the limit of detection for Q1 and Q2.

Plasmids pNVmcp, pNV-APC Q1, and pNV-APC Q2 were used to determine the ASe for Q1, Q2, and C1. For the Q2 test, plasmids pMRSIVmcp, pBCWSVmcp, pWSIVmcp, and pSNSVmcp were also used. The tests were positive with plasmid DNA present between $10^{8.7}$ and $10^{0.7}$ copies, or 6 to 8 orders of magnitude (Figs. 5 & 6). Reaction efficiencies of Q2 varied from −3.3 to −3.5, and the *y*-intercept values varied from 39 to 41, indicating similar limits of detection for NV, BCWSV, MRSIV, and SNSV (Fig. 6). The lower ASe of Q2 for WSIV is reflected in the higher *y*-intercept value (Fig. 6E). The observed 50% limit of detection for Q1 was 3 copies of pNVmcp (5 of 6 replicates tested positive; 35.84 ± 2.11 Cq) and for Q2 it was 6.25 copies (4 of 6 replicates tested positive; 37.02 ± 1.58 Cq; Figs. 5B & 6B). The 100% limit of detection for Q1 (33.66 ± 0.75 Cq) and Q2 (35.78 ± 1.14 Cq) was observed to be 25 copies of pNVmcp. The presence of host DNA (1500 ng) and VIC-labeled probe pAPC in each reaction did not significantly alter the limit of detection for either test. The 95% limit of detection estimates were nearly identical to those measured, and for Q1, the copies and Cq associated with the estimated 95%

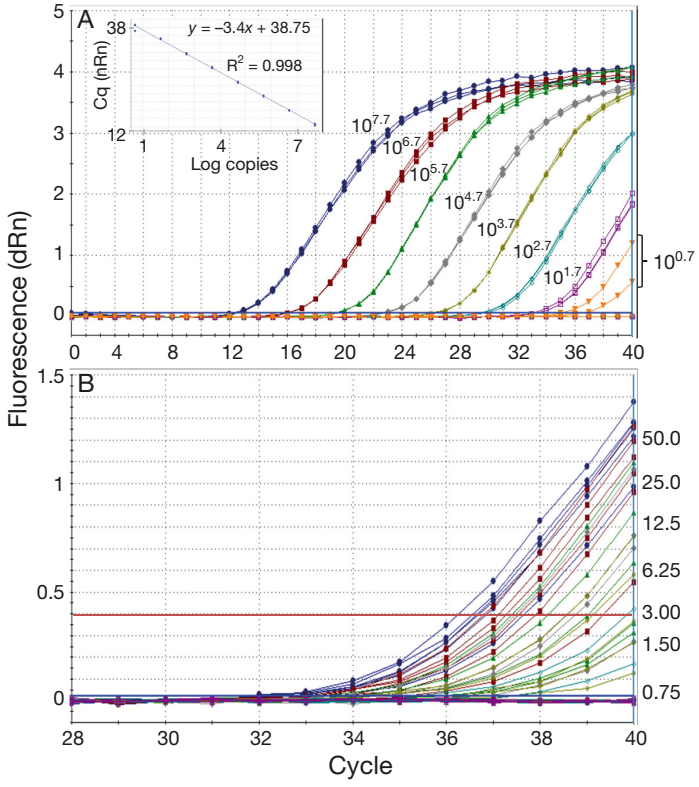


Fig. 5. Analytical sensitivity of the Q1 qPCR test. Quantification cycle (Cq) values for the FAM-labeled probe were generated using pNVmcp DNA diluted (A) from $10^{7.7}$ to 5 copies or (B) from 50 to 0.75 copies. Linear regression for panel A is shown in the inset. The limit of detection is indicated by the horizontal red line in panel B

limit of detection were 1.13 and 39.39, whereas for Q2 it was 1.42 and 38.93, respectively (Fig. S3 in the Supplement).

The ASp of C1, Q1, and Q2 was determined using the panel of viruses in Table 1. AciHV-1 and -2 (Kelley et al. 2005, Kurobe et al. 2008), which are not members of the *Megavirales*, were also included since they have been concurrently diagnosed with WSIV (Georgiadis et al. 2000) and iridovirus-like infections in shortnose sturgeon (LaPatra et al. 2014). None of the tests, C1, Q1, or Q2, recognized the sturgeon herpesviruses (data not shown). The ASp of the 3 tests was ranked according to the level of agreement between the observed and expected status. For exclusivity (including 95% confidence intervals), C1 (76% [57–90%]) < Q1 (100% [81–100%]) = Q2 (100% [81–100%]) whereas for inclusivity, Q1 (80% [28–99%]) < Q2 (100% [48–100%]) = C1 (100% [48–100%]). Q1 tests results were positive with pNVmcp, pMRSIVmcp, pBCWSVmcp, and pSNSVmcp but not pWSIVmcp or plasmids encoding target MCP nucleic acid sequences from other viruses within *Megavirales* (Table 1). Q2 test results were positive for the 5 sturgeon viruses and negative for other members of *Megavirales* (Table 1). C1 test results were positive for the sturgeon viruses and select viruses belonging to the families *Mimi-*, *Phycodna-*, or *Iridoviridae* but not *Marseille-*, *Asco-* or *Asfarviridae* (Table 1).

Repeatability of each qPCR assay was evaluated with 10-fold serial dilutions of pNVmcp plasmid DNA from $10^{7.7}$ to 5 copies per reaction analyzed in replicates of 5 per run in 5 independent runs. Estimates of intra- and inter-assay repeatability of Cq values for each dilution were generated using the CV. The average intra-assay CV for Cq values obtained with samples from each dilution within a run ranged from 0.59 to 3.74, with the highest CV values associated with samples having the lowest plasmid copy number of 5 copies (Fig. 7). The inter-assay CV for Cq values obtained with samples from each dilution between the 5 runs varied from 0.68 to 3.79 (Fig. 7). In this case, the highest CV values were associated with samples with the lowest and highest plasmid copy numbers of 5 and $10^{7.7}$ plasmid copies, respectively.

Repeatability of Q1 was also evaluated with positive control samples P1 and P2. The average intra-assay CV for Cq values obtained with P1 was 1.4, whereas the inter-assay CV was 13.85 (Fig. 8). The average intra-assay CV for Cq values obtained with samples from each dilution of P2 within a run ranged from 0.78 to 2.71 (Fig. 8). The highest CV values of 2.46 and 2.71 were again associated with samples with the lowest and highest plasmid copy numbers of 5 and $10^{7.7}$ plasmid copies, respectively. The variability in Cq values obtained for P1 was statistically significant ($p < 0.05$) between batches (+1.45 Cq) and runs (+0.034 Cq). For P2, variability was only significant at 5 copies between batches (+0.18 Cq) and runs (0.01 Cq).

Application of Q1

Q1 has been used to test for sturgeon viruses NV, MRSIV, BCWSV, and SNSV in endangered populations of lake sturgeon within the Winnipeg River or Nelson River drainage systems of Manitoba. Positive test results with Q1 were obtained with lake sturgeon from either river drainage systems (Table 3). In addition, positive test results were obtained from larvae, juvenile, sub-adult, and adult lake sturgeon somatic tissues, but not from reproductive products (Table 3).

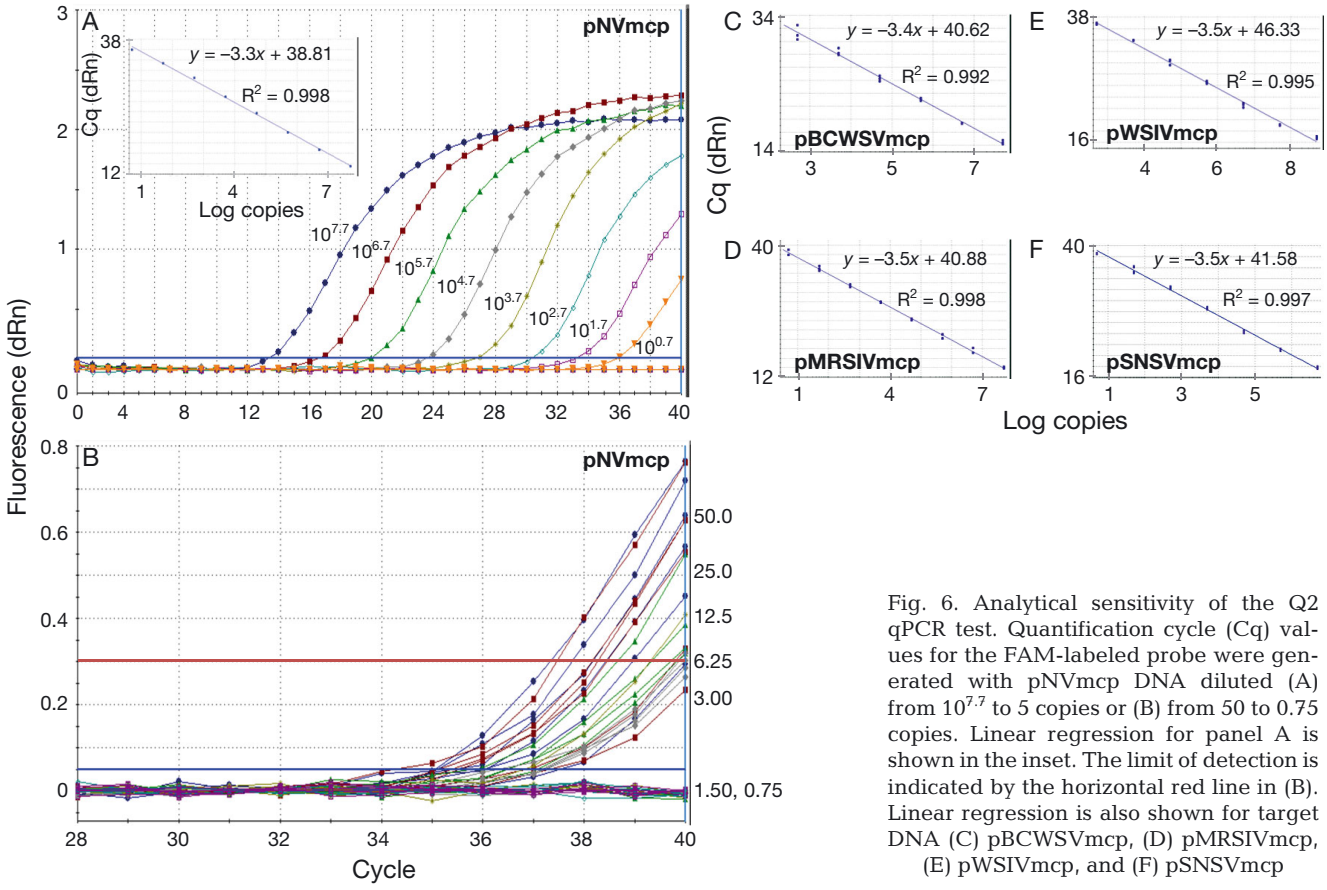


Fig. 6. Analytical sensitivity of the Q2 qPCR test. Quantification cycle (Cq) values for the FAM-labeled probe were generated with pNVmcp DNA diluted (A) from $10^{7.7}$ to 5 copies or (B) from 50 to 0.75 copies. Linear regression for panel A is shown in the inset. The limit of detection is indicated by the horizontal red line in (B). Linear regression is also shown for target DNA (C) pBCWSVmcp, (D) pMRSIVmcp, (E) pWSIVmcp, and (F) pSNSVmcp

Table 3. Application of qPCR test Q1 and histology for detection of sturgeon nucleo-cytoplasmic large DNA viruses in Manitoba lake sturgeon *Acipenser fulvescens*. Values shown are the number positive/total number tested

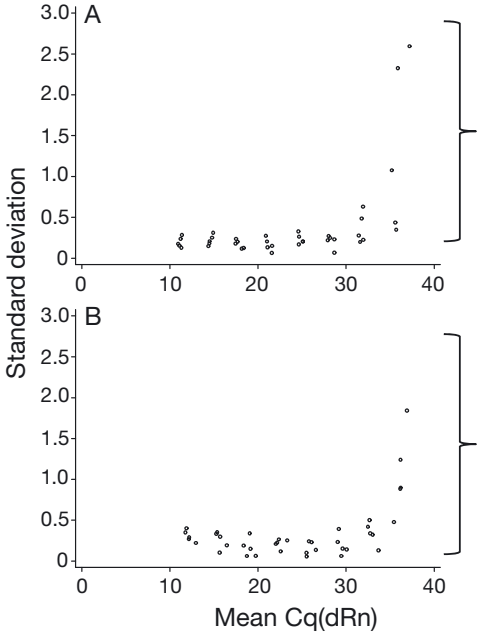
Sample source	Winnipeg River drainage		Nelson River drainage	
	Q1	Histology	Q1	Histology
Broodstock	1/47	0/47	5/22	0/12
Gametes	0/40	–	0/20	–
Larvae	0/154	0/104	1/139	0/5
Juveniles	167/171	28/34	0/469	4/86
Sub-adults	3/9	–	–	–

Broodstock tested positive (1 of 12 fish) at Pointe du Bois Falls during the 2013 spawning season, but samples (N = 35) collected during the 2010 to 2012 spawning season all tested negative (Table 3). Broodstock tested positive (5 of 22) from the Nelson River drainage system in 2012, 2013, and 2014: at Landing River, 2 of 4 tested positive in 2012, at Burntwood River, 1 of 4 tested positive in 2013, and at Birthday Rapids, 2 of 3 tested positive in 2014. Broodstock (N = 11) collected from Landing River in 2011 and 2014 tested negative. Gametes (N = 60) collected from broodstock never tested positive by Q1 (Table 3); this was the case even when a parent tested positive. For example, virus was not detected in the gametes from 6 broodstock (2 males, 4 females) whose fin tissue tested positive by Q1. Larvae (N = 293) generated from mating crosses of all broodstock tested negative, except on 1 occasion in which a single hatchery-reared larva tested positive during virus screening (Table 3). In this case, the fish was the offspring of 2 parents whose fin tissue tested positive by Q1.

Broodstock fin tissue testing positive by Q1 (N = 6) contained 2 to 830 equivalent plasmid copies per µg of total DNA. For comparison, hatchery-reared, moribund juvenile sturgeon (N = 130) collected during a 2009 epizootic occurring at UMAAHF contained 136 033 to 388 089 equivalent plasmid copies per µg DNA.

Histological examination was used as a confirmatory diagnostic assay for broodstock (N = 59), larvae (N = 109), and juvenile (N = 120) lake sturgeon from

the Winnipeg River or Nelson River drainage systems (Table 3). Samples testing negative by histology (N = 256) sometimes tested positive by Q1 (N = 9). Pathognomonic changes consistent with the presence of these viruses were only observed in samples from populations of juvenile sturgeon undergoing a viral epidemic (i.e. broodstock were negative by histology).

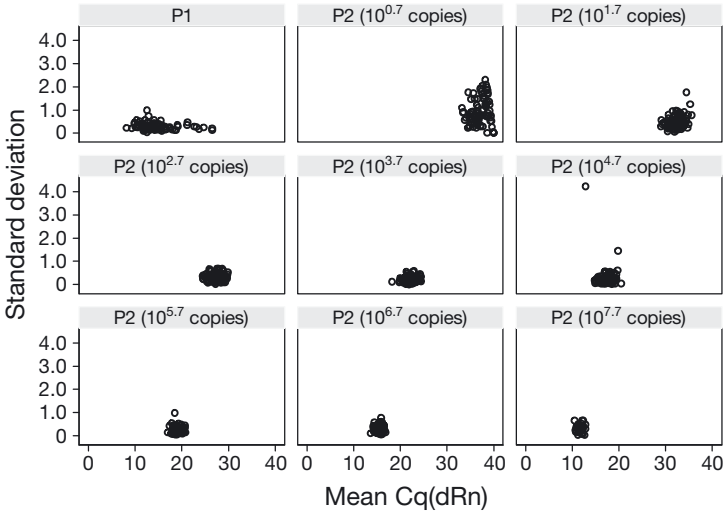


Plasmid copies	Cq ± SD[a]	Coefficient of variation	
		Intra-assay[b]	Inter-assay
5×10^0	35.92 ± 0.76	3.74	2.12
5×10^1	31.74 ± 0.22	1.14	0.68
5×10^2	28.30 ± 0.35	0.73	1.23
5×10^3	24.83 ± 0.25	0.94	1.02
5×10^4	21.25 ± 0.32	0.78	1.51
5×10^5	17.84 ± 0.40	0.96	2.26
5×10^6	14.62 ± 0.23	1.49	1.58
5×10^7	11.17 ± 0.18	1.74	1.61

Plasmid copies	Cq ± SD[a]	Coefficient of variation	
		Intra-assay[b]	Inter-assay
5×10^0	36.19 ± 0.52	2.93	1.45
5×10^1	32.93 ± 0.47	1.04	1.42
5×10^2	29.48 ± 0.40	0.67	1.34
5×10^3	25.90 ± 0.46	0.59	1.76
5×10^4	22.50 ± 0.51	0.95	2.28
5×10^5	19.02 ± 0.52	0.85	2.71
5×10^6	15.69 ± 0.47	1.64	2.97
5×10^7	12.19 ± 0.46	2.53	3.79

[a]Cq, mean of mean Cq/run; SD, mean of Cq standard deviation/run
[b]Average of within-assay coefficient of variation for all runs

