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CHELONID ALPHAHERPESVIRUS 5 IN CUTANEOUS TUMORS OF GREEN TURTLES (CHELONIA MYDAS) STRANDED IN NORTHEASTERN BRAZIL

(*Chelonid alphaherpesvirus* 5 em tumores cutâneos de tartarugas verdes (*Chelonia mydas*) encalhadas no nordeste do Brasil)

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ABSTRACT - Green turtles (*Chelonia mydas*) are affected by fibropapillomatosis (FP) by causing tumors and this disease represents a threat to the species. In this study, we described the histopathological features of FP tumors and survey for the presence of *Chelonid alphaherpesvirus 5* (ChHV5) DNA in FP tumors collected from 26 green turtles stranded in the Potiguar Basin (northeastern Brazil). The animals were found during daily monitoring. The FP tumors were collected via biopsy or necropsy and were quantified and analyzed according to anatomical distribution, size, and Southwest Atlantic Fibropapillomatosis Score (FPS_{SWA}). We examined 26 histological sections and analyzed 26 formalin-fixed paraffin-embedded samples by polymerase chain reaction (PCR) to detect ChHV5 DNA. We analyzed 292 tumors (1-42 per individual), most of them between 1-4 cm (74%; 218/292 tumors) occurred in the forelimbs (48.6%; 142/292 tumors). According to FPS_{SWA}, most green turtles were mildly affected (53.8%; 14/26). Histopathological examination revealed features described in the literature (e.g., papillary projections of the epithelial layer, hyperkeratosis, basal layer hyperplasia, well-differentiated proliferating fibroblasts), classifying the FP tumors as papillomas or fibropapillomas. The PCR analyses showed that 76.9% of tumors were positive for ChHV5

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and sequences were obtained from all amplified samples. Our UL30 sequences were identical to several sequences described for sea turtles in Brazil, while the UL18 and UL27 sequences were more similar to the findings for green turtles in Florida (USA). Our study reports molecular detection and characterization of ChHV5 in juvenile green turtles stranded in the Potiguar Basin, an important feeding area with high FP frequency.

Key words - Potiguar Basin; Neoplastic disease; Herpesvirus; Sea turtles.

RESUMO - As tartarugas-verdes (*Chelonia mydas*) são afetadas pela fibropapilomatose (FP), doença que pode ser uma ameaça para elas. O trabalho teve como objetivo descrever as características histopatológicas dos tumores sugestivos de FP e pesquisar a presença de DNA de *Chelonid alphaherpesvirus 5* (ChHV5) em lesões cutâneas de 26 tartarugas verdes encalhadas na Bacia Potiguar (nordeste do Brasil). As tartarugas verdes foram encontradas durante o monitoramento diário, e os tumores de FP foram coletados por biópsia ou durante necropsia, quantificados e analisados de acordo com distribuição anatômica, tamanho e Escore de Fibropapilomatose do Atlântico Sudoeste (FPS_{SWA}). Vinte e seis cortes histológicos foram examinados, e 26 amostras fixadas em formalina e embebidas em parafina foram analisadas por reação em cadeia da polimerase (PCR) para detectar o DNA do ChHV5. Um total de 292 tumores foi analisado (1-42 por indivíduo), sendo a maioria com tamanho de 1-4 cm (74%; 218/292 tumores) e presentes nos membros anteriores (48,6%; 142/292 tumores). De acordo com o $FPS_{SWA,}$ a maioria das tartarugas verdes estava afetada de forma leve (53,8%; 14/26). O exame histopatológico revelou características previamente descritas na literatura (projeções papilares da camada epitelial, hiperceratose, hiperplasia da camada basal, fibroblastos em proliferação bem diferenciados) classificando os tumores como papilomas ou fibropapilomas. Com relação aos resultados de PCR, 76,9% das amostras de tumor testadas foram positivas para ChHV5 e as sequências foram obtidas em todas as amostras amplificadas. Nossas sequências UL30 são idênticas a várias sequências descritas em tartarugas marinhas do Brasil, enquanto as sequências UL18 e UL27 são mais semelhantes as de tartarugas verdes na Flórida. Nosso estudo relata detecção e caracterização molecular do ChHV5 em tartarugas verdes encalhadas na Bacia Potiguar, importante área de alimentação com alta frequência de ocorrência de FP.

