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Fibropapillomatosis in green turtles *Chelonia mydas* in Brazil: characteristics of tumors and virus

C. R. Rodenbusch[1,*], C. Baptistotte[2], M. R. Werneck[3], T. T. Pires[4], M. T. D. Melo[5], M. W. de Ataíde[6], K. D. H. L. dos Reis[6], P. Testa[1], M. M. Alieve[6], C. W. Canal[1]

[1]Laboratory of Virology, Veterinary Medicine College, Federal University of Rio Grande do Sul, Av. Bento Gonçalves 9090, Porto Alegre, 915640-000 RS, Brazil

[2]Projeto TAMAR/ICMBio — Av. Paulino Muller 1111, Vitória, 29040-715 ES, Brazil

[3]Bw Consultoria Veterinária — R. Ponciano Eugênio Duarte 203, Ubatuba, 11680-000 SP, Brazil

[4]Fundação Pro-Tamar — Rua Rubens Guelli 134 / 307, Salvador, 41815-135 BA, Brazil

[5]Fundação Pro-Tamar — Acesso Projeto TAMAR 151, Almofala, 62592-000 CE, Brazil

[6]Veterinary Medicine Hospital, Veterinary Medicine College, Federal University of Rio Grande do Sul, Av. Bento Gonçalves 9090, Porto Alegre, 91540-000 RS, Brazil

ABSTRACT: Fibropapillomatosis (FP) is a benign neoplasia that affects physiological functions of sea turtles and may lead to death. High prevalence of FP in sea turtle populations has prompted several research groups to study the disease and the associated herpesvirus, chelonid herpesvirus 5 (ChHV5). The present study detected and quantified ChHV5 in 153 fibropapilloma samples collected from green turtles *Chelonia mydas* on the Brazilian coast between 2009 and 2010 to characterize the relationship between viral load and tumor characteristics. Of the tumor samples collected, 73 and 87 % were positive for ChHV5 in conventional PCR and real-time PCR, respectively, and viral loads ranged between 1 and 118.62 copies cell^{-1}. Thirty-three percent of turtles were mildly, 28 % were moderately and 39 % were severely affected with FP. Skin samples were used as negative control. High viral loads correlated positively with increasing FP severity in turtles sampled on the Brazilian coast and with samples from turtles found dead in the states of São Paulo and Bahia. Six viral variants were detected in tumor samples, 4 of which were similar to the Atlantic phylogenetic group. Two variants were similar to the western Atlantic/eastern Caribbean phylogenetic group. Co-infection in turtles with more than one variant was observed in the states of São Paulo and Bahia.

KEY WORDS: Green turtle · *Chelonia mydas* · Fibropapillomatosis · Chelonid herpesvirus 5 · Real-time PCR · Genotyping

INTRODUCTION

The green turtle *Chelonia mydas* is circumglobally distributed in tropical and subtropical oceans, normally between latitudes 40° N and 40° S (Hirth 1997). Currently the species ranks as 'Endangered' in the International Union for Conservation of Nature (IUCN) Red List of Endangered Species (IUCN 2011).

In Brazil, anthropogenic waste and accidental fishing are the main threats to sea turtles (Bugoni et al. 2001). Apart from anthropogenic development initiatives in coastal areas, which negatively influence local ecosystems and may cause the exclusion or even the extinction of species (Daszak et al. 2000, Worm et al. 2006), diseases such as fibropapillomatosis (FP) may pose an additional hazard to green tur-

*Corresponding author: carlarodenbusch@yahoo.com.br

tles (Herbst 1994, Aguirre et al. 1998). Cutaneous FP was first described in *Chelonia mydas* 80 yr ago, and today the disease is considered a pandemic, with infection rates above 70% in some regions (Aguirre & Lutz 2004). In recent decades, FP emerged as a significant epizootic disease around the world, with prevalence reaching 92% in some turtle populations (Balazs 1991, Herbst 1994). FP is a benign but debilitating neoplastic disease, which may cause the death of sea turtles. Tumors may occur on flippers, axillar and inguinal regions, neck, mouth, eyes and head, affecting motion, feeding, sight and buoyancy. Tumors have also been diagnosed as visceral fibromas that may lead to organ failure and death (Norton et al. 1990, Work et al. 2004). The etiology of FP has yet to be precisely established. Studies have discussed the association between FP and the presence of ectoparasites (Greenblatt et al. 2004), pollution (Santos et al. 2010, Torezani et al. 2010), ingestion of microalgae (Van Houtan et al. 2010), and water temperature (Haines & Kleese 1977).

However, evidence shows that a herpesvirus is closely associated with presence of disease. Recent molecular studies (polymerase chain reaction, PCR) have revealed a strong correlation between this virus and FP (Lu et al. 2000, Quackenbush et al. 2001). The virus detected in fibropapillomas belongs to the *Herspesviridae* family, *Alphaherpesvirinae* subfamily, *Scutavirus* genus, and was called *Chelonid herpesvirus 5* (ChHV5) (ICTV 2011). In a pioneering study that recorded FP in Brazil, in the state of Espírito Santo, Baptistotte (2007) reported growing percentages of samples positive for the disease: 3.2% in 1997, 10.8% in 1998, 10.9% in 1999, and 12.4% in 2000. In the same survey, it was shown that mean FP occurrence in green turtles between 2000 and 2005 was 15.41% (1288/8659) in the country, of which 36.94% (181/490) of cases were in the state of Ceará (CE), 31.43% (33/105) