Isolation and characterization of the fall Chinook aquareovirus

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Abstract

Background: Salmon are paramount to the economy, ecology, and history of the Pacific Northwest. Viruses constitute one of the major threats to salmon health and well-being, with more than twenty known virus species that infect salmon. Here, we describe the isolation and characterization of the fall Chinook aquareovirus, a divergent member of the species Aquareovirus B within the family Reoviridae.

Methods: The virus was first found in 2014 as part of a routine adult broodstock screening program in which kidney and spleen tissue samples from healthy-appearing, adult fall Chinook salmon (Oncorhynchus tshawytscha) returning to a hatchery in Washington State produced cytopathic effects when inoculated onto a Chinook salmon embryo cell line (CHSE-214). The virus was not able to be confirmed by an RT-PCR assay using existing aquareovirus pan-species primers, and instead was identified by metagenomic next-generation sequencing. Metagenomic next-generation sequencing was used to recover the full genome and completed using 3′ RACE.

Results: The genome of the fall Chinook aquareovirus contains 11 segments of double-stranded RNA totaling 23.3 kb, with each segment flanked by the canonical sequence termini found in the aquareoviruses. Sequence comparisons and a phylogenetic analysis revealed a nucleotide identity of 63.2% in the VP7 gene with the Green River Chinook virus, placing the new isolate in the species Aquareovirus B. A qRT-PCR assay was developed targeting the VP2, which showed rapid growth of the isolate during the initial 5 days in culture using CHSE-214 cells.

Conclusions: This sequence represents the first complete genome of an Aquareovirus B species. Future studies will be required to understand the potential pathogenicity and epidemiology of the fall Chinook aquareovirus.

Keywords: Aquareovirus, Aquareovirus B, Chinook, Fall chinook, Salmon virus

Background

Members of the family Reoviridae include viruses with genomes having between 9 to 12 segments of double-stranded RNA [1]. Reoviruses infect a variety of hosts from mammals to fungi and have a wide geographic distribution [1]. The family Reoviridae is composed of two sub families: Spinareovirinae and Sedoreovirinae with 15 different genera between them [2]. Members of the genus Aquareovirus have been isolated from a wide variety of aquatic animal hosts in which they produce infections that range from benign to severe hemorrhagic disease [3–5].

Among the seven established species of aquareoviruses (A–G), isolates representing the species Aquareovirus A and B are known to infect a wide variety of salmonids in the Pacific Northwest. They are typically isolated at low prevalence and intensity from populations of returning adult salmonids in which they produce no identified pathology. However, experimental infections of several species of juvenile salmonids have demonstrated Aquareovirus A causes a focal necrotizing hepatitis [6]. In other studies, infections of trout with Aquareovirus A were shown to increase resistance to the highly pathogenic fish rhabdovirus, infectious hematopoietic necrosis virus (IHNV), presumably by the induction of interferon [7].
While aquareoviruses generally grow well in cell culture producing a characteristic plaque-like or syncytial type of cytopathic effect (CPE), their identification is often performed by molecular means such as RT-PCR. The reliance on molecular detection necessitates the availability of complete genome sequences to improve PCR primer design and diagnostic test accuracy and to understand virus diversity and their molecular evolution. Aquareoviruses all contain 11 dsRNA segments that encode for seven structural proteins (VP1-VP7) and 5 nonstructural proteins [2, 8]. While their distribution among the seven aquareovirus species is uneven, approximately 15 aquareovirus genomes have been completed and several strains have been partially completed [2].

Here, we describe the isolation, characterization, and complete genome sequencing of the fall Chinook aquareovirus, a new member of species Aquareovirus B detected in routine surveillance of salmonids. The virus grew in CHSE-214 cell cultures, but failed to be confirmed by diagnostic RT-PCR assays primed with sets of degenerate Aquareovirus pan-species primers. Our results show the genome sequence of the fall Chinook aquareovirus to be the most divergent member of an existing aquareovirus species and will aid in the redesign of diagnostic molecular assays for salmonid aquareoviruses.