Palavras-chave - Bacia Potiguar; doença neoplásica; herpesvírus; tartarugas marinhas.

INTRODUCTION

Green turtles (*Chelonia mydas*) are exposed to numerous threats (e.g. pollution, habitat loss, climate change, fisheries bycatch, and marine debris intake) (Van Houtan et al., 2010). Conservation efforts to reduce these threats have helped to recover green turtle populations (Chaloupka et al., 2008). However, pathogens and disease outbreaks also contribute significantly to morbidity and mortality in this vulnerable species (Jones et al., 2016).

Fibropapillomatosis (FP) is a neoplastic disease (Hamann et al., 2010) characterized by cutaneous tumors that can be sessile or pedunculated with smooth or verruciform texture with varying coloration and size, arising anywhere on the body surface of affected turtles, including the eyes (Zwarg et al., 2014; Rossi et al., 2016). Internal fibromas, myxofibromas, and fibrosarcomas have been also reported (Work, Balazs et al., 2004; Dutra et al., 2012).

Data on FP prevalence in green turtles have been collected along the coast and oceanic islands of Brazil, ranging from 0.04% (between 2004 and 2014; n = 2431) to 32.59% (between 2000 and 2014; n = 2940) in Rio Grande do Sul and Ceará States (Brazil), respectively (Baptistotte, 2016). Research on sea turtle strandings, recorded in the Potiguar Basin (Rio Grande do Norte and Ceará States, Brazil), revealed that green turtles represented 99% (604/610) of all FP-affected individuals and an increase of relative FP frequency from 13.2% in 2012 (65/494) to 35.3% in 2015 (228/646) was observed (Silva-Junior et al., 2019).

Chelonid alphaherpesvirus 5 (ChHV5) is considered the primary etiological FP agent (Quackenbush et al., 2001; Cárdenas et al., 2019; Domiciano et al., 2019). The epidemiology and pathogeny of ChHV5, including its infection route, clinical manifestations and association with lesions are still under discussion (Page-Karjian et al., 2015, 2017). The detection of ChHV5 DNA in clinically healthy tissues has been regarded as indicative persistent or suggestive latent infections (Alfaro-Núñez et al., 2016; Page-Karjian et al., 2017), as described for other alphaherpesviruses (Chen e Hudnall, 2006; Gilden et al., 2011). Besides ChHV5 infection, other factors possibly associated to FP occurrence and prevalence include environmental factors, such as water quality, land use, and pollutants (Santos et al., 2010; Van Houtan et al., 2010; Keller, 2013; Jones et al., 2016).

Due to the life history of sea turtles and their aggregation on foraging lands, interspecific co-infection by well-differentiated geographic variations of ChHV5 has been suggested. Moreover, evidence of ChHV5 evolution via recombination was observed between different strains from Hawaii and Florida (USA), which may influence ChHV5 virulence (Morrison et al. 2018). In Brazil, ChHV5 DNA detection in green turtles has been only performed in samples from southern and southeastern regions (Rodenbusch et al., 2014; Monezi et al., 2016; Domiciano et al., 2019); however, to our knowledge, there is no information available from the northeastern part of Brazil. The northeastern cost of Brazil is considered important habitat of green turtles (Farias et al., 2019), since most sea turtles stranded are green turtles (Poli et al., 2014; Farias et al., 2019). In this study, we aimed to describe the histopathological features of FP tumors and detect ChHV5 DNA in these lesions from green turtles stranded in the Potiguar Basin, northeastern Brazil, using polymerase chain reaction (PCR) protocols (Alfaro-Núñez e Gilbert., 2014; Page-Karjian et al., 2015; Chaves et al., 2017).

MATERIAL AND METHODS

Green turtles and study site

We examined 26 stranded green turtles found by a trained team during daily monitoring during 2010–2016 in an area known as the Potiguar Basin, northeastern Brazil, covering roughly 300 km.

