High prevalence of subclinical frog virus 3 infection in freshwater turtles of Ontario, Canada

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ABSTRACT

Ranaviruses have been associated with chelonian mortality. In Canada, the first two cases of ranavirus were detected in turtles in 2018 in Ontario, although a subsequent survey of its prevalence failed to detect additional positive cases. To confirm the prevalence of ranavirus in turtles in Ontario, we used a more sensitive method to investigate if lower level persistent infection was present in the population. Here we report results via a combination of qPCR, PCR, Sanger sequencing and genome sequencing from turtles from across Ontario, with no clinical signs of illness. We found 2 positives with high viral load and 5 positives with low viral load. Histopathology found subtle histological changes. DNA sequences identified two types of frog virus 3 (FV3), and genome sequencing identified a ranavirus similar to wild-type FV3. Our results show that the virus has been present in Ontario's turtles as subclinical infections.

1. Introduction

Wildlife diseases are global emerging threats to species persistence and are one of the key underlying causes for species decline (Daszak et al. 2000). The invasion of new pathogens from anthropogenic influences have been associated with declining populations (Cunningham et al. 2003; Daszak et al. 2003; Duffus et al. 2015) and as such require surveillance to assess these threats. Long-lived reptile species, like turtles, are of special concern, as stressors like raising temperatures, pollution, and habitat loss can increase their susceptibility to disease and hence the prevalence of diseases in these species (Keller et al. 2014; Kimble et al. 2015; Allender et al. 2018).

Ranavirus is a genus of double stranded DNA viruses within the *Iridoviridae* family, which infect a wide range of ectothermic vertebrates on all continents except Antarctica (Duffus et al. 2015). *Frog virus 3* is the type species and most studied *Ranavirus* (Mao et al. 1997). In Ontario, Canada, ranavirus was first reported in amphibians in 2004, and has been responsible for mass mortality events as high as 90–100% (Greer et al. 2005; Duffus et al., 2008; Duffus and Andrews, 2013). Ranavirus infection causes marked systemic disease, although susceptibility can be species-specific (Hoverman et al. 2011). Susceptibility and disease intensity are also shown to vary depending on the ranavirus

lineage. For example, recombinants between two lineages derived from ranaculture facilities exhibited higher mortality rates in amphibians than non-recombinant lineages (Majji et al. 2006), possibly due to an acquisition of more virulent genes.

In North America, at least three different lineages of ranavirus are known to infect wild and captive amphibians. The most common species, *Frog virus 3* (FV3) can infect both amphibians and turtles (Grant et al. 2019; McKenzie et al. 2019). The other two ranavirus lineages, *Ambystoma tigrinum virus* (ATV) and *Common midwife toad virus* (CMTV) are reported in salamanders and frog farms in the United States (US), respectively (Epstein and Storfer, 2016; Claytor et al. 2017). In Ontario, previous studies have shown a high prevalence of ranavirus in waterbodies, based on environmental DNA assessments (Vilaça et al. 2019b). Further, genome sequences of FV3 isolated from amphibians (tadpoles and adults) show a high recombination frequency between CMTV-like and FV3-like ranaviruses. Specifically, in southern Ontario, two FV3-like viruses isolated from tadpoles were observed to have two recombination events (Vilaça et al. 2019a) involving Open Reading Frames (ORFs) with important roles in viral activities (Vilaça et al. 2019a). These include ORFs 5R, 6R, 24R, 25R and 26R, of which 26R is the viral homolog of eIF-2α and is a putative ranavirus immune invasion gene (Andino et al., 2015). ORFs 5R and 6R are within the US22
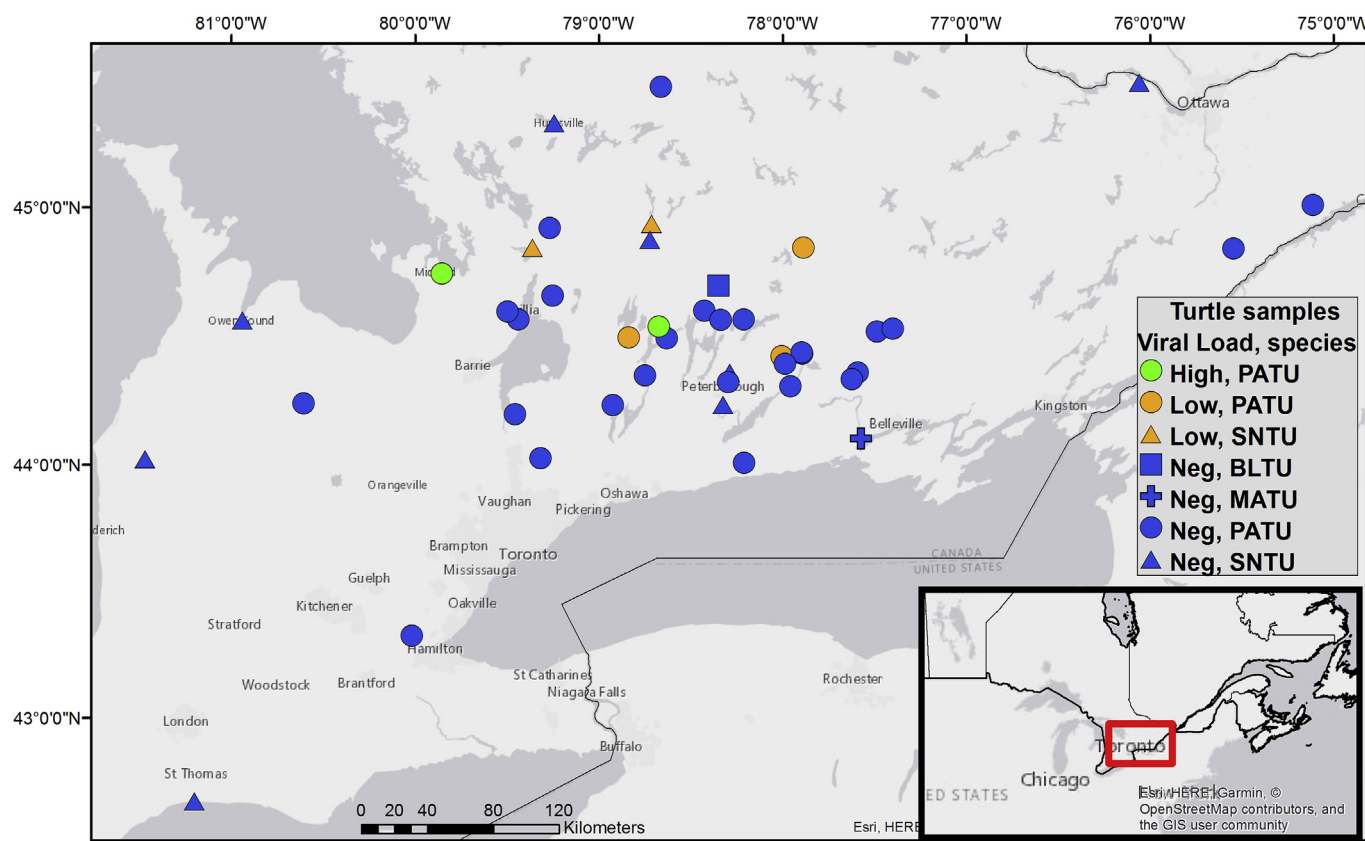
Fig. 1. Map showing the turtle samples used in this study. Abbreviations: BLTU: Blanding's turtle, MATU: Map Turtle, PATU: Painted turtle, SNTU: Snapping turtle. Colors follow viral load (high: green, low: orange, negative: blue), and shapes follow the turtle species (circle: painted turtle, triangle: snapping turtle, square: blanting's turtle, cross: map turtle).

orthologous gene cluster and are involved in antiviral cell stress responses in hosts (Zhang et al., 2011). These recombination events might indicate that wild amphibians are being infected by increasingly virulent viruses (Vilaça et al. 2019a).

Ranavirus has been reported to infect several turtle species in North America and Europe (Duffus et al. 2015). In the US, six turtle and tortoise species have been reported as infected by ranaviruses (Duffus et al. 2015), and another two turtle species were reported in Canada (Canadian Wildlife Health Cooperative blog 2018-05-07, McKenzie et al. 2019). Similar to amphibians, susceptibility to ranavirus infections and manifestation of clinical signs in turtles vary depending on developmental stages (Duffus et al. 2015), species, and temperature (Allender et al. 2018). A study of Eastern box turtles (*Terrapene carolina carolina*) in the US reported a prevalence less than 5% in a population without abnormal mortality events (Allender et al. 2013), while in asymptomatic wild Eastern painted turtles (*Chrysemys picta picta*) the reported prevalence was between 4.8-31.6% in ponds without mortality events (Goodman et al. 2013). While ranavirus cases have been confirmed in North America, the few reports in Canada are potentially from a combination of a lack of surveillance in turtles, as ranavirus surveys are focused on amphibians, and subtle phenotypic manifestations of disease in turtles in general. Ranavirus can be transmitted via the water between amphibians and turtles (Brenes et al. 2014) so they can potentially act as reservoirs for each other. Understanding the ecology of this pathogen in populations of turtles is vital to their conservation and can have far reaching effects to other ectotherms.

All eight of Ontario's freshwater turtle species are listed as at risk federally where anthropogenic threats remain the main overarching reason for population declines (Ontario Endangered Species Act, 2007). In Canada, ranavirus presence in turtles was first identified in 2018 in a snapping turtle (*Chelydra serpentina*) from southern Ontario (McKenzie

et al. 2019). This turtle showed classical signs of disease (i.e., ulcerative conjunctivitis, necrotizing stomatitis, splenomegaly), and PCR and histopathology confirmed the ranavirus infection (McKenzie et al. 2019). However, a subsequent survey of cases using PCR showing similar clinical signs, as well as a survey of turtle road mortalities, showed zero prevalence of ranavirus infections (Carstairs, 2019).

In this study, we investigated the prevalence and ranavirus load in a random sample of turtles from southern Ontario who succumbed to road injuries. We used a quantitative PCR (qPCR) TaqMan assay, a more specific and sensitive method than the conventional PCR used in previous studies (Miller et al. 2015). TaqMan assays via qPCR are faster, more specific, and can be orders of magnitude more sensitive than conventional PCR (Balamurugan et al. 2009). Furthermore, we aimed to characterize the ranavirus lineages infecting turtles to compare with lineages known to infect amphibians. Our specific goals were to (1) use a more sensitive molecular technique (qPCR) to identify ranavirus infection in turtles, (2) compare the results of qPCR and PCR assays and infer if previous studies have underestimated ranavirus infections in turtles, (3) assess if positive individuals present tissue damage and other classical signs of ranavirosis, (4) assess which ranavirus lineages are present in positive individuals, and (5) to determine if the same lineages cross-infect both ectothermic organisms or whether different ranavirus lineages infect amphibians and reptiles. Our study adds to the knowledge of this virus by identifying whether a subclinical carrier state exists, and evaluating the sensitivity to infection and disease.

2. Materials and methods

2.1. Sampling

Turtles are admitted to Ontario Turtle Conservation Centre's

hospital (OTCC, operating name of the Kawartha Turtle Trauma Centre) from across their home range in Ontario, and beyond. The majority of admissions are due to road injuries, but OTCC also admits those found with any clinical signs of disease. More than 1000 turtles are admitted and treated annually, under Ministry of Natural Resources and Forestry Wildlife Custodian Authorization number 20025217. Veterinary work was carried out by Sue Carstairs (College of Veterinarians of Ontario licence 3649). The turtles who succumb to their injuries provide an opportunity to collect organ tissues for subclinical infection testing.

Forty-six turtles belonging to four different species were tested for ranavirus (Fig. 1). A total of 36 painted turtles (*Chrysemys picta*), 12 snapping turtles (*Chelydra serpentina*), 1 map turtle (*Graptemys geographica*) and 1 Blanding's turtle (*Emydoidea blandingii)* were tested. The tests were carried out on kidney and liver samples of a random sample of turtles with no clinical signs of disease and that had succumbed to traumatic injuries between 2014 and 2018. Samples from deceased turtles were collected from liver and kidney post-mortem using aseptic technique. Samples were stored in sterile containers containing no additives, and immediately frozen.

2.2. Laboratory methods and analysis

Tissue samples were extracted by magnetic bead using a MagneSil® Blood Genomic Max Yield System (Promega Corporation) on a Janus 96-well automated liquid handler (PerkinElmer). Three negative controls were included with each set of extractions. qPCRs were performed following Grant et al. (2019) and Picco et al. (2007), using the TaqMan Universal Master Mix (Applied Biosystems). Standard curves for the qPCR assay were generated using 10-fold dilutions between 10^4 to 10^{-3} plaque forming units (pfu/µL) of FV3-like cultured in *Epithelioma papulosum cyprini* (EPC) cells (Fijan et al. 1983). All samples were initially run as duplicates, and positive amplifications in at least one replicate were run again in duplicate. A sample was considered as a positive if at least three replicates showed amplifications at Ct < 34. A positive FV3 sample and blanks (PCR and extraction) were included in duplicates in each qPCR run. Three dilutions with known DNA concentrations (8 x 10^{-3} pfu/µL, 1.6 x 10^{-4} pfu/µL, 3.2 x10^{-4} pfu/µL) were also run in duplicates alongside samples. Total pfu per sample was considered as an average of all positive qPCRs.

Positive samples were amplified via PCR for the Major Capsid Protein (MCP, FV3 ORF 90R) gene and the viral homolog of eIF-2 (vIF-2α, FV3 ORF 26R) following Grant et al. (2019). Samples with positive amplifications were purified using ExoSAP (New England Biolabs) then sequenced using a Big Dye® Terminator version 3.1 cycle sequencing kit (Life Technologies) and run on an ABI 3730 sequencer. The same primers used for amplification were used in the sequencing reactions. Sequences were manually verified for errors in Geneious 8.1 (Kearse et al. 2012) and sequence similarity was determined through a Nucleotide BLAST search against the Nucleotide collection of *Iridoviridae* sequences. To evaluate if the same strains infecting amphibians are infecting turtles, we also sequenced the full genomes of positive samples. Long range PCRs, sequencing, and genome assembly were performed as described in (Vilaça et al. 2019a). To estimate recombination events within the sequenced genome, the software RDP4 (Martin et al. 2015) was used. The same parameters and reference genomes used by Vilaça et al. (2019a) were used. To estimate the phylogenetic relationships between previously sequenced genomes and the newly sequenced ranavirus genomes, we ran a Bayesian tree using MrBayes 3.2.6 (Ronquist et al. 2012), with a GTR + G evolutionary model, a total chain length of 1,100,000 generations sampled every 200, and 10% burn-in. Convergence (Effective Sample Size > 200) was calculated in Tracer 1.7 (Rambaut et al. 2018). The same genomes used in the phylogenetic tree in Vilaça et al. (2019a) were used.

To cross-validate our results, we sent a subsample (n = 6) of positive and negative samples (as per qPCR results; 1 positive and 5 negatives) to be tested using conventional PCR by an independent

laboratory. The samples were kept frozen and sent to the Animal Health Centre of British Columbia for viral testing via PCR. Samples were evaluated for ranavirus infection using the same method as described in Carstairs (2019). Analytical sensitivity of this PCR was estimated at approximately 3000 copies of genomic DNA (Dr. Tomy Joseph, personal communication). A positive control, an epizootic haematopoietic necrosis virus (EHNV) received from the University of Saskatchewan, was run along with the samples. PCR products were visualized in a 2% agarose gel stained with ethidium bromide under a UV documentation system.

To investigate histopathological changes in our samples as a consequence of ranavirus infection, formalinized liver and kidney samples from the two cases with high viral loads, were prepared routinely for histological examination.

A previous study by our group investigated the presence of ranavirus in waterbodies in southern Ontario (Vilaça et al. 2019b). To associate if areas with higher presence of ranavirus (as number of positive filtered capsules per waterbody) were associated with presence of turtles positively infected with ranavirus, we interpolated the results of sampled waterbodies by Vilaça et al. (2019b) with our qPCR results. Although the eDNA samples were more geographically limited when compared to the turtle's samples, 18 turtle locations overlapped with 79 sampled waterbodies for eDNA. Interpolation was done using the exact interpolator Inverse distance weighting method (IDW, Shepard, 1968). The resulting interpolated map showed ranavirus intensity based on the number of positive capsules (0 to 10) as determined by Vilaça et al. (2019b). IDW considers the geographical distance between sampled sites and interprets closer sites as having a stronger weight on ranavirus presence, with weight diminishing as distance between sites increases (Bataille et al. 2013). Interpolation was performed in ArcMap v10.4.1.

3. Results

Of the 46 individuals tested via qPCR, 7 individual turtles tested positive based on the established threshold of detection, with a total of 10 tissue samples positive (Table 1 and S1). Among the positive individuals, 5 were painted turtles and 2 were snapping turtles. Most individuals had a low viral load (< 0.2 pfu/µL), while two samples had high viral load (222.99 and 432.39 pfu/µL). These two samples were from painted turtles sampled in 2018 and 2014, respectively. For both samples, the highest viral load was for kidney samples, while liver samples were positive but had lower pfu values (24.73 and 0.21, respectively). The individuals with lower viral load were sampled in 2014 (N = 4) and 2018 (N = 1). Two were snapping turtles, while the remaining were painted turtles. The average viral load for these samples was 0.0098 pfu/µL (st dev = 0.0048), with a maximum value of 0.0165 (18–298, kidney) and a minimum of 0.0044 (14–022, liver). Only samples with high viral load tested positive for both liver and kidney tissues, the other samples tested positive for only one tissue. The three dilutions with known viral DNA concentrations (8 x 10^{-3} pfu/µL, 1.6 x 10^{-4} pfu/uL, 3.2 x10^{-4} pfu/µL) successfully amplified although with Ct > 34, as expected. This result indicates that even lower viral concentration thresholds could be used for positive turtle ranavirus infection detection.

Out of the six samples that were sent to be tested by a different laboratory using PCR, only one sample was deemed positive (sample, 14–255), and had the highest viral load of all of our samples. The remaining five sampleswere found to be negative via PCR were also negative in our qPCR tests (Table 1 and S1).

Among the 7 samples deemed positive by qPCR, we amplified and sequenced the viral major capsid protein (MCP) gene. All sequences for the MCP gene were identical to the FV3 reference (GenBank accession number AY548484). Four samples had a truncated vIF-2α gene, characteristic of FV3-like ranaviruses, and two had a non-truncated gene, characteristic of CMTV-like ranaviruses. A BLAST search revealed the full (non-truncated) vIF-2α allele was identical (100% identity) to the

Table 1
Description of results per sample. Asterisks in the "ID" column shows individuals with qPCR amplification below our detection threshold but were amplified and sequenced with PCR. Numbers in parenthesis in column "Average pfu (qPCR)" show the number of positive qPCRs (Ct < 34).PATU: Painted turtle, SNTU: Snapping turtle.

ID	Species	Age	Sex	Year	PCR	Average pfu (qPCR)	Tissue	Latitude	Longitude	MCP	vIF
14–022	PATU	Adult	M	2014	–	0.0044 (4)	Liver	44.496842	−78.837851	FV3	
14–180	PATU	Adult	F	2014	–	0.0007*	Kidney	44.424279	−78.005075		Non-truncated
14–194	PATU	Adult	F	2014	–	0.0029 (3)	Liver	44.844429	−77.888603	FV3	Truncated
14–255	PATU	Adult	F	2014	Positive	432.3907 (4)	Kidney	44.539835	−78.674741	FV3	Non-truncated (RCV)
14–255	PATU	Adult	F	2014	–	0.2139 (4)	Liver	44.539835	−78.674741		
14–262	PATU	Adult	F	2014	–	0.0051(4)	Kidney	44.433136	−77.894619	FV3	Truncated
14–262	PATU	Adult	F	2014	–	0.0133(4)	Liver	44.433136	−77.894619		
14–310	SNTU	Adult	F	2014	–	0.0114 (4)	Liver	44.935762	−78.713794	FV3	Truncated
18–298	SNTU	Adult	M	2018	–	0.0165 (3)	Kidney	44.8432	−79.3621	FV3	Non-truncated
18–452	PATU	Adult	F	2018	–	0.0081*	Kidney	44.659562	−79.25385	FV3	Non-truncated
18–600	PATU	Adult	M	2018	–	0.0057*	Kidney	43.3264686	−80.01912	FV3	Non-truncated
18–692	PATU	Adult	F	2018	–	222.9974 (4)	Kidney	44.7450043	−79.855802	FV3	Truncated
18–692	PATU	Adult	F	2018	–	24.7317 (4)	Liver	44.7450043	−79.8558026		

Chinese salamander iridovirus (CGSIV, KF512820) and Andrias davidianus ranavirus (ADRV, KF033124). The truncated vIF-2α alleles were identical (100%) to FV3 isolate spotted salamander Maine (SSME, KJ175144, (Morrison et al., 2014), Terrapene carolina carolina ranavirus (MG953518), and Trioceros melleri ranavirus (MG953519).

