Short Communication

Novel reovirus associated with epidemic mortality in wild largemouth bass (*Micropterus salmoides*)

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Reoviruses (family *Reoviridae*) infect vertebrate and invertebrate hosts with clinical effects ranging from inapparent to lethal. Here, we describe the discovery and characterization of *Largemouth bass reovirus* (LMBRV), found during investigation of a mortality event in wild largemouth bass (*Micropterus salmoides*) in 2015 in WI, USA. LMBRV has spherical virions of approximately 80 nm diameter containing 10 segments of linear dsRNA, aligning it with members of the genus *Orthoreovirus*, which infect mammals and birds, rather than members of the genus *Aquareovirus*, which contain 11 segments and infect teleost fishes. LMBRV is only between 24 % and 68 % similar at the amino acid level to its closest relative, *Piscine reovirus* (PRV), the putative cause of heart and skeletal muscle inflammation of farmed salmon. LMBRV expands the known diversity and host range of its lineage, which suggests that an undiscovered diversity of related pathogenic reoviruses may exist in wild fishes.

The family *Reoviridae* has a worldwide distribution and a host range spanning fungi, plants, insects, molluscs, birds, mammals and fishes (Attoui *et al.*, 2012). Many reoviruses cause clinical disease, such as rotaviruses that cause gastro-intestinal illness in children and young animals (Dhama *et al.*, 2009; Kotloff *et al.*, 2013). In fishes, reoviruses are associated with diseases of aquaculture (Blindheim *et al.*, 2015; Lupiani *et al.*, 1995; Seng *et al.*, 2002; Yan *et al.*, 2014). For example, *Grass carp haemorrhagic reovirus* emerged in the 1970s in China, causing high mortality in fingerling and yearling grass carp (*Ctenopharyngodon idellus*) (Lupiani *et al.*, 1995). Threadfin reovirus was isolated in 1998, following a mortality event in threadfin (*Eleutheronema tetradactylus*) fingerlings in Singapore (Seng *et al.*, 2002). Experimental infection of both threadfin and barramundi (*Lates calcarifer*) fingerlings resulted in high mortality, confirming the pathogenicity of the virus and demonstrating its ability to infect multiple host species

(Seng *et al.*, 2002). *Piscine reovirus* (PRV) was discovered in association with heart and skeletal muscle inflammation (HSMI) of farmed Atlantic salmon (*Salmo salar*) and, later, rainbow trout (*Oncorhynchus mykiss*) in Norway (Olsen *et al.*, 2015; Palacios *et al.*, 2010), although epidemiological and experimental evidence for PRV as the cause of HSMI has remained equivocal (Garseth *et al.*, 2013b; Garver *et al.*, 2016; Lovoll *et al.*, 2012; Marty *et al.*, 2015).

Members of the family *Reoviridae* are non-enveloped and icosahedral with 9–12 segments of linear, dsRNA (Auguste *et al.*, 2015). The family *Reoviridae* is divided into two sub-families based on the presence (*Spinareovirinae*) or absence (*Sedoreovirinae*) of turret proteins on the viral capsid (Attoui *et al.*, 2012; Auguste *et al.*, 2015). The *Spinareovirinae* contain the named genera *Aquareovirus* and *Orthoreovirus* and one unnamed genus with PRV as the sole member (Kibenge *et al.*, 2013). All aquareoviruses described to date have 11 genome segments and infect fishes, crustaceans and shellfish (Attoui *et al.*, 2012; Lupiani *et al.*, 1995). In contrast, all known orthoreoviruses contain 10 genome segments and infect mammals and birds (Kibenge *et al.*, 2013; Olsen *et al.*, 2015). PRV is genetically distinct from the orthoreoviruses and aquareoviruses, but its phylogenetic

position and genomic architecture (10 genome segments) align it more closely with the orthoreoviruses (Kibenge *et al.*, 2013; Palacios *et al.*, 2010). The commercial importance of HSMI has spawned studies of PRV, showing generally low genetic diversity among viruses from widely separated locations, consistent with anthropogenic spread through activities related to salmonid aquaculture (Garseth *et al.*, 2013a; Kibenge *et al.*, 2013).

On 4 May 2015, a fish mortality event was reported in Pine Lake, Forest County, northern WI (latitude 45.676729, longitude −88.980851, 6.8 km^2, maximum depth of 4.6 m, water temperature 15.6 °C at the time of the event). The case was investigated by the Wisconsin Department of Natural Resources (WDNR; case number 2015–95). On 5 May 2015, biologists observed several hundred dead or moribund largemouth bass between approximately 20 and 46 cm in length and in good body condition. Some fish appeared to have died recently, whereas others had been dead for longer, as evidenced by extensive autolysis and exterior saprophytic fungi. Small numbers of dead black crappie (*Pomoxis nigromaculatus*), bluegill (*Lepomis macrochirus*), northern pike (*Esox lucius*) and walleye (*Sander vitreus*) were observed as well, but these were in poor body condition, consistent with 'winterkill' (hypoxia), frequently observed at this location.

Ten bass were collected in individual bags, held overnight on ice and transported the following day to the WDNR Fish Health Laboratory (Madison, WI) for diagnostic evaluation.
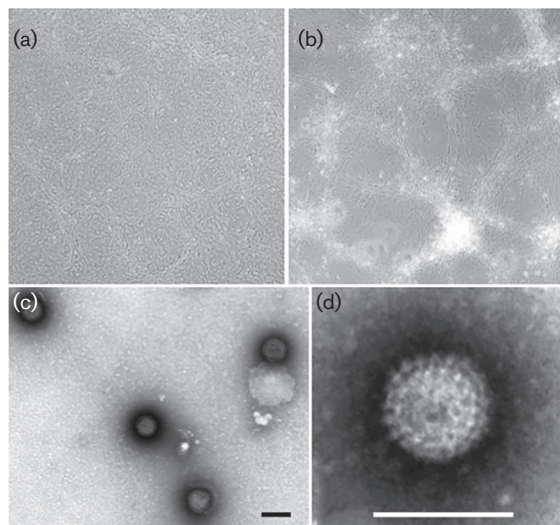

Fig. 1. Images of novel reovirus recovered from culture of largemouth bass tissues on BF-2 cells. (a) Uninfected monolayer of BF-2 cells (×40). (b) BF-2 cells 4 days post-inoculation, showing syncytia, elongated cells at plaque margins and large multinucleated cells (×40). (c) Ultramicrograph of spherical, double-shelled particles varying in diameter from 76.27 to 89.73 nm (1 % phosphotungstic-acid-negative contrast; bar, 100 nm). (d) Ultramicrograph of single virion showing core, capsid and concentric layering (1 % phosphotungstic-acid-negative contrast; bar, 100 nm).

Upon gross examination, bass showed congestion of the skin, mouth and base of fins; brown pale livers and kidneys; fibrinous coelomitis and severe adhesion between visceral (spleen, gastrointestinal tract and hepatopancreas) and parietal serosa. Incidental parasite infections common to fish in this region (e.g. 'black spot', caused by trematodes) were observed in the muscle, spleen and kidney of several fish.

For diagnosis of bacteria, sterile kidney swabs were cultured on trypticase soy agar (TSA) and Hsu–Shotts agar for 1 week at 20 °C or room temperature, respectively. Resulting bacterial colonies were submitted to the Wisconsin Veterinary Diagnostic Laboratory (WVDL, Madison, WI) for identification. Mixed bacterial cultures were subcultured at 20 °C on blood agar and tryptic soy agar or tryptic soy agar and Hsu–Shotts agar to obtain pure colonies, which were identified at WVDL by their characteristic protein signatures using matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) MS on an in-house instrument (MALDI Biotyper; Bruker Corporation). Analyses identified only common opportunistically pathogenic bacteria: motile aeromonads in all 10 fish and *Pseudomonas* spp. in four fish.