Beach Monitoring Project in the Potiguar Basin (Projeto de Monitoramento de Praias) is conducted by the Projeto Cetáceos da Costa Branca - Universidade do Estado do Rio Grande do Norte (PCCB-UERN) in compliance with the environmental constraints enforced by the Brazilian Institute of the Environment and Renewable Natural Resources (IBAMA) for the activities operated by PETROBRAS (Petróleo Brasileiro S.A., agreement number 2500.005657510.2).

Stranded green turtles found alive were admitted to a sea turtle rehabilitation center of PCCB-UERN in Areia Branca/Rio Grande do Norte (RN), Brazil, while complete post-mortem examinations were conducted on turtles found dead and mildly or moderately decomposed (condition codes D2 and D3; Flint et al., 2009) and on turtles that died during rehabilitation. At the sea turtle rehabilitation facility, stringent quarantine measures were enacted to prevent FP transmission, including safety equipment for individual protection of the team and restricted area to house only turtles with FP (far from the site of FP-free turtles). Moreover, all activity involving FP-free turtles was carried out by a specific technical team or after the management of FP-free individuals. The green turtles were housed individually in tanks with independent water supplies (148 cm diameter x 74 cm depth; 25–26°C), fitted with bead filtration and ambient light periodicity ranging between 7 and 16 h.

Flexible tape was used to measure the curved carapace length (CCL) from the nuchal to notch between supra-caudal scales (Bolten, 1999) for all examined individuals. All tumors were evaluated, quantified, and classified according to (1) anatomic distribution (e.g., eyes, head, cervical region, forelimbs, hindlimbs, carapace, plastron, inguinal region); (2) size (A: < 1 cm; B: 1–4 cm; C: > 4–10cm; D: >10 cm) (Work e Balazs, 1999); and (3) Southwest Atlantic Fibropapillomatosis Score - FPS_{SWA}: mild, moderate, and severe (Rossi et al., 2016).

Sampling collection and tissue processing

The FP tumor samples were obtained from (1) turtles found dead. Samples were collected in the stranding site or during necropsies (13 and 8 individuals, respectively) and (2) individuals found alive and kept in a local sea turtle rehabilitation center (n = 5): sedation with meperidine + midazolan 1 mg/kg SID/ intramuscularly, and lidocaine 2 mg/kg by intramuscular injection at the tumor sites. Tumors from live green turtles were

excised using an electric scalpel and cauterizer with 1-cm margins on all sides to help prevent neoplasia recurrence. Antisepsis was achieved with povidone iodine and ethyl alcohol and hemostasis was achieved using a gauze compress for several minutes. After tumor excision, surgical wounds were treated with Alantol® (Vetnil, São Paulo, Brazil) for healing; Bactrovet® Prata AM (König S.A. Argentina) for systemic antimicrobial therapy, healing and anti-inflammatory; and Terra-Cortril® Spray (Zoetis, São Paulo, Brazil) as a topical antimicrobial and anti-inflammatory. We fixed 26 FP tumor samples, one from each turtle (n = 26), in 10% neutral buffered formalin, routinely processed, and stained with hematoxylin and eosin for the light microscopy evaluation.

Molecular analysis

Sectioning: we sectioned 26 formalin-fixed paraffin-embedded (FFPE) skin lesions from 26 examined green turtles (the same samples used in histopatological analysis) using a microtome Leica RM2035 (20 serial 10 µm sections) and transferred to 1.5 mL sterilized tubes (Navas-Suárez et al., 2018). The microtome was cleaned with 2% hypochlorite between each case. Every case required new gloves and a new knife.

Deparaffinization and DNA extraction: Deparaffinization of selected FFPE samples was achieved by consecutive 1 mL xylene washes (thrice) intercalated by incubation during 10 min at 65°C followed by vortexing and centrifugation at 15,000 rpm for 5 min. Subsequently, the samples were washed with ethanol (rising in 100%, 95% and 70%) and collected by centrifugation at 15,000 rpm for 5 min after each step. After the final wash, ethanol was aspirated and the tissue pellets were rehydrated with sterile ultrapure water over 10 min. This step was repeated twice. Subsequently, total DNA was extracted using the DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany). The obtained DNA was quantified using a Biotek Epoch Microplate Spectrophotometer (Agilent Technologies, California, USA) and the samples were submitted to end-point PCR for ChHV5 DNA detection.