The formalinized samples of liver and kidney from the two positives with high viral load were examined histologically (Fig. 2); both individuals were painted turtles. Autolysis caused a loss of nuclear morphology in one sample, making interpretation challenging. There were also accumulations of postmortem bacteria. Multifocal acute cortical necrosis with heterophilic inflammation can be seen in the kidney of sample 18–692 (Fig. 2a). In addition, this case showed severe multifocal presumed hepatocellular necrosis with hepatocellular inflammation and possible intravascular thrombosis (Fig. 2b). The second case (14–255) showed improved morphology, but with subtle lesions that included acute, mild-moderate, locally extensive fibrinous and heterophilic peritonitis (Fig. 2c) and the kidney showed mild perivascular hilar fibrinous and heterophilic inflammation (Fig. 2d).

Only sample 18-692 amplified cleanly with the long-range PCRs, and was thus the only sample that had its genome sequenced. A total of 5,957,519 paired reads were obtained for one sample, and 6,112,190 reads mapped to the SSME (KJ175144) genome. The consensus sequence showed similarity to FV3 genomes, and a phylogeny confirmed that the closest genome was wild type FV3 (AY548484, Fig. 3). Of the recombinant events, one recombination involving ORFs 19R to 24R was detected (Fig. 4). The major parent was from a FV3-like ranavirus isolated in Canada (Vilaça et al. 2019a), and the minor parent was a CMTV-like virus isolated in the USA. While ORFs 20R, 23R and 24R have no predicted function, ORF 19R encodes an uncharacterized protein similar to ORF 10L from the lymphocystis disease virus 1 (LCDV1), ORF 21L is similar to ORF 56L from the infectious spleen and kidney necrosis virus (ISKNV), ORF 22R corresponds encodes a D5 family NTPase, which is a component of the DNA.

The geographic comparison of disease presence between the eDNA assessments for ranavirus (Vilaça et al. 2019b) and the locations of the turtles for this study showed concordance between number of positive capsules and presence of positive turtles (Fig. 5). A previous ranavirus survey in Ontario's waterbodies using eDNA (Vilaça et al. 2019b) showed a high prevalence of ranavirus near Peterborough, Ontario, Canada. The interpolated heat map showed that in regions with more positive filtered capsules for ranavirus eDNA (reddish regions), there were also more FV3 positive turtles, including a turtle with high viral load.

4. Discussion

Herein, we used a more sensitive technique (qPCR) that was able to identify lower viral loads in turtles in Ontario than assessed in previous surveys, and therefore showed a higher prevalence of ranavirus than previous reports. We also identified two samples with high viral load,
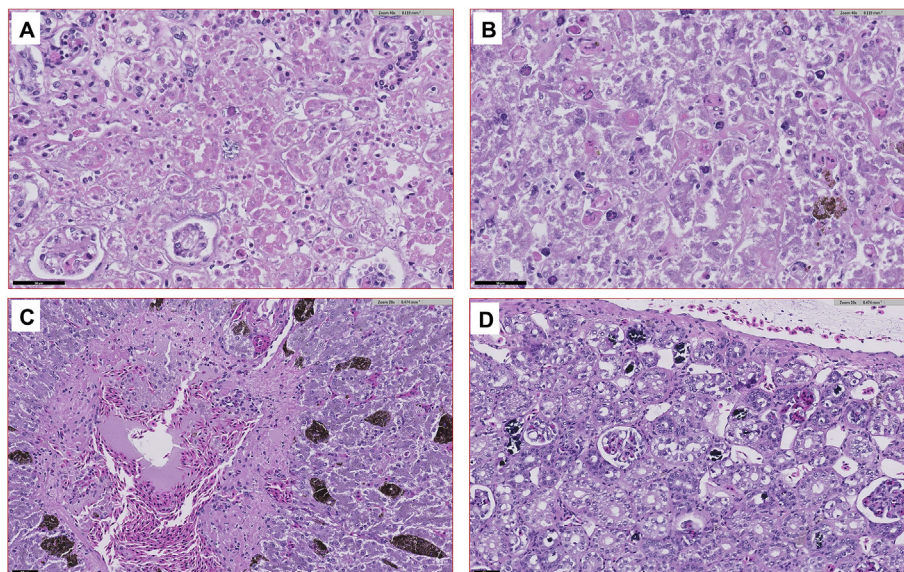