Methods
Cell culture and Aquareovirus RT-PCR
Tissue samples from adult fall Chinook salmon (Oncorhynchus tshawytscha) were collected from fish returning to the Washington Department of Fish and Wildlife (WDFW) Glenwood Springs Hatchery in Washington State in October 2014. Five fish pools of kidney and spleen were homogenized and diluted 1:10 by weight in minimum essential medium (MEM). Antibiotic incubation media consisting of 1000 µg/mL penicillin-streptomycin, 50 µg/mL Fungizone and 0.5 µg/mL gentamicin in MEM was used to prepare a 1:4 dilution of the sample and incubated 2 h at 15 °C. A 24- well tissue culture plate was seeded with Chinook salmon embryo (CHSE-214) cells 24 h prior and allowed to grow until a monolayer of >90% confluency was reached. CHSE-214 cells were inoculated with 200 µL per well of sample in duplicate. The inoculum was kept on cells for 1 h at 15 °C before cells were overlayed with MEM containing 5% fetal bovine serum (MEM-5), 0.5 µg/mL gentamycin, 0.5 µg/mL fungizone and 100 µg/mL penicillin-streptomycin and incubated at 15 °C. For 28 days, cells were observed for CPE 2–3 times per week.

RNA from the tissue culture supernatants were extracted with Trizol according to the RNA extraction miniprep protocol (DirectZol, Zymo Research) and screened using sets of degenerate aquareovirus pan-species RT-PCR primers (Table 1). For each test, known positive control RNA from isolates of Aquareoviruses A and B were included along with deionized water as a negative control. Forward and reverse primers as shown in Table 1 for either Aquareovirus A (1811F/1812R) or Aquareovirus B (1807F/1809R) were tested in separate reactions. A nested PCR without the reverse transcription step, reverse transcriptase or RNasin enzymes was conducted using internal primers (Table 1) for Aquareovirus A (1813F/1814R) or for Aquareovirus B (1808F/1810R). Because the CPE produced by the fall Chinook aquareovirus on the CHSE-214 cells appeared somewhat different from that in a companion flask of cells infected with an Aquareovirus B isolate from a different location, we performed another RNA extraction and RT-PCRs with primers 1807F/1809R and 1811F/1812R. Additionally, after 13 days post infection, a further RNA extraction and PCR run was tested in combination with several isolates of putative aquareoviruses from locations near the Glenwood Springs Hatchery. The RT-PCR was performed in 50uL reactions using 28uL deionized water, 10uL 5X PCR buffer, 3uL 25 mM MgCl2, 1uL 10 mM dNTP, 1uL of each 20uM each primer, 0.25uL GoTaq, and 0.5uL AMV reverse transcriptase, and 0.25uL RNasin with 5uL of RNA template. RT-PCR

| Table 1 | Pan-specific degenerate RT-PCR primers used for (A) identification of isolates of Aquareovirus species A or B in an attempt to identify the fall Chinook aquareovirus (Batts, unpublished data). (B) Modified primers for identification of isolates of species Aquareovirus B based upon sequence analysis that includes the fall Chinook aquareovirus |
| A. | Aquareovirus A | Aquareovirus B |
| 1811F | C RCC ATG GAG ACC AAA CC | C GCC ATG GAT ACC AAG CC |
| 1812R | CT STG RTT CAT CAT AGC GTG | TG GGA CAG CAG GGC GTG |
| 1813F | AY ATC ACY CAY SAG TGT CA | AY WW C M CT CAC GAG TGT CA |
| 1814R | GC TAG ATC TTT GCC ATA GAA | GM KAG ATC CCT GCC GTA RAA |
| 1807F | 1809R | 1808F | 1810R |
| B. | 4370F | ACC ATC GGY ATG GAT AYY AAG CC | ACC ATC GCY ATG GAT AYY AAG CC |
| 4371R | CG GTT SGA SCC ATG ACG RTT CTC | CG TT SGA SCC ATG ACG RTT CTC |
| 4372F | ACC GTW GCC AAC GCA CCT TGC GA | ACC GTW GCC AAC GCA CCT TGC GA |
| 4373R | G CGC GAT TGS WSS TAG AGA GGA | G CGC GAT TGS WSS TAG AGA GGA |
conditions were 50°C 30 min; 95°C 2 min; 30 cycles of 95°C 30s, 50°C 30s, 72°C 1 min; followed by 72°C for 7 min and a 15°C hold. PCR products were visualized on an ethidium bromide stained 1.5% agarose gel.

