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NOTE

Chelonid alphaherpesvirus 5 qPCR of archived blood and skin samples underpredicts infections in juvenile green turtles *Chelonia mydas*

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ABSTRACT: Chelonid alphaherpesvirus 5 (ChHV5) is the putative pathogen of fibropapillomatosis, a tumor-forming sea turtle disease. Quantitative polymerase chain reaction (qPCR) techniques are commonly applied to evaluate the presence and quantity of ChHV5 in blood and tissue samples. However, samples can have little or no virus present despite coming from infected individuals. In addition, sample age and storage may further impact ChHV5 detection. This creates uncertainty in the interpretation of ChHV5 qPCR results for archived (>1 yr old) blood and skin because a negative sample does not imply an individual was free of ChHV5 infection, but rather that the sample itself was free of viable ChHV5 DNA. To assess the ability of qPCR to detect ChHV5 infections from archived samples, we tested blood, skin, and tumor samples collected from juvenile green turtles *Chelonia mydas* captured in the Indian River Lagoon, Florida, USA, between 1995 and 2018. Sample ages ranged from 1 to 24 yr old at the time of molecular analyses. We found very low overall prevalence of ChHV5 in blood (1.4% positive, n = 513) and skin (6.5% positive, n = 123) samples, and high prevalence in tumor samples (89.7% positive, n = 39). These results suggest that qPCR of archived blood and/or skin samples provides little inference on individual-level infection status.

KEY WORDS: Fibropapillomatosis · Chelonid alphaherpesvirus 5 · ChHV5 · *Chelonia mydas* · Indian River Lagoon · Disease ecology · Pathogen detection · qPCR

1. INTRODUCTION

Fibropapillomatosis (FP) is a tumor-forming sea turtle disease that commonly affects juvenile green turtles *Chelonia mydas* in coastal habitats (Herbst 1994, Work et al. 2004). Chelonid alphaherpesvirus 5 (ChHV5) is associated with FP tumors and is the putative pathogen of FP (Herbst et al. 1995, Kang et al. 2008). While there is strong evidence that ChHV5 infection is necessary for FP to occur, individuals can be infected with ChHV5 and present no signs of disease (Alfaro-Núñez et al. 2016, Lawrance et al. 2018, Page-Karjian et al. 2020). Therefore, the relative role of ChHV5 in the development of FP is unclear. Investigations of the infection dynamics of ChHV5 are necessary to better understand the cause of FP (Dupont et al. 2024, Whilde et al. 2024).

ChHV5 presence can be detected in blood and skin samples via quantitative polymerase chain reaction (qPCR) techniques (Quackenbush et al. 2001, Page-Karjian et al. 2015). This method has commonly been applied to assess ChHV5 infection dynamics in groups of sea turtles (Mashkour et al. 2021, Perrault et al. 2021). While qPCR is highly sensitive (Page-

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Karjian et al. 2015), samples collected from infected individuals may have little or no ChHV5 present due to latency and localization of herpesviruses (Kang et al. 2008, Alfaro-Núñez et al. 2016, Farrell et al. 2021), making it difficult to detect infection status of ChHV5 in non-tumored turtles.

Sample age and storage techniques may also impact the results of ChHV5 molecular analyses. Older archived samples may have some DNA degradation that can limit qPCR efficiency, especially when target DNA exists in low quantities, as would be expected for a pathogen like ChHV5 (King et al. 2009, Harkins et al. 2014, Muletz et al. 2014). Different sample storage techniques can lead to varying levels of DNA degradation (Kilpatrick 2002, Permenter et al. 2015). Older samples can also be more impacted by PCR inhibitors that limit the effectiveness of qPCR (King et al. 2009, Harkins et al. 2014). Sea turtle blood and skin sample archives are often available from previous turtle genetic studies and/or general sampling from long-term monitoring programs and could be used to assess ChHV5 infection dynamics. However, these sample archives are often composed of older samples with inconsistent sample collection and storage over time. Therefore, it is important to consider whether sample age and storage methods affect ChHV5 qPCR for different sample types, especially commonly collected blood and skin samples.

In this study, we assessed the viability of using qPCR of archived blood and skin samples to assess ChHV5 infection dynamics in an aggregation of juvenile green turtles. We leveraged a large temporal sample archive (1995–2018) of blood, skin, and tumor tissues collected from juvenile green turtles with and without FP by the University of Central Florida Marine Turtle Research Group (UCFMTRG) in the Indian River Lagoon (IRL), Florida, USA. This site has high FP prevalence (~50%; Hirama & Ehrhart 2007, Kelley et al. 2022) and therefore would be expected to also have high ChHV5 infection prevalence. Here, we summarized qPCR results for ChHV5 viral presence and quantity from juvenile green turtles for sample groups of varying tissue types (blood, skin, and tumor), sample ages, and storage methods.

2. MATERIALS AND METHODS

2.1. Sample collection and storage

The UCFMTRG conducted periodic sea turtle sampling sessions (typically twice each month) in the IRL (approximately 27.8312, −80.4395; WGS84) starting in 1982 (Ehrhart et al. 2007). Turtles were captured using large-mesh entanglement nets, and the presence or absence of external FP tumors was recorded for each turtle captured (Ehrhart et al. 2007, Kelley et al. 2022). Blood and tissue sample collection effort varied over the course of the study. From about 1995 to 2013, blood samples were collected primarily for genetic studies, and skin was typically only collected if a blood sample could not be obtained. Therefore, each individual would only have one sample type collected: blood or skin. Starting in 2014, both blood and skin sample collection were attempted for every individual turtle. Blood samples were collected from the dorsal cervical sinus and typically stored in 2 ways: (1) a few drops of blood were stored at room temperature in a lysis buffer solution (lysis buffer storage); and (2) the remaining blood was spun to separate the plasma layer, with both the plasma and remaining blood stored in a freezer in separate vials (frozen storage). Blood samples prior to 2006 were typically stored both ways, while samples after 2006 were typically only stored frozen, with a few also stored in lysis buffer. Skin samples prior to 2006 were biopsied from the proximal fleshy portion of either shoulder, and thereafter were biopsied from the proximal fleshy portion of the trailing edge of either rear flipper. All skin samples were stored at room temperature in vials with 95% ethanol. Starting in 2015, tumor samples were collected as was done for skin.

Given the variable handling and storage procedures used on archived samples over time, we grouped samples as 'blood: lysis buffer, 18–24 yr' (blood in lysis buffer collected during 1995–1999, ~18–24 yr old at processing, n = 121), 'blood: frozen, 11–19 yr' (frozen blood collected during 2000–2006, ~11–19 yr old at processing, n = 243), 'blood: lysis buffer, 4–9 yr' (blood in lysis buffer collected during 2010–2013, ~4–9 yr old at processing, n = 51), 'blood: frozen, 1–3 yr' (frozen blood collected during 2016–2018, ~1–3 yr old at processing, n = 98), 'skin: shoulder, 18–20 yr' (shoulder skin samples collected in 1999, ~18–20 yr old at processing, n = 33), and 'skin: rear flipper, 1–3 yr' (rear flipper skin samples collected during 2016–2018, ~1–3 yr old at processing, n = 90). We also evaluated a subset of 39 turtles with external FP tumors captured between 2016 and 2018 (~1–3 yr old at processing) that had samples of blood, skin, and tumor tissues to allow for direct comparisons across sample types. Overall, using molecular analyses, we assessed 513 blood samples, 123 skin samples, and 39 tumor samples from 573 green turtles captured between 1995 and 2018.

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2.2. Molecular analyses

We used Qiagen DNeasy Blood and Tissue Kits to extract genomic DNA (gDNA) from each sample. For frozen blood samples, we followed the standard protocol for nucleated blood DNA extraction with the DNeasy Blood and Tissue Kit. For lysis buffer blood samples, we modified the first step of the nucleated blood extraction protocol by using 200 µl of the lysis buffer sample instead of 10 µl of blood with 190 µl of phosphate-buffered saline (PBS). For skin and tumor samples, we followed the standard DNA extraction protocol for tissue. We obtained 2 eluates for each DNA extraction: a first eluate using just 50 µl of buffer AE instead of the standard 200 µl to increase DNA concentration, and a second eluate with 200 µl of buffer AE to create a less concentrated extraction. All DNA extractions were stored frozen (−80°C) until later analyses, with all molecular analyses occurring within 2 yr of DNA extraction.