Fig. 2. Histopathology results for painted turtle samples with high viral load. A) Kidney sample of a painted turtle (18–692) with multifocal acute cortical necrosis with heterophilic inflammation. B) Liver sample (18–692) with severe multifocal presumed hepatocellular necrosis with hepatocellular inflammation and possible intravascular thrombosis. C) The second case (sample 14–255) showed improved morphology, but with subtle lesions that included acute, mild-moderate, locally extensive fibrinous and heterophilic peritonitis. D) Kidney sample of individual 14–255 with mild perivascular hilar fibrinous and heterophilic inflammation.
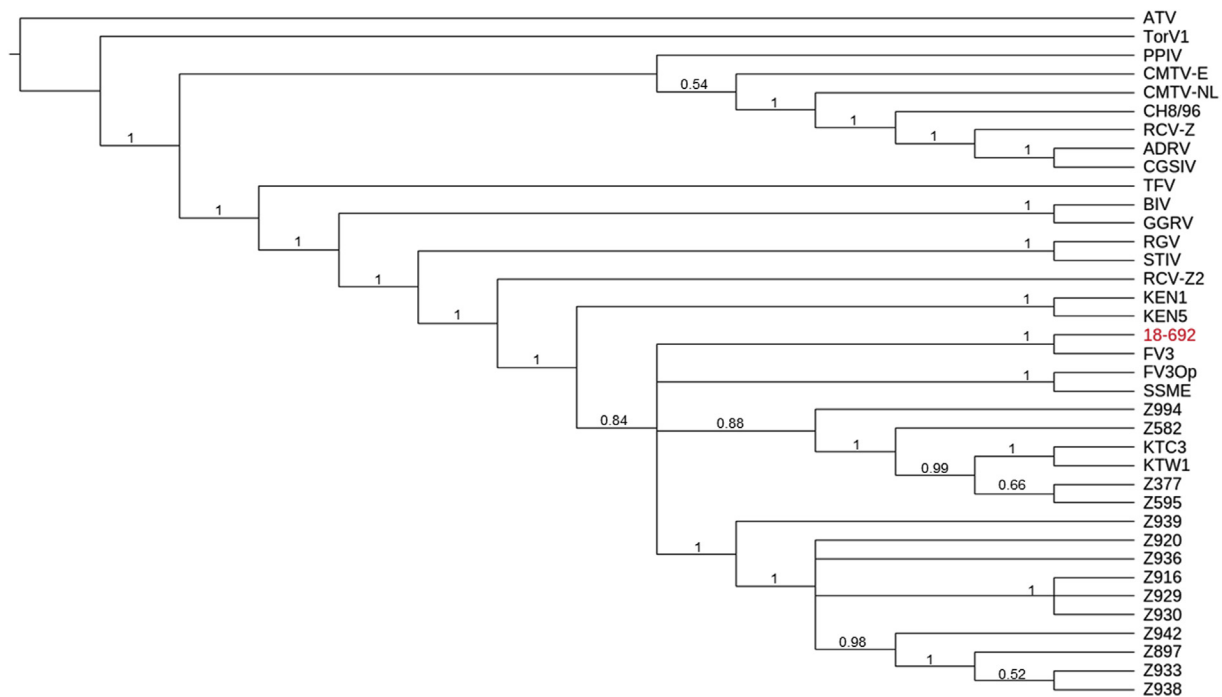
Fig. 3. Bayesian consensus tree depicting the newly sequenced genome (18–692) in red. All recombinant regions identified by RDP4 were excluded. Posterior Probabilities are shown by numbers in branches. GenBank references for all genomes can be found in Vilaça et al. (2019a). The ATV ranavirus was used to root the tree, following Jancovich et al. (2015).

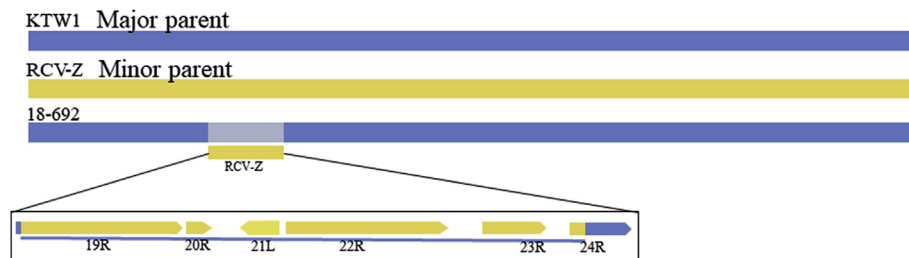**Fig. 4.** Schematic representation of the significant recombination event in sample 18–692 detected by RDP4. Colors in sample names represent the lineage of previously sequenced genomes: CMTV-like (RCV-Z, yellow) and FV3-like (KTW1, blue). The recombination event is represented by a colored rectangle, where colors reflect the minor parent. Below: Detailed representation of the recombination event. ORF colors correspond to minor (CMTV-like, yellow) and major (FV3-like, blue) parents. The blue bar represents the extension of the recombination event and is equivalent to colored rectangle.

although with no signs of ranavirosis. Our results were subsequently validated by PCR and sequencing to further assess the prevalence of ranavirus infection in turtles. We report seven individuals as positive for ranavirus with varying viral loads, beyond the 2 positive turtles previously reported in Ontario. Furthermore, we sequenced a partial and a complete genome from FV3 positive turtles, demonstrating that the same virus infecting amphibians in southern Ontario is also responsible for infecting turtles.

Subclinical infections are known to occur in amphibians and turtles (Gray et al. 2007; Allender et al. 2013). Conventional PCR testing is routinely done in amphibians and reptiles suspected to be infected with ranavirus (Miller et al. 2015; McKenzie et al. 2019), given rapid results and relative low cost when compared to other molecular methods. We show that qPCR, a more sensitive test, gives more accurate results as lower viral loads can be detected and is more appropriate for surveillance than conventional PCR despite the higher cost. The samples identified by qPCR as having a high viral load (greater than 220 pfu/μL) were subsequently also detected as positive by the PCR method employed by the previous studies, however the samples showing low (< 0.02 pfu/μL) or no viral load tested negative by PCR in the same facility that previous studies were done. Although all turtles tested were asymptomatic for disease, it is unknown whether these cases would have progressed to clinical disease, or whether the virus would subsequently be cleared from the body. To answer this question, a series of

qPCR tests on live positive turtles should be performed to determine if this infection may remain static, progress, or may be eliminated. This is important to determine in order to decide whether positive cases may be 'cured' and subsequently released.

The location of the positive turtle cases overlaid with the location of waterbodies previously shown to be carrying the virus show the high prevalence of ranavirus presence in southern Ontario (Vilaca et al. 2019b). This, in combination with the relatively large number of subclinical cases with very low prevalence of clinical disease (zero), suggests that these turtles may carry a low sensitivity to clinical infection. Unfortunately, since the virus may be transmitted via the water between ectotherm species (Bandín and Dopazo, 2011; Brenes et al. 2014), the ongoing carrier state does presents opportunities to perpetuate the virus in the water bodies and therefore act as a reservoir for other species. While amphibians have been suggested as potential reservoir hosts for chelonians (Johnson et al. 2008), other studies suggested that reptiles can act as asymptomatic carriers of ranaviruses (Goodman et al. 2013, 2018; Brenes et al. 2014). Reptiles can act as host and transmit ranavirus to amphibians, and while reptiles do not experience mortality, infected amphibian larvae can experience up to 100% mortality (Brenes et al. 2014). Tadpole die-offs have been observed around the Peterborough region in Ontario, Canada and ranavirus presence has been shown to increase in late summer when tadpoles and metamorphizing amphibians are more prevalent (Vilaça et al.
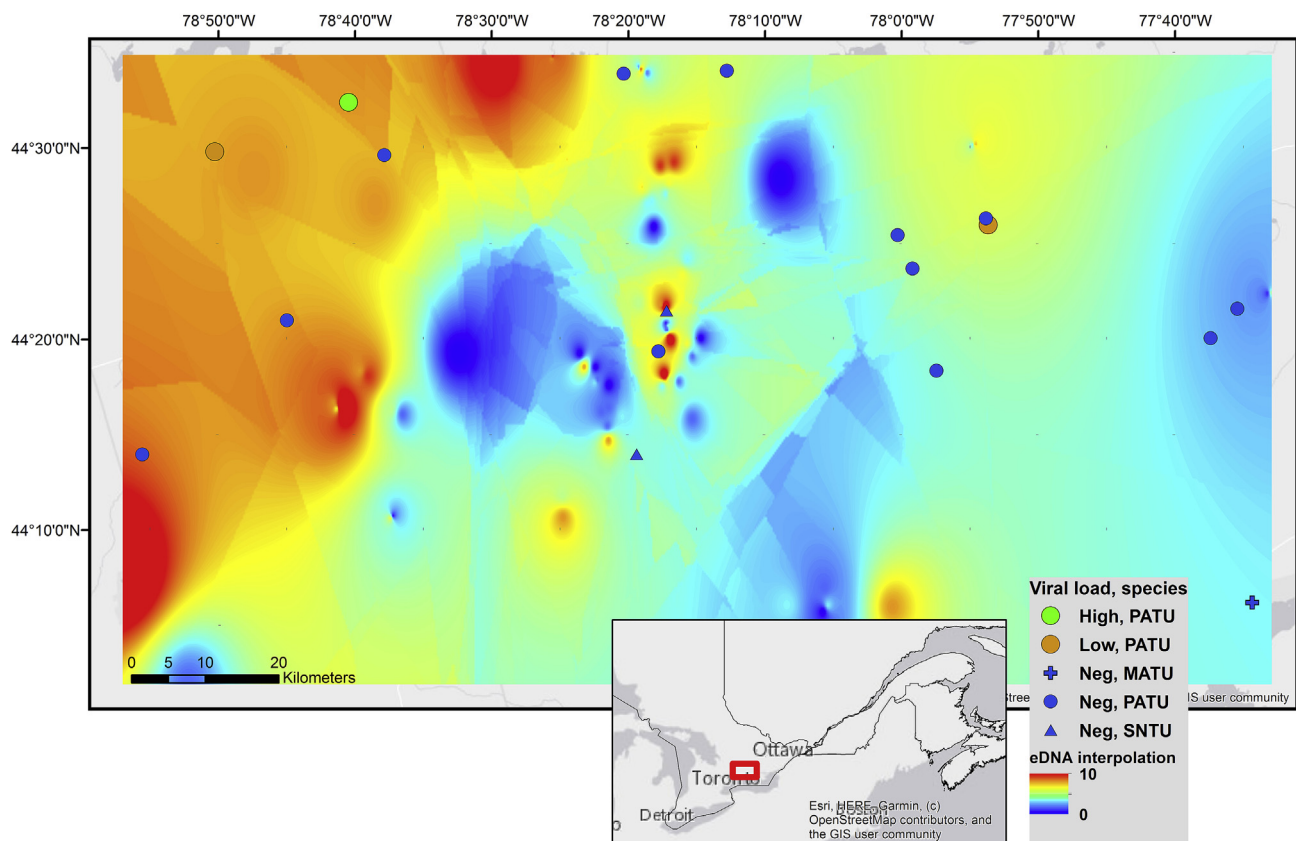
Fig. 5. Interpolation of spatial variation in ranavirus prevalence. Interpolation was generated with IDW method. Intensity values are based on the number of positive capsule samples from each site out of 10, and follow a gradient of colour-coded values from 0 (darker blue) to 10 (darker red). Turtle samples are also shown, and colors follow Fig. 1.