The end-point PCR: A combination of previously published and custom primers (Table 1) was used to amplify fragments of the ChHV5 unique long (UL) genes: UL30 (DNA polymerase), UL18 (capsid protein), and UL27 (Glycoprotein B). The end-point PCR was performed using Platinum Taq DNA polymerase (Invitrogen, California, USA) in a 25 µL reaction volume and the mixtures were amplified in a thermal cycler (Bio-Rad, California, USA).

The gene UL30 was amplified according to Rodenbusch et al. (2014), and UL18 and UL27 according to Alfaro-Núñez e Gilbert (2014). The PCR products were visualized in 2% agarose gels and bands were excised and purified using the GFX gel extraction kit (GE

Healthcare, Illinois, USA). The positive samples were confirmed by direct (Sanger) sequencing. The sequences obtained were aligned with ChHV5 partial genome sequences available at GenBank with the CLUSTAL/W method, followed the p-distance analysis by the MEGA 7 program to determine the identity percentage. A green turtle ß-actin gene segment (Page Karjian et al., 2015) was used as an endogenous control. The PCR amplification of the ß-actin gene was performed according to Page Karjian et al. (2015).

Table 1 – Polymerase chain reaction primer sequences targeting DNA segments of *Chelonid alphaherpesvirus* 5 (ChHV5) and green turtle (*Chelonia mydas*) partial genomes.

Prime list	UL / Gene name	Primer sequence	Product size	Reference
GTHV	UL30 (DNA polymerase)	F:GACACGCAGGCCAAAAAGCGA R:AGCATCATCCAGGCCCACAA	480 bp	Quackenbush et al. (2001)
UL18	UL18 (capsid protein)	F:GTGGAACCCCGCCGGGTAAT R:TGATCCGGGCCGAGTAGCGG	140 bp	Alfaro-Núñez and Gilbert (2014)
UL27	UL27 (glycoprotein B)	F:CTAGATACATACTGGCCRTGCTCGTC R:GCCAGCGACCATCCGGAG	143 bp	Alfaro-Núñez and Gilbert (2014)
ß-actin	ß-actin	F:TGGTACAGTCTCCCATTCCA R:AGGCATACAGGGACAACACA	232 bp	Page-Karjian et al. (2015)

RESULTS

Morphometrics

The mean ± standard deviation for CCL was 51.55 ± 14.6 cm (range: 35–59.4 cm), indicating that all green turtles examined were classified as juveniles based on the value 90 cm CCL, which was the smallest size registered for nesting females in the Brazilian nesting areas (Almeida et al. 2011).

Macroscopic and histological descriptions of FP tumors

We counted 292 tumors in the 26 examined green turtles, with an average of 15 tumors per individual ranging from 1 to 42 tumors per green turtle. The forelimbs were the most affected anatomical region (142 tumors), followed by the hindlimbs (n = 69), and cervical region (n = 64) (Table 2). The FP tumors were sessile, pedunculated, and smooth to verruciform, with variable coloration (white, pink, gray, and black).

Most tumors (n = 218) were classified as size B, followed by C (n = 46), and A (n = 27), and only one turtle had a tumor classified as size D. Considering the FPS_{SWA}, 53.8% (n = 14) of the cases were classified as mild, 42.3% (n = 11) as moderate, and 3.8% (n = 1) as severe.

Table 2 - Number of tumors in 26 juvenile green turtles (*Chelonia mydas*) stranded in the Potiguar Basin during 2010–2016, according to tumor size and anatomical location.