Library preparation and next generation sequencing
For genome recovery, RNA from supernatant was extracted using the Zymo Viral RNA Kit (Zymo Research) and treated with Turbo DNase I (Thermo Fisher) [9]. cDNA synthesis was performed using random hexamers with SuperScript III reverse transcriptase and second-strand synthesis was performed using Sequenase v2.0 (Thermo Fisher). Double-stranded DNA was cleaned using a Zymo DNA-5 Clean and Concentrator column (Zymo Research) and library preparation was performed using 0.4X volumes of Nextera XT (Illumina), 16 cycles of dual-indexed library amplification, purification with 0.75X Ampure XP beads (Beckman Coulter). The library was quantified on Qubit 3.0 and Agilent Bioanalyzer 2100 and sequenced using a portion of a 1x180bp sequencing run on an Illumina MiSeq [10]. Sequencing reads were adapter and quality trimmed using cutadapt, repaired using pairfq, and de novo assembled using SPAdes v3.8 [11]. The resultant contigs were aligned by megablast to the NT database and the unaligned contigs were aligned by DIAMOND to the NR database [12]. The alignment files were taxonomically assigned and visualized using MEGAN 2.0, while contigs were visualized in Geneious v9.1 and phylogenies were performed using MAFFT alignments and MrBayes for phylogenetic trees [13–15].

Genome finishing
Follow-up RT-PCR was performed to close a gap in the NS1 gene using NS1–1386R and NS1–374F primers (Table 2) and quarter-volumes of the Qiagen One-Step RT-PCR kit. 3'RACE was performed as described previously [16]. Briefly, post-DNAsed RNA was polyadenylated by using E. coli Poly(A) Polymerase (NEB). Poly(A) RNA was reverse transcribed using Super Script III RT with Q_T primer and 30 cycles of Phusion PCR with a T_m of 55 °C with the Q_outer primer and GSP1 (Table 2) [16]. Nested PCR was performed using 1uL product from the first round PCR with 35 cycles using the Q inner primer and associated GSP2. PCR products were visualized on a 1.5% agarose gel and bands were gel-extracted using the QIAquick Gel Extract kit and sequenced via Sanger sequencing.

Development of a qRT-PCR assay for assessing virus growth kinetics
After the appearance of initial CPE in the primary culture, three, 25 cm² flasks were seeded with CHSE 214 cells and allowed to incubate at 15 °C overnight with MEM containing 10% fetal bovine serum (MEM-10) until cells were 90–100% confluent. When the cell monolayer was confluent, growth medium was removed and 200 μL of primary culture was filtered through a 0.2 μM membrane into two of the three flasks. The negative control was 200 μL of MEM-10 into a third flask. Flasks were gently swirled and allowed to incubate for 1 h at 15 °C. After 1 h, 7 mL of MEM-5 was added to each flask. A 200uL aliquot for PCR was taken to quantify the starting inoculum. On days 1–11, flasks were observed for CPE and 200 uL aliquots were collected from each flask and frozen at −20 °C.