Fig. 7. Analytical repeatability of the qPCR tests (A) Q1 and (B) Q2. Quantification cycle (Cq) values for the FAM-labeled probes were generated with pNVmcp DNA diluted from $10^{7.7}$ to 5 copies. Measurements were performed in replicates of 5 in 5 independent runs. Intra- and inter-assay coefficient of variation for Cq values as well as the standard deviation and mean Cq values for each of 5 runs are presented for each dilution in tables to the right of each graph



Control sample	Number of runs	Cq ± SD[a]	Coefficient of variation	
			Intra-assay[b]	Inter-assay
P1	83	20.91 ± 2.9	1.40	13.85
P2				
5×10^0	88	37.87 ± 1.33	2.46	3.50
5×10^1	88	34.28 ± 1.11	1.44	3.25
5×10^2	88	30.60 ± 1.02	1.10	3.32
5×10^3	88	26.65 ± 0.89	0.78	3.34
5×10^4	88	23.16 ± 1.03	1.28	4.44
5×10^5	88	19.23 ± 0.83	1.27	4.29
5×10^6	88	15.51 ± 0.63	1.66	4.07
5×10^7	32	11.77 ± 0.64	2.71	5.41

[a]Cq, mean of mean Cq/run; SD, mean of Cq standard deviation/run
[b]Average of within-assay coefficient of variation for all runs

Fig. 8. Analytical repeatability of the Q1 qPCR test with P1 and P2 (see 'Materials and methods' for details of P1 and P2). Quantification cycle (Cq) values for the FAM-labeled probe were generated with $10^{7.8}$ copies of pNV-APC Q1 DNA in 50 mg tissue (P1) or pNV-APC Q1 DNA diluted from $10^{7.7}$ to 5 copies (P2). Measurements were performed in replicates of 3 in 32 to 88 independent runs. Intra- and inter-assay coefficient of variation for Cq values as well as the standard deviation and mean Cq values are presented for P1 and each dilution of P2 in the table

DISCUSSION

The analytical performance of 3 PCR tests were validated for detection of sturgeon NCLDVs NV, WSIV, MRSIV, SNSV, and BCWSV. This group of viruses form a monophyletic clade that, based on our results, we hypothesize represents a new virus genus and family in the order *Megavirales*. The viruses appear to be endemic in populations of Acipenseridae found throughout North America.

Phylogenetic analysis of MCP sequences has been a reliable tool for taxonomic classification of NCLDVs to the species, genus, and family level (Jancovich et al. 2012). In general, our tree topology mirrored those reported in other studies (Boyer et al. 2009, Fischer et al. 2010, Colson et al. 2011, 2012). Its internal node structure places the sturgeon NCLDVs outside of both the small and large mimiviruses comprising the *Mimiviridae* as well as all viruses belonging to the *Iridoviridae* and *Marseilleviridae* families. An alternative hypothesis is that the sturgeon NCLDVs arose by genetic homoplasy. In this instance, an existing member of the NCLDV group would have acquired MCP or serpin sequences from an unknown virus. Based on this information, reference to iridovirus in the common name of the virus should be revised to avoid confusion in the scientific literature.

Each of the sturgeon NCLDVs forms a unique taxonomic unit located at the tips of the phylogenetic tree (Fig. 4). This was the basis for using the MCP sequence as a tool for virus genotyping. We found that the 5 virus genotypes were distributed across 6 river basins in North America. Whether viruses with different genotypes co-circulate within a water drainage system is not known. The clustering of taxa was not strictly isomorphic with respect to phylogeography since some isolates from the Pacific, central, and Atlantic regions of North America were more closely related to one another than to isolates from the central region. The virus MCP genotypes were not exclusively associated with host species, but our results suggest that they may be host family- or genus-specific. For instance, BCWSV and WSIV were isolated from white sturgeon, while MRSIV was found in both shovelnose and pallid sturgeon.

By inference, our data show that viruses with any of the 5 MCP genotypes were capable of causing pathognomonic changes consistent with sturgeon NCLDVs. For example, each of the viruses has exhibited the potential for a complex lifecycle involving an acute, lethal disease or a persistent, subclinical infection (Watson et al. 1998a,b, Raverty et al. 2003, Kurobe et al. 2010, 2011, Clouthier et al. 2013, LaPatra et al. 2014). This life history is an interesting feature, since virus persistence can influence virus–host dynamics across decades given the long lifespan of members of the Acipenseridae.