Anatomical region of the tumor(s)	Number of affected green turtles	Number of tumors	Tumor size category			
			A	B	C	D
Eyes	1	5	0	5	0	0
Head	1	2	2	0	0	0
Cervical region (neck)	15	64	7	45	12	0
Carapace	1	2	0	0	2	0
Plastron	1	1	0	1	0	0
Right forelimb	18	58	3	47	7	1
Left forelimb	18	84	9	63	12	0
Right hindlimb	16	38	4	26	8	0
Left hindlimb	10	31	0	26	5	0
Inguinal region/tail	7	7	2	5	0	0
Total		292	27	218	46	1

Slides from these 26 individuals (but not all tumors) were examined and they presented features consistent with FP tumors. We noted papillary projections of the epithelial layer, ballooning degeneration, hyperkeratosis, and basal layer hyperplasia. In the dermal portion, we found loose connective tissue with well-differentiated proliferating fibroblasts arranged in fine and disorganized collagen matrix, resulting in increased dermal thickness (Figures 1A-D). According to the histopathological analysis, the cutaneous growths corresponded to papillomas and fibropapillomas depending on the extent of epithelial and/or stromal proliferation.

End-point PCR amplification and the phylogenetic analysis

ChHV5-positive amplification was observed for UL30 (480 bp), UL27 (143 bp), and UL18 (140 bp). Overall, 20/26 samples (76.9%) were positive for ChHV5 DNA by the PCR. The detection sensitivity for ChHV5 DNA varied according to the PCR assay. The UL18 assay yielded positive results for 16/20 (80%) of the positive samples, UL27 for 10/20 (50%), and UL30 for 3/20 (15%). We obtained sequences from all amplified samples and the amplification of the ß-actin gene was positive in all samples, confirming the presence of amplifiable DNA in green turtle. Representative nucleotide sequences of the UL18 and UL27 partial genes were submitted to the DNA Data Bank of Japan (DDBJ) under accession numbers LC506443 and LC509014, respectively. The representative nucleotide sequence of the UL30 gene segment was submitted to the GenBank under accession number MN563740.

Our UL30 (MN563740) sequences were identical (100% nucleotide identity) to each other and to several sequences described for sea turtles of Brazil, including those from the southeastern (a herpesvirus-positive fibropapillomatous skin lesion in a green turtle from São Paulo state [JN938585]) and northeastern regions (sequences amplified in

a pulmonary fibromyxoid sarcoma of a leatherback turtle (*Dermochelys coriacea*) from Sergipe State [MK357710], and in a cutaneous fibropapilloma of a green turtle from the archipelago of Fernando de Noronha (Brazil) [MH101748]. The UL18 (LC506443) sequences were identical to each other and more similar (98% nucleotide and 97% amino acid identities, respectively) to sequences from a green turtle in Florida (USA) (AY644454), also presenting high amino acid similarity (97%) with sequences from green turtles in Hawaii (USA) (HQ878327) and Taiwan (KY933585). The UL27 (LC509014) sequences were identical to each other (100% nucleotide identity) and to UL27 ChHV5 sequences identified in green turtles from Florida (USA) (AY390406 and AY644454), Puerto Rico (JN580286), and Barbados (AY390404).

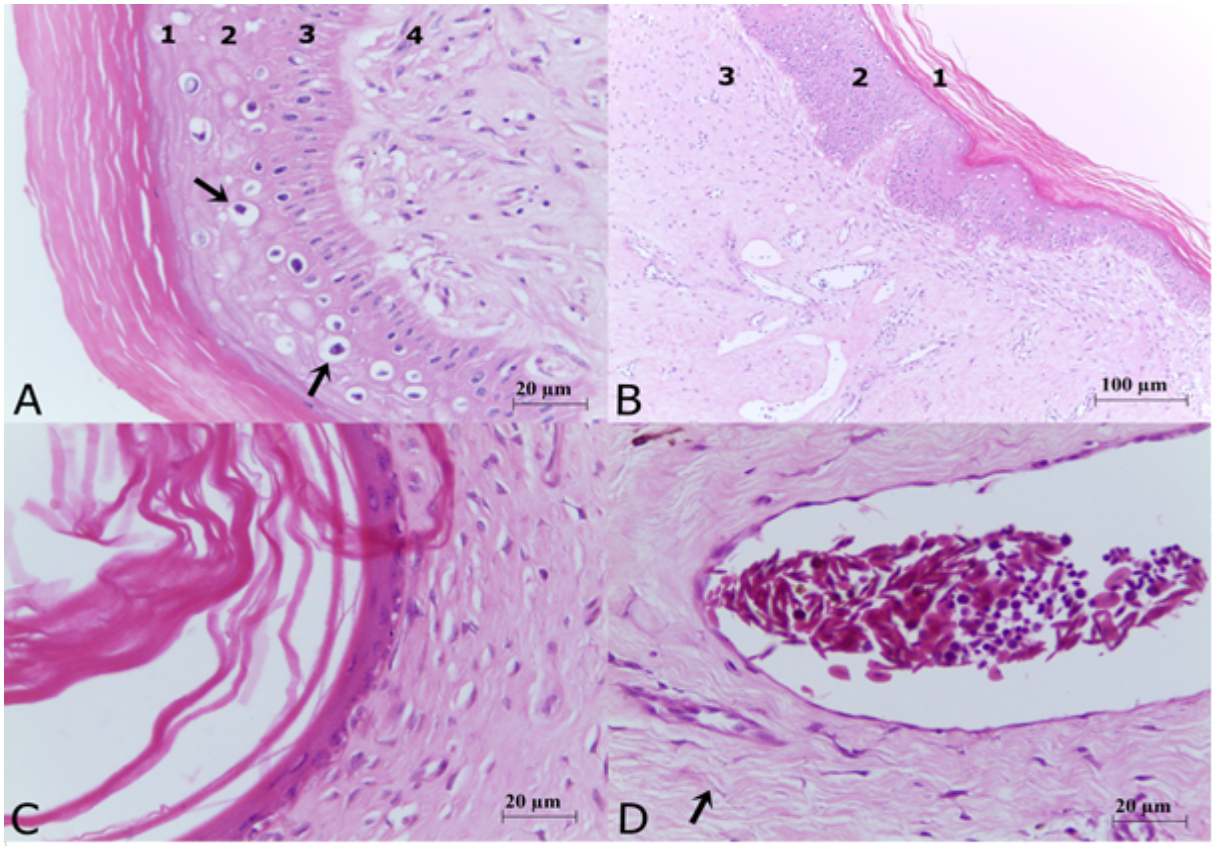

Figure 3 - Histological section of fibropapilloma obtained from a studied green sea turtle, HE. (A) Granulosa layer (1), *Stratum spinosum* (2), basal layer (3), and loose connective tissue (4); note vacuolated cells (arrows). (B) Thickening of the *stratum corneum* (1), stratified squamous epithelium (2), and loose connective tissue with proliferating fibroblasts arranged in fine and disorganized collagen fibers (3). (C) Well-differentiated proliferating fibroblasts. (D) Blood vessel with erythrocytes and leukocytes; note the disorganized collagen fibers (arrow).

DISCUSSION

Most tumors were found on the forelimbs (n = 142), hindlimbs (n = 69), and cervical region (n = 64) of the green turtles, as described by studies in Brazil (Hirama e Ehrhart, 2007; Mascarenhas e Iverson, 2008; Santos et al., 2010; Rossi et al., 2016) and in the United States (Page-Karjian et al., 2014). On the other hand, the posterior part of body was more affected in green turtles studied in Indonesia (Adnyana et al., 1997). The FP tumors were sessile or pedunculated and varied in color and texture. Most tumors were classified as size B (n = 218), followed by C (n = 46), and A (n = 27); and according to $FPS_{SWA,}$ most green turtles were mildly (53.8%) and moderately affected (n = 11). These results are in accordance with a study conducted in the Potiguar Basin (Silva-Junior et al., 2019). Vilca et al. (2018) used the same severity score and found more green turtles classified as moderate, followed by mild.