We tested DNA extractions for the presence and quantity (number of gene copies; viral load) of ChHV5 using the qPCR protocol developed in Page-Karjian et al. (2015). The qPCR protocol targets a 173 bp sequence of the DNA polymerase gene UL30 (forward primer: AAC GCT TGC TTT TGG ACA AG; reverse primer: CCA GCG GGT GTG AAT AAA AT; probe: 5′-6-FAM-TGG CCA TCA-ZEN-AGC TGA CGT GCA-3′). We modified the qPCR protocol to have 21.80 µl reactions containing 10 µl of SsoAdvanced Universal Probes Supermix (Bio-Rad), 0.80 µl of 10 µM forward primer, 0.80 µl of 10 µM reverse primer, 2.00 µl of 1 µM ChHV5 pol probe, and 8.20 µl of DNA extraction template. For blood and skin samples, which are likely to have low levels of ChHV5 if present, we used the first, more concentrated elution of DNA. For tumor samples, which likely have high ChHV5 concentrations, we used the second, more dilute elution. Each qPCR plate included serial dilutions of a gBlock standard solution of the target sequence, a positive control (known ChHV5-positive FP tumor DNA extraction), and a negative control (water). Reactions followed the conditions in Page-Karjian et al. (2015): 10 min at 95°C, then 40 cycles of 30 s at 95°C and 1 min at 55°C. Reactions were run on a Bio-Rad CFX96 Real-Time System, and results were analyzed using Bio-Rad CFX Manager software (v.3.1). Each sample was initially tested once via qPCR. Samples that amplified before cycle 40 were considered potentially positive, and all others were considered negative. Potentially positive samples were tested a second time via qPCR and were only considered positive if the sample again amplified before cycle 40, and at least one of the 2 runs amplified before cycle 38, with the rest of the samples being considered negative. For positive samples, ChHV5 viral loads were determined by averaging the results of both qPCR runs.

These are relatively relaxed standards for considering a sample as positive that deviate from standard qPCR guidelines (Bustin et al. 2025). We used these criteria because blood samples were likely to have low viral loads, possibly near the reported limit of detection for this qPCR assay (50 gene copies; Page-Karjian et al. 2015), and we expected many samples to be considered negative by strict standards due to these low viral loads. We preferred to classify samples with some evidence of viral amplification as positive to give an upper limit to the extent to which archived blood and skin samples are viable in detecting a ChHV5 infection.

3. RESULTS

We tested for ChHV5 via qPCR in 675 samples (513 blood, 123 skin, 39 tumor) from 573 juvenile green turtles captured between 1995 and 2018, including 292 without and 281 with external FP tumors at the time of sampling. Overall, just 1.4% and 6.5% of blood and skin samples tested positive for ChHV5, respectively, while 89.7% of tumor samples tested positive (Table 1). Only one of the 292 blood and skin samples from turtles without FP tested positive for ChHV5: a skin sample from a 44.8 cm SCL turtle captured in September 2018.

Blood sample groups for every storage type and age had very low positive rates for ChHV5, with the highest rate being 5.4% from FP turtles in the 'blood: lysis buffer, 18−24 yr' group. For skin samples, there was a small sample size for the oldest skin sample group ('skin: shoulder, 18−20 yr'), with none of the 33 samples testing positive. The sample group 'skin: rear flipper, 1−3 yr' had the highest ChHV5 positive rate of all non-tumor samples, with 15.6% of samples from FP turtles testing positive, along with producing the only positive from a non-FP turtle. For positive samples, tumors had the highest viral loads, followed by skin and then blood.

Of the 39 turtles collected in 2016−2018 for which skin, blood, and tumors were tested, 2 blood samples (5.1%), 6 skin samples (15.4%), and 35 tumor samples (89.7%) tested positive (Fig. 1). Both turtles with positive blood samples also had positive skin and tumor samples. Five of the 6 positive skin samples also had

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Table 1. Summary of chelonid alphaherpesvirus 5 (ChHV5) qPCR test results for archived blood, skin, and tumor samples of juvenile green turtles *Chelonia mydas* with and without fibropapillomatosis (FP) captured from 1995 to 2018 in the Indian River Lagoon, FL, USA. Approximate sample ages for each group are noted in the sample group description and ranged from 1 to 24 yr old. Viral load is shown in log genome equivalents

Sample	Sample group description	ChHV5 prevalence			Average viral load for positives (range)
		FP turtles	Non-FP turtles	All turtles	
Blood	Blood in lysis buffer (~18–24 yr)	4/74 (5.4%)	0/47 (0%)	4/121 (3.3%)	13.1 (5.83–22.57)
	Blood frozen (~11–19 yr)	0/122 (0%)	0/121 (0%)	0/243 (0%)	
	Blood in lysis buffer (~4–9 yr)	1/28 (3.6%)	0/23 (0%)	1/51 (2.0%)	
	Blood frozen (~1–3 yr)	2/54 (3.7%)	0/44 (0%)	2/98 (2.0%)	
	All blood samples	7/278 (2.5%)	0/235 (0%)	7/513 (1.4%)	
Skin	Shoulder skin (~18–20 yr)	0/21 (0%)	0/12 (0%)	0/33 (0%)	538.8 (31.2–1739)
	Rear flipper skin (~1–3 yr)	7/45 (15.6%)	1/45 (2.2%)	8/90 (8.9%)	
	All skin samples	7/66 (10.6%)	1/57 (1.8%)	8/123 (6.5%)	
Tumor	All tumors (~1–3 yr)	35/39 (89.7%)	–	35/39 (89.7%)	3.83×10^5 ($68.6–2.69 \times 10^6$)