ASp of the C1 test was relatively broad, being inclusive of the sturgeon NCLDVs as well as synthetic targets derived from viruses in the *Phyco-*, *Mimi-*, or *Iridoviridae* families. The inclusivity of the test was likely a consequence of the deep evolutionary conservation of the MCP, being 1 of 5 orthologous genes of NCLDVs (Yutin et al. 2009). Further, the C1 target is located in 1 of 2 evolutionarily conserved protein domains found in all known NCLDVs (Marchler-Bauer et al. 2015). In contrast, genetic information derived from the sequences located between the 2 domains is less conserved. It is possible that this region could be used to genotype sturgeon NCLDVs rather than using the entire MCP sequence.

ASp of the Q1 and Q2 tests was narrower than that of C1 and inclusive of only the sturgeon NCLDVs. The Q2 qPCR test detected all 5 sturgeon NCLDVs, whereas Q1 did not detect WSIV. Q2 is the preferred test if the genotype of the sturgeon virus in a sample is not known *a priori*. In a diagnostic setting, the qPCR tests could be used sequentially with the conventional PCR test to screen for sturgeon NCLDVs and confirm presumptive positive samples. The limit of detection for C1 was 50 plasmid copies which was one-tenth of that recorded for the qPCR tests. These performance characteristics make the C1 test suitable for confirmatory diagnostic testing or the discovery of new sturgeon NCLDVs.

The Q2 test is pan-specific for North American sturgeon NCLDVs, unlike the isolate-specific qPCR assays designed for detection of WSIV (Kwak 2006) or MRSIV (Kurobe et al. 2011). The Q1 and Q2 tests described in this study and the WSIV qPCR assay described previously target DNA encoding the MCP. The former 2 tests were designed to detect conserved regions within the sequence, whereas the WSIV test was designed to detect a variable region. Thus the utility of the WSIV test is limited to detection of some but not all isolates of sturgeon NCLDVs from white sturgeon. The sequence targeted by the MRSIV qPCR test encodes a serine/cysteine proteinase inhibitor or serpin, and the test is limited to detection of sturgeon NCLDVs from pallid or shovelnose sturgeon. The false negative results obtained with the WSIV and MRSIV qPCR tests when DNA from tissues infected with the BC white sturgeon virus, the shortnose sturgeon virus,

or NV from lake sturgeon were tested illustrates the need for a diagnostic test capable of detecting all isolates of sturgeon NCLDVs.

The analytical performance characteristics of Q1 and Q2 were validated according to international standards (OIE 2014). In this context, the ASe and repeatability of the assays were determined in addition to their aforementioned ASp. Both assays were linear over 8 orders of magnitude with reaction efficiencies of 93% or higher. Thus the assays' performance was consistent and reliable regardless of the virus load in the sample. The observed 50 to 95% limit of detection for both assays for NV was between 3 and 25 plasmid copies. The other isolates were detected between 5 and 500 plasmid copies. The Q1 and Q2 tests also yielded highly repeatable results both within and between assay runs. Since the ASe and repeatability of Q1 and Q2 were similar, the tests are considered to be interchangeable if WSIV is not suspected. In these situations, higher levels of probe fluorescence are obtained with the Q1 test.

We hypothesize that NV is endemic in wild lake sturgeon in the Nelson River basin, specifically the Nelson River and the Winnipeg River in Manitoba. These sturgeon are listed as endangered by the Committee on the Status of Endangered Wildlife in Canada (www.cosewic.gc.ca/eng/sct0/rpt/rpt_csar_e.pdf). The virus has been maintained in these populations without causing extinction. The last NV disease outbreak in the province occurred in the 2009 year class of sturgeon from the Winnipeg River and the Nelson River. Since then, 5 different year classes of juvenile sturgeon (2010 to 2014) tested negative for the presence of NV by Q1 except for fish originating from broodstock collected in 2014 from Birthday Rapids on the Nelson River. When offspring held at Grand Rapids Hatchery were tested 2 mo later, there was no evidence of virus.

The Q1 test was also used to measure relative virus abundance in infected sturgeon. Virus was more abundant in tissues from diseased fish relative to those from spawning wild adults. This pattern was also observed by Kurobe et al. (2010, 2011) with MRSIV and pallid or shovelnose sturgeon. In those studies, moribund fish from experimental challenge trials had consistently higher virus loads relative to wild adult fish or convalescent fish 8.5 mo after initial exposure. We can hypothesize from this that sturgeon NCLDV abundance is proportional to virus virulence or host mortality. In cases where low levels of virus were detected, clinical disease was not observed.

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