The histopathological analysis showed thickened epithelium, hyperkeratosis, vacuolated cells, and loose connective tissues with well-differentiated proliferating fibroblasts arranged in fine and disorganized collagen matrix with inflammatory infiltrate. Our findings are similar to previous descriptions for tumor samples from green (Jacobson et al., 1989; Herbst et al., 1999; Work et al., 2004; Zwarg et al., 2014) and loggerhead (*Caretta caretta*) sea turtles (Rossi et al., 2015). We also found highly vascularized hyperplastic stroma, as described by Rossi et al. (2015). Vascularization is important for neoplastic cells to obtain nutrition and it is necessary to keep the blood supply in tumors > 2 mm diameter (Anneroth et al., 1986).

The etiology of FP tumors remains questioned; however, ChHV5 is the leading candidate. Molecular differentiation of ChHV5 strains has revealed that variants are typically present at specific locations, even within sympatric sea turtle species, indicating that FP originates regionally (Jones et al., 2016). Many studies demonstrate the association of ChHV5 with the tumors, using end-point and quantitative PCR (qPCR) *in situ* hybridization as well as immunohistochemistry assays (Quackenbush et al., 2001; Ene et al., 2005; Kang et al., 2008; Work et al., 2017). In the current study, ChHV5 DNA detection was based on three partial genes (UL18, UL27, and UL30), providing UL18 and UL27 sequences of ChHV5 infecting green turtles of Brazil. The prevalence of ChHV5 DNA was 76.9% (20/26).

In Brazil, the ChHV5 detection rate in cutaneous tumors of green turtles varies depending on the geographical region. Rodenbusch et al. (2014) carried out a study between 2009 and 2010 in Bahia, Ceará, Espírito Santo, and São Paulo States and reported a ChHV5 detection rate of 73% (128/175 tumors of 139 green turtles) by the

conventional PCR and 87% (153/175 tumors of 139 turtles) by qPCR (Quantitative PCR). Monezi et al. (2016) conducted a study in São Paulo State between 2001 and 2012 and obtained a ChHV5 detection rate of 58.14% (25/43 tumors from 17 green turtles). On the other hand, Domiciano et al. (2019) found a ChHV5 detection rate of 100% (12/12 tumors of 7 green turtles from Paraná State). However, comparisons of the ChHV5 prevalence among Brazilian regions are limited by relatively small sample sizes and by the various molecular diagnostic techniques applied by these studies. Additionally, the DNA extraction from FFPE typically has low efficiency, limiting the comparison of our prevalence data and those obtained using better preserved (e.g., frozen) tissue samples.

Among the ChHV5 DNA-positive cases, the PCR assay targeting UL18, presented the highest detection rate (16/20, 80%), followed by the assay targeting UL27 (10/20, 50%). In contrast, Alfaro-Núñez e Gilbert (2014) found a higher prevalence using the PCR assay targeting UL27. The low detection rate (15%) observed for the PCR protocol amplifying 480 bp fragment of UL30 when compared to the protocol that amplifies shorter fragments (less than 200 pb) suggest that detection methodologies, based on minor differences in amplification of DNA fragment sizes, present higher ChHV5 detection sensitivity and are especially useful for poorly preserved samples, such as FFPE tissues (Alfaro-Núñez et al., 2016). We applied tests to detect short fragments of the UL18 and UL27 genes to improve the sensitivity of ChHV5 detection (Cárdenas et al., 2019).

The UL30 sequences of our study (MN563740) were identical to those previously described in sea turtles from Brazil, suggesting that that the same ChHV5 variant circulates along the southeastern and northeastern coasts, between São Paulo and Sergipe States and Fernando de Noronha Islands. Our molecular results for UL27 showed that the ChHV5 variant in our study is highly similar to those circulating in the Caribbean (Greenblatt et al., 2005). The short size of our UL18 sequence may preclude differentiation of the ChVH5 variants circulating in Asia.

CONCLUSION

Our study provides a technique applied to ChHV5 DNA extraction from FFPE FP tumors and reports molecular characterization of ChHV5 in tumors of green turtles from Potiguar Basin, an important green turtle foraging area with high frequency of FP tumors. The use of the PCR protocols designed to amplify short fragments of UL27 and UL18 is recommended for future studies to establish ChHV5 prevalence in sea turtles.

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