For diagnosis of viruses, kidney, liver, spleen and heart tissue were collected from each fish and combined into two pools of five for virus isolation. Tissue (~0.5 g) was placed in 4.5 ml Hank's balanced salt solution (pH 7.6) and homogenized before transport on ice to the United States Fish and Wildlife Service La Crosse Fish Health Center (case no. 15–145). Homogenized tissue was inoculated onto epithelioma papulosum cyprini CRL-2872 cells at 15 °C, chinook salmon embryo (CHSE-214) CRL-1681 cells at 15 °C, and bluegill fry (BF-2) CCL-91 cells at 25 °C according to standard procedures (USFWS & AFS-FHS, 2014). Cytopathic effect (CPE) was observed on BF-2 cells inoculated with one of two pooled tissue-sample homogenates after a single blind passage at 14 days post-infection. CPE was characterized by syncytium formation, elongated cells at plaque margins and large multinucleated cells, progressing to lysis and detachment (Fig. 1), as has been reported in BF-2 cells for reoviruses (Seng *et al.*, 2002; Blindheim *et al.*, 2015).

Electron microscopy was performed at the Minnesota Veterinary Diagnostic Laboratory. Cell culture supernatant was placed in 1.8 ml centrifuge tubes (Eppendorf) to which 1 ml of double distilled water was added. Tissues were homogenized into an opalescent suspension, and homogenates were centrifuged at 19 802 *g* (Eppendorf). Supernatant was then centrifuged at 80 000 *g* using an Airfuge centrifuge (Beckman Coulter) for 10 min. Supernatant was removed and the pellet was reconstituted with 10 µl of double distilled water. Five microlitres of sample were then placed on paraffin film, and formvar-coated copper grids (Electron Microscopy Sciences) were placed on the top of the drop for 10 min. Excess liquid was wicked and the grids were stained with 1 % phosphotungstic acid (Electron Microscopy Sciences) for 1 min prior to microscopy. Images revealed numerous non-

enveloped viral particles with icosahedral symmetry, 80.8 (±6.06) nm in diameter, with a visible core surrounded by a single layer of capsid proteins, and some particles appearing to contain either one or two concentrically distinct to poorly defined layers (Fig. 1). Virion size and morphology were consistent with viruses of the family *Reoviridae*, subfamily *Spinareovirinae* and were similar morphologically to PRV particles in Atlantic salmon erythrocytes visualized using transmission electron microscopy (Finstad *et al.*, 2014).

PCR assays for known reoviruses all yielded negative results, so 'shotgun' deep sequencing was performed for viral genome characterization. Cell culture supernatant (200 µl) from BF-2 cells was centrifuged (10 000 *g*, 10 min) to remove cellular debris, and viral RNA was isolated using the QIAamp MinElute Virus Spin kit (Qiagen), omitting carrier RNA. RNA was denatured at 95 °C (5 min) in the presence of random hexamers, and dsDNA was synthesized using the SuperScript Double-Stranded cDNA Synthesis kit (Invitrogen). Resulting cDNA was then purified using Agencourt Ampure XP beads (Beckman Coulter), and approximately 1 ng DNA was prepared for sequencing on an Illumina MiSeq instrument (Illumina) using the Nextera XT DNA Sample Preparation kit (Illumina). Sequence data were analysed using CLC Genomics Workbench version 8.5 (CLC bio). Briefly, low-quality bases were trimmed (Phred quality score<30) and short reads (<100 bp) were discarded, and the remaining reads were subjected to *de novo* assembly. Assembled contiguous sequences (contigs) were analysed for nucleotide- (BLASTN) and protein-level (BLASTX) similarity against GenBank databases.

Sequence analysis yielded 10 contigs with similarity to the 10 previously characterized genomic segments of PRV (BLASTX *E*-values 10^{-23} to 0). Other high-coverage contigs were most similar to known bacterial contaminants of cell culture media. Original deep sequencing data were used to generate complete ORF sequences for all 10 segments (Table 1). Like PRV, all *Largemouth bass reovirus* (LMBRV)

segments were monocistronic except for S1, which contained two fully overlapping ORFs. Sequence similarity between LMBRV and PRV across ORFs ranged from 30 % to 62 % and 25 % to 66 % on the nucleotide and amino acid levels, respectively.