2019b). Further, diverse anuran and caudate species are infected with FV3 in the Peterborough region (Duffus et al., 2008). Our observations correlate with results from Brenes et al. (2014), suggesting amphibian larval hosts act as amplification hosts while reptiles are reservoirs for other species.

The clinical signs of ranavirus infection in reptiles can be variable, which reinforces the need of multiple and reliable diagnostic tests such as PCR, qPCR, ELISA, and histopathology (Miller et al. 2015). Ranavirus has been responsible for high mortality in other turtle species, with one group of Mediterranean tortoises (*Testudo hermannii*) reported to have 100% mortality (Marschang et al. 1999). In this species, reported clinical signs included fibrinonecrotic oral ulceration, conjunctivitis, cellulitis, and subcutaneous edema (Marschang et al. 1999; De Voe et al. 2009). Internally, esophagitis, fibrinous and necrotizing splenitis, and multicentric fribrinoid vasculitis were also observed (Johnson et al. 2008), as well as necrotizing tracheitis and pneumonia. Obvious and nonspecific external lesions include marked blepharitis, and cervical edema (Miller et al. 2015). Ranavirus clinical signs are not pathognomonic (i.e., distinctive characteristics of the disease) and include sudden onset of severe illness or sudden death with no premonitory signs (Allender et al. 2011). These indicators can appear similar to those of other infectious agents such as mycoplasma and herpesvirus infections, bacterial infection secondary to trauma, as well as non-infectious issues such as Vitamin A deficiency.

Although the hepatic and renal histopathology results obtained from the two positive samples with high viral load were not as definitive or marked as those observed by McKenzie et al. (2019), and we cannot be sure that the changes seen were caused by ranavirus, they appeared to be consistent with lesions typical for ranavirus infection, including intravascular thrombosis and necrotizing hepatitis (Johnson et al. 2008; Allender et al. 2013; Adamovicz et al., 2018Zhang et al.,

2011). These more subtle lesions tie in with the lack of clinical signs observed in these turtles. The gross clinical signs of ulcerative stomatitis and ulcerative skin lesions, that are typical of ranavirus, as well as herpesvirus, have been seen at the Ontario Turtle Conservation Centre (OTCC) as both fresh lesions and older, scarred lesions, without evidence via PCR or qPCR of infection (unpublished results). Further qPCR testing in turtles received at the OTCC will help determine if these lesions are symptoms from ranavirus infection.

Recombinants between FV3 x CMTV were previously detected in the same region of our samples (Grant et al. 2019; Vilaça et al. 2019a) using the same methods used in this study. Recombinant ranaviruses between FV3- and CMTV-like were deemed as more virulent than non-recombinant (wild type) FV3 (Claytor et al. 2017). The fact that similar ranaviruses found in amphibians in Ontario, Canada were also found infecting turtles demonstrates that ranaviruses are being transmitted between different classes of vertebrates. The majority of samples from 2014 had a truncated allele in the viF-2α gene, with the exception of the sample with high viral load, while samples from 2018 all had non-truncated copies in the viF-2α gene. While the sample size within the current study is relatively small, it is possible that the recombinant strain (as defined by Grant et al. 2019) is becoming more common in southern Ontario. The presence of a more possible more virulent lineage in southern Ontario should be further monitored and its impact in wild amphibians and turtle communities evaluated.

The genome sequence showed a single recombination event. This event was previously detected in the wild type FV3 genome (AY548484), although it was not detected in genomes derived from tadpoles isolated in the same region (Vilaça et al. 2019a). Our results suggest that different viruses are circulating in southern Ontario, including a ranavirus similar to wild type FV3.

Our results have important implications for understanding pathogen

dynamics in southern Ontario. The presence of subclinical infections in turtles suggests that diverse hosts are involved in ranavirus transmission and may be acting as reservoirs. Future studies should assess if turtles are capable of clearing ranavirus infection and if disease manifestation is dependent on the individual ranavirus load. Furthermore, future studies should use more sensitive molecular techniques (like qPCR) combined with DNA sequencing (possibly genome sequencing) in order to avoid false negatives and allow lineage identification in positive samples.

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Database linking

Sequences are available upon request.

CRediT authorship contribution statement

Sue Jacqueline Carstairs: Conceptualization, Funding acquisition, Investigation, Methodology, Writing - original draft. **Christopher J. Kyle:** Conceptualization, Methodology. **Sibelle Torres Vilaça:** Conceptualization, Formal analysis, Investigation, Methodology, Writing - original draft.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Sue Jacqueline Carstairs is employed as the Executive and Medical Director of the Ontario Turtle Conservation Centre (Kawartha Turtle Trauma Centre). The other authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.virol.2020.01.016.

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