A quantitative PCR was designed based on alignment of the nucleotide sequences of selected segments of other aquareoviruses. Primers to conserved regions of

| Table 2 | RT-PCR primers used for sequencing the complete genome (A) and for a novel qRT-PCR assay to quantify virus load (B) |
|---------|---------------------------------------------------------------------------------------------------------------------------------|
| A.      |                                                                                                                                |
| VP1-F   | TGT GAC ATC GGC GAC AAC                                                                                                        |
| VP1-R   | CTG ACG TGA CCA TGC CAA T                                                                                                       |
| VP2-F   | TGG ATG CCC CTA GAC TTT G                                                                                                       |
| VP2-R   | TGT CCG AAT TAT GAC CAA AGT C                                                                                                   |
| VP3-F   | CCG ATG GTC AGC TCT CG                                                                                                          |
| VP3-F-outer | CAC CCA GAA CAA CAA TGG                                                                                                        |
| VP3-R   | GGG GAC TTC GTC TCC GA                                                                                                          |
| VP4-F   | TAC TTC CCG TGA GTC                                                                                                              |
| VP4-R   | TGG AGT CTA GAA CCA AGC GG                                                                                                       |
| VP5-F   | CCG CAA TTA TTA CCA CTA CTG                                                                                                      |
| VP5-R   | CAG CCG TTC CCA TGA GT                                                                                                          |
| VP6-F   | GCG ATG GAA CTC GCT C                                                                                                           |
| VP6-R   | CCA TTA AGA TGG GCA CCG                                                                                                          |
| NS2-F   | TGA TGT GGC AGA AGC CTG                                                                                                          |
| NS2-R   | TGG CCG CAT TGA GAC C                                                                                                            |
| VP7-F   | TCT AGC AGC AAT GGC GC                                                                                                          |
| VP7-R   | ATA CCT GTG ACT GTC CAG                                                                                                          |
| NS3-F   | GTT AAA TGG TAC GGC GGC                                                                                                          |
| NS3-R   | TAT GGG TCT GCA GGC TG                                                                                                          |
| NS4-F   | GCC TGG TTC GAC TAC ATC                                                                                                          |
| NS4-R   | GCA CTT CCA ACC TTC TTG                                                                                                          |
| NS1–2-F | TAC GGC CAA GGT CAG TTC                                                                                                         |
| NS1–2-R | GGT TGG GAA TGA AGC GAA GA                                                                                                       |
| NS1–1-F | CAC AGC TCG GGG TCA C                                                                                                            |
| NS1–1-R | TAA GTG ATT GTC CAG GTG G                                                                                                        |
| B.      |                                                                                                                                |
| qVP2-F  | GGC GTA ATC CAG CCG C                                                                                                            |
| qVP2-R  | GCT AGT GAA GGG ATC GTC                                                                                                          |
the viral polymerase (VP2) were designed (Table 2) and
a positive control amplicon for use in developing a
standard curve was created using the Qiagen One-Step
RT-PCR kit.

Double-stranded viral RNAs were extracted from
culture supernatant using the Pure Link RNA mini Kit
(Thermo Fisher) and eluted with 60uL RNAse free water
then frozen at −20 °C until used. Reverse transcription
was completed using SuperScript II (Thermo Fisher)
following manufacturer’s instructions. cDNA was frozen
at −20 °C until qPCR could be completed. Primers pro-
vided were optimized using the SYBR Green recommenda-
tion for a final primer concentration of 0.3uM. The control
standard was quantified using a Qubit (Thermo Fisher)
and a known stock solution prepared (10^6 copies/μL) for
use in creating a standard curve.

PCR master mix consisted of a 1X final concentra-
tion SYBR Green Master mix (Applied Biosystems),
0.3uM of the forward and reverse primer and 1uL of
cDNA template for a final volume of 10uL/ rxn. Sam-
ples were run in triplicate using a 384 well plate in a
Quant studio 6 Flex PCR machine (Applied Biosys-
tems). The standard curve was prepared as a dilution
series of 10^6 to 10^1 copies/ rxn.