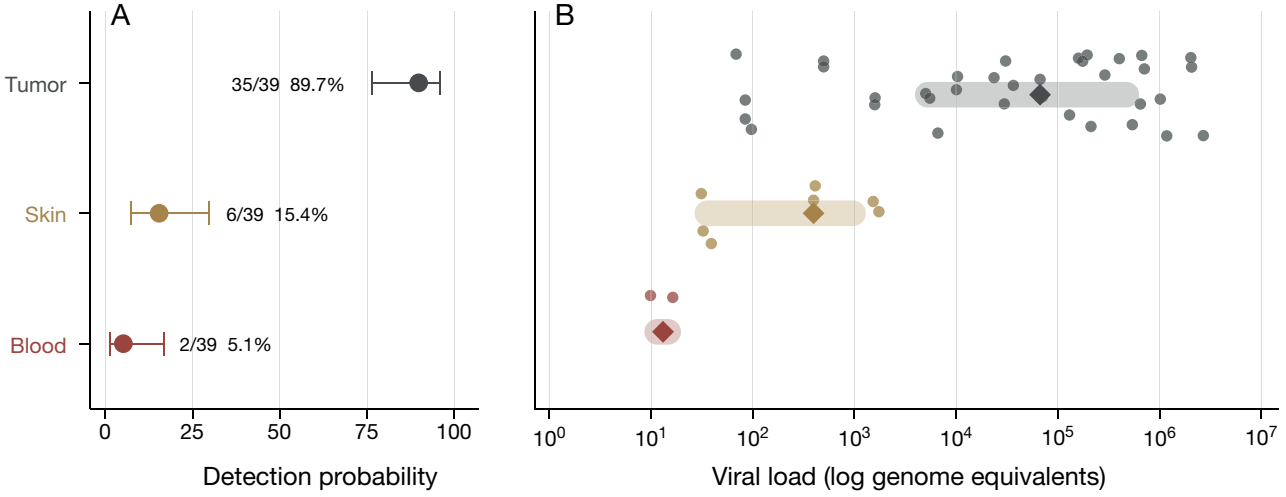


Fig. 1. Results of chelonid herpesvirus 5 (ChHV5) qPCR testing of blood, skin, and tumor samples collected from 39 juvenile green turtles *Chelonia mydas* with fibropapillomatosis (FP) captured during 2016–2018 in the Indian River Lagoon, FL, USA. Samples were 1–3 yr old at time of processing. (A) ChHV5 detection probability via qPCR for each sample type. Points: proportions of positive samples for each sample type; error bars: Wilson 95% confidence intervals. (B) ChHV5 viral loads of positive samples for each sample type. Points: individual samples; diamonds: median values; shaded areas: interquartile ranges

positive tumor samples, but one positive skin sample came from a turtle with a negative tumor sample.

4. DISCUSSION

Our results suggest that qPCR testing of archived blood or skin samples for ChHV5 presence is likely not reliable for detecting whether an individual turtle is infected. Our large sample set had very few samples test positive for ChHV5 despite coming from an aggregation of turtles with high FP prevalence. For turtles without FP, no blood samples and only one skin sample tested positive for ChHV5, suggesting limited use of these archived samples to characterize ChHV5 infections in non-FP turtles.

When comparing blood and skin samples, blood samples were particularly unreliable for detecting ChHV5, as there were few positive samples and all positives had viral loads below the reported limit of detection for this qPCR technique (50 gene copies; Page-Karjian et al. 2015). Skin samples did show some evidence of consistency for detecting ChHV5 infections, with all positives having viral loads well above the limit of detection. Tumor tissues consistently tested positive for ChHV5 (35 out of 39, 89.7%)

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with high viral loads, consistent with previous analyses (Quackenbush et al. 2001, Page-Karjian et al. 2015, Alfaro-Núñez et al. 2016). Given that tumors have very high viral loads, there are sufficient quantities of viral DNA in archived samples despite some degradation.

Low ChHV5 detection in older sample groups brings into question the viability of using archived samples for ChHV5 qPCR testing. The uncertainty of DNA quality for older samples makes it difficult to determine whether degraded or inhibited samples led to false negatives or whether there were simply very few actual positives, a common issue in assessing pathogen presence in older samples (Harkins et al. 2014, Muletz et al. 2014, Stone & Ozga 2019). In addition, archived samples like those in this study do not align with established qPCR minimum standards for sample storage and handling (rapid freezing storage of samples, avoiding thawing and refreezing, short interval from collection to qPCR analysis; see Bustin et al. 2025), limiting robust assessments that are comparable to other studies. Therefore, we recommend using caution in interpreting ChHV5 infection dynamics from qPCR of archived samples.

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