The ends of the reovirus RNA-dependent RNA polymerase (RdRp) segment, L3, were sequenced using template-switching and step-out PCR (Picelli *et al.*, 2014; Pinto & Lindblad, 2010). Briefly, 1 µL of RNA was denatured (95 °C, 5 min) in the presence of dNTPs (1 mM final concentration) and two gene-specific primers (5′GSP-1, 5′-CTTCAC TGATGCCATGCTGATAG-3′ and 3′GSP-1, 5′-GGA TTTGCACACACCACTCAAT-3′, 200 nM final each) and cooled to 4 °C for 2 min. Template switching reactions were performed in 20 µl volumes using the SuperScript IV First-Strand Synthesis System (Invitrogen), with the additions of DMSO (4 % final concentration), MgCl$_2$ (6 mM final concentration) and the template-switching oligonucleotide, /5B iosG/AAGCAGTGGTATCAACGCAGAGTACATrGrGrG/ 3SpC3/ (500 nM final concentration), incubated as follows: 56 °C, 10 min; 42 °C, 60 min and 80 °C, 10 min. Subsequently, 10 µl of first-strand product was amplified in two separate reactions (KAPA HiFi HotStart ReadyMix kit; KAPA Biosystems) with nested primer 5′GSP-2, 5′-GC TTACCAGATTTAGCCCCAGAG-3′ or 3′GSP-2, 5′-GAG TCGACACGCACTTATTTCG-3′, (300 nM final each) and ISPCR primer (Picelli *et al.*, 2014), 5′-AAGCAGTGGTA TCAACGCAGAGT-3′ (300 nM). Touchdown PCRs were performed using the following cycling parameters: one cycle of 95 °C, 3 min; seven cycles of 98 °C, 15 s; 72 ° to 65 °C, 15 s; 72 °C, 15 s (−1 °C per cycle); 35 cycles of 98 °C, 15 s; 65 °C, 15 s; 72 °C, 15 s and a final extension of 72 °C, 2 min. RACE products were visualized by gel electrophoresis and amplicons were sequenced using the Sanger method on ABI 3730xl DNA Analyzers at the University of Wisconsin–Madison Biotechnology Center.

Table 1. Nucleotide and amino acid similarity between LMBRV and PRV across segments and ORFs

Segment	ORFs	ORF length (aa)	Nucleotide identity (%)	Amino acid identity (%)	Protein name*	GenBank accession number
L1	1	1291	61	64	Core shell	KU974953
L2	1	1309	49	43	Core turret	KU974954
L3	1	1286	61	64	Core RNA-dependent RNA polymerase	KU974955
M1	1	757	50	44	Core NTPase, pfam07781	KU974956
M2	1	661	62	66	Outer shell, pfam05993	KU974957
M3	1	733	37	28	Non-structural (NS) factory	KU974958
S1	2	328, 134	46, 41	36, 30	Outer clamp; NS p13	KU974959
S2	1	425	52	47	Core clamp	KU974960
S3	1	355	54	47	NS RNA, pfam01518	KU974961
S4	1	314	36	25	Outer fibre	KU974962

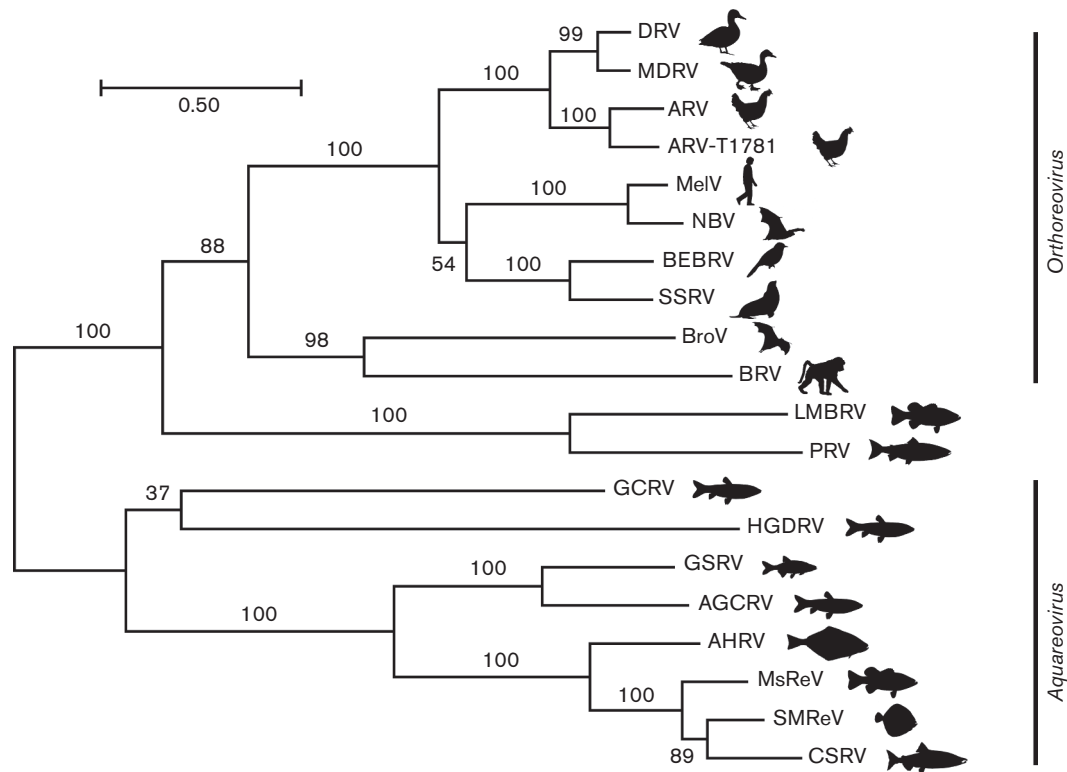*Protein names are from Kibenge *et al.* (2013).

Fig. 2. Phylogeny of the reovirus clade containing the genera *Aquareovirus* and *Orthoreovirus*, based on full coding sequences of the RdRp gene (segment L3). The tree was constructed using the maximum likelihood method with MEGA7 (best-fit substitution model GTR+Γ, estimated from the data) and was rooted at the midpoint of the longest branch. Numbers beside branches indicate bootstrap values. Bar, 0.50 nucleotide substitutions per site. The 20 viruses included in the analysis were chosen to capture the maximum diversity within known major clades of the genera *Aquareovirus* and *Orthoreovirus* and the unnamed genus containing PRV and LMBRV, available in GenBank as of February 2016. Silhouettes indicate the host species in which each virus was originally detected, which is not necessarily the natural or typical host of that virus. Viruses included in the analysis (abbreviation, country of origin, GenBank accession number) are: *Largemouth bass reovirus* (LMBRV, USA, KU974955); *Muscovy duck reovirus* (MDRV, China, KC508648); *Duck reovirus* (DRV, China, JX478251); *Avian orthoreovirus* T1781 (ARV-T1781, Hungary, KC865787); *Avian orthoreovirus* (ARV, USA, NC_015127); *Nelson Bay orthoreovirus* (NBV, Australia, JF342673); *Melaka orthoreovirus* (MelV, Malaysia, NC_020447); *Steller sea lion orthoreovirus* (SSRV, USA, HM222980); *Brown-eared bulbul orthoreovirus* (BEBRV, Japan, AB914761); *Baboon orthoreovirus* (BRV, USA, NC_015878); *Broome virus* (BroV, Australia, NC_014238); *Piscine reovirus* (PRV, Norway, GU994015); *Hubei grass carp disease reovirus* (HGDRV, China, JN967630); *Grass carp reovirus* (GCRV, China, KC201167); *American grass carp reovirus* (AGCRV, USA, NC_010585); *Golden shiner reovirus* (GSRV, USA, NC_005167); *Atlantic halibut reovirus* (AHRV, Norway, KJ499467); *Micropterus salmoides reovirus* (MsReV, China, KJ740726); *Chum salmon reovirus* (CSRV, Japan, NC_007583); *Scophthalmus maximus reovirus* (SMReV, China, HM989931).

The lengths of the 5′ and 3′ UTRs as determined by RACE were 10 and 44 nt, respectively, for segment L3. LMBRV shared the 3′ terminal sequence, UCAUC-3′, with both aquareoviruses and orthoreoviruses, whereas the 5′ terminal sequence, 5′-GACAU, was distinct from the aquareoviruses and orthoreoviruses. Both the lengths of the L3 UTRs and the complementary nature of the 5′ and 3′ terminal bases (G-C) conform to expectations established for previously sequenced aquareoviruses and orthoreoviruses (Kibenge et al., 2013).

Complete RdRp ORF sequences for 20 viruses were included in phylogenetic analyses to represent the maximum diversity within known major clades of the genera *Aquareovirus* and *Orthoreovirus* available in GenBank as of February 2016. Codon-guided sequence alignments were generated via MAAFT (Katoh et al., 2002) with the removal of poorly aligned regions using Gblocks (Talavera & Castresana, 2007) in the computer program TranslatorX (Abascal et al., 2010). A maximum likelihood phylogenetic tree was constructed from the resulting 3440-character alignment using MEGA7 (Kumar et al., 2016), with a best-fit substitution model estimated from the data and 1000 bootstrap replicates. Sequence similarity (p-distance, or proportion of nucleotide or amino acid differences) was also measured using MEGA7.

Analyses confirmed that LMBRV and PRV are sister taxa (Fig. 2). The lineage containing PRV and LMBRV was phylogenetically intermediate between the aquareoviruses and the orthoreoviruses, although more closely related to the orthoreoviruses, as has been previously shown (Kibenge *et al.*, 2013; Palacios *et al.*, 2010). Repeating the phylogenetic analysis with outgroup taxa [*Spissistilus festinus reovirus* (NC_016874) and *Aedes pseudoscutellaris reovirus* (NC_007667)] generated identical topologies (not shown).

LMBRV, isolated during a fish mortality event in northern WI, USA, is only the second described member of the proposed genus to which it belongs, which also contains PRV, the putative cause of HSMI in farmed salmon. Unlike PRV, LMBRV was discovered in a non-salmonid fish species (largemouth bass are in the family *Centrarchidae*), and it was found in association with a mortality event in wild, native fish, rather than in farmed fish. LMBRV is only distantly related to PRV, and it and PRV are more divergent from each other than are other species pairs within the genera *Aquareovirus* and *Orthoreovirus* (Fig. 2). Like PRV, LMBRV contains 10 segments, distinguishing it from the genus *Aquareovirus*, members of which contain 11 segments. These features, in combination with its host and geographic location of origin, are consistent with classification of LMBRV as a new viral species.

We note that a distantly related *Aquareovirus*, *Micropterus salmoides reovirus* (MsReV), has also been detected in largemouth bass (Chen *et al.*, 2015; Fig. 2). However, this virus was recovered from largemouth bass in Hubei province, China, which is far from the North American native range of *M. salmoides* (Philipp & Ridgway, 2002), suggesting that largemouth bass may not, in fact, be the natural host of MsReV. We, therefore, anticipate that as more aquatic and marine reoviruses are discovered, nomenclature referring to the species from which the virus was first isolated will become increasingly confusing. A taxonomic revision appears to be warranted, in which viruses are named according to criteria other than host of origin (e.g. geography).

Our results do not indicate definitively whether LMBRV was the cause of the mortality event in Pine Lake, WI. Mortality events at this location have occurred previously in early spring and attributed to the combined stresses of hypoxia, spawning activity, poor water quality and opportunistic infections with bacteria and fungi (WDNR, unpublished data). However, large fish kills dominated by a single species have not previously been recorded at this location. LMBRV may, therefore, be an incidental finding, an opportunistic virus or a frank pathogen. Its isolation from only one of two tissue homogenate pools, and only after blind passage, may indicate either low prevalence/concentration of the virus or that it is refractory to isolation on standard diagnostic cell lines. Further studies, including experimental inoculation of largemouth bass, will be required to determine the virulence of LMBRV alone and in combination with other agents and stressors.

As deep sequencing technologies become more generally accessible, we anticipate the discovery of other aquatic and marine reoviruses in the LMBRV/PRV lineage. In the case of PRV, its genetic homogeneity across time and space (Kibenge *et al.*, 2013; Siah *et al.*, 2015) strongly suggests rapid clonal expansion as a result of aquaculture-related activities. The presence of LMBRV in wild fish could have resulted from anthropogenic factors (e.g. movement of fish or contaminated water), or it could indicate the presence of hitherto unrecognized naturally occurring endemic reoviruses in wild fish populations. While the pathogenicity of these viruses is being investigated, we urge caution in the movement of wild fish for such purposes as stock enhancement or habitat restoration, and we recommend broader testing than is typically performed for members of the family *Reoviridae*. The inadvertent movement of unknown viruses could complicate epidemiological inferences about viral origins while also threatening the health and sustainability of wild, naive fisheries.

Acknowledgements

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