Results
Isolation of the fall Chinook aquareovirus
During the initial incubation of samples at the
WDFW laboratory, CPE appeared on day 28 in only
1 of 2 wells of CHSE-214 cells inoculated with 1 of
the 13 pools of kidney/spleen samples (Fig. 1a), indic-
ating the virus was present in the population at low
prevalence and intensity of infection. Beginning on
day 4, flasks of CHSE-214 cells inoculated with fil-
tered cell culture fluid from the affected well devel-
oped CPE typical of most aquareoviruses consisting
of plaque-like areas of syncytia (Fig. 1b). Viral stocks
were prepared by filtering culture fluid into cryovials
that were frozen at −80 °C and sent to the United
States Geological Survey Western Fisheries Research
Center for testing. RT-PCR assays using several pairs of
aquareovirus pan-species primers failed to amplify the
extracted RNA (Fig. 2). Subsequently, the unidentified
isolate was submitted to the Department of Laboratory
Medicine at the University of Washington for next-
generation sequencing.

Genome characterization of Fall Chinook reovirus
A total of 187,569 adapter-trimmed reads were re-
covered from the RNA-sequencing libraries from the
original cell culture supernatant. De novo assembly
of the RNA-sequencing reads yielded 42 contigs
longer than 1000 bp. BLASTN and DIAMOND align-
ment indicated the presence of a novel aquareovirus,
as alignments to various aquareoviruses over all
eleven segments were found among the top 100
contigs.

Because of the dsRNA nature of the reovirus genome,
3′RACE was performed to finish both 5′ and 3′ ends.
The fall Chinook aquareovirus genome contained 11
segments totaling 23.3 kb, ranging in size from 3947 bp
to 728 bp (Fig. 3a). 5′ NTR length ranged from 11 to
30 bp while the 3′ NTR length ranged from 30 to
157 bp. All segments began and ended with the cano-
onical aquareovirus sequence (5′-GUUUA and UCAUC-3′,
Fig. 3b). All segments contained a single ORF, except S7
which contained two overlapping ORFs, suggesting that
the fall Chinook aquareovirus encodes for 12 proteins.

In total, 39,633 of the 187,569 adapter-trimmed reads
(21.1%) mapped to the finished fall Chinook aquareo-
virus genome.

Phylogenetic analysis of fall Chinook aquareovirus
The fall Chinook aquareovirus VP7 shares a 63% nu-
cleotide identity with the Green River Chinook virus
(KC588384) and 47% amino acid identity with the
Coho salmon aquareovirus (AAC59609). Phylogenetic
analysis of both the VP4 and VP7 segments revealed
clustering of the fall Chinook aquareovirus within
the species Aquareovirus B (Fig. 4). Of note, the fall

Fig. 1 Characteristics of the fall Chinook aquareovirus. a Virus was recovered from kidney-spleen homogenates based on cytopathic effect on
CHSE-214 cells. b Syncytia formation characteristic of Aquareovirus was visible as early as day 4 on CHSE-214. Plaques from day 6 on inoculated
CHSE-214 cells are shown compared with uninoculated CHSE-214 cells.
Chinook aquareovirus demonstrates the most divergence of any new aquareovirus that does not constitute a new species based on the ICTV definition as well as the first complete genome sequence for an aquareovirus B species. Examination of the VP2 coding sequence revealed the canonical amino acid motifs for polymerase (DXXXXD, SG, and GDD) present in members of the *Aquareovirus* and *Orthoreovirus* genera.

Pairwise nucleotide identity between the Green River chinook aquareovirus and the fall Chinook aquareovirus ranged from 63% (VP7) to 73% (VP3). The existing pan-Aquareovirus B primers had 3 mismatches in each of the outer primers and 9 mismatches in the hemi-nested second round primer in the VP7 segment, consistent with their inability to amplify the fall Chinook aquareovirus, and leading to the design of a novel primer set for confirmation of isolates of species Aquareovirus B (Table 1).

**Virus growth kinetics of fall Chinook aquareovirus**

Recovery of the full genome sequence of the fall Chinook aquareovirus allowed development of a qRT-PCR assay that was used to study viral growth kinetics in flasks containing monolayer cultures of CHSE-214 cells. Samples of culture fluid removed from the flasks at daily intervals showed a strong increase in virus titer during the initial incubation period (Fig. 5).

**Discussion**

We describe the identification, culture, and complete genome sequencing of a novel aquareovirus in fall Chinook salmon from Washington State. We have named this new virus the fall Chinook aquareovirus. Like all members of the *Aquareovirus* genus, the fall Chinook aquareovirus contains 11 dsRNA segments. Its culture in CHSE 214 cells was associated with the canonical large plaque-like syncytia CPE. The ICTV defines a new aquareovirus species based a variety criteria, including sequence alignment of the
VP7 segment as having less than 55% nucleotide sequence identity and less than 36% amino acid sequence identity [17]. The fall Chinook aquareovirus thus constitutes a novel member of the species Aquareovirus B.

The virus was recovered from kidney-spleen pools of asymptomatic fall Chinook salmon in Washington State. Although they commonly cause cytopathic effect in cell culture, aquareoviruses of Pacific salmon are typically found in low prevalence among returning adult fish without illness and it is unclear what, if any, loss of function or sequelae they are associated.

The discovery and characterization of this virus will allow for the development of improved diagnostic markers as the existing aquareovirus B RT-PCR primers and represents the first complete genome sequence of an aquareovirus B.

This virus also illustrates the importance of routine surveillance for discovering new fish viruses that may not be associated with pathogenicity. The added sequence diversity here will inform the molecular mechanism by which aquareovirus induce interferon and why certain species are more pathogenic than others. Interferon induction by aquareoviruses has shown to be a potential protective mechanism against other more pathogenic viruses such as IHNV [7]. Future studies will also be required to understand the geographical and temporal breadth and evolution of fall Chinook aquareovirus, and whether it has pathogenicity in other fish species [8].

**Conclusion**

We describe the isolation, genome sequencing, and characterization of the fall Chinook aquareovirus, a novel aquareovirus B member, during routine surveillance of salmon in Washington State. This virus failed to be amplified by existing aquareovirus B RT-PCR primers and represents the first complete genome sequence of an aquareovirus B.
The authors declare that they have no competing interests. Competing interests Not applicable. Consent for publication Not applicable. Ethics approval and consent to participate approved the final manuscript. Funding This work was funded by the University of Washington Department of Laboratory Medicine and United States Geological Survey. Availability of data and materials Genome sequences are publicly deposited in NCBI Genbank under accessions KX891216-KX891226. Statement of consent Not applicable. Authors’ contributions NJM performed the sequencing and 3’ RACE. NJ performed the culture and qRT-PCR. WNB performed the aquareovirus RT-PCR. KRJ and KHH supervised the experimentation. ALG and KHH conceived of the study. ALG analyzed the sequencing data. JRW and ALG wrote the paper. All authors read and approved the final manuscript. Ethics approval and consent to participate Not applicable. Consent for publication Not applicable. Competing interests The authors declare that they have no competing interests.

Abbreviations
CPE: Cytopathic effect; dsRNA: Double-stranded ribonucleic acid; MEM: Minimum essential medium; RT-PCR: Reverse transcription polymerase chain reaction; WDFW: Washington Department of Fish and Wildlife

Acknowledgements Portions of this work were funded by the Fisheries and Wildlife Program of the US Geological Survey. Any use of trade, firm, or product names is for descriptive purposes only and does not imply endorsement by the U.S. Government.

Funding This work was funded by the University of Washington Department of Laboratory Medicine and United States Geological Survey.

Availability of data and materials Genome sequences are publicly deposited in NCBI Genbank under accessions KX891216-KX891226.

Statement of consent Not applicable.

Authors’ contributions NJM performed the sequencing and 3’ RACE. NJ performed the culture and qRT-PCR. WNB performed the aquareovirus RT-PCR. KRJ and KHH supervised the experimentation. ALG and KHH conceived of the study. ALG analyzed the sequencing data. JRW and ALG wrote the paper. All authors read and approved the final manuscript.

Ethics approval and consent to participate Not applicable.

Consent for publication Not applicable.

Competing interests The authors declare that they have no competing interests.

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Received: 25 May 2017 Accepted: 29 August 2017 Published online: 05 September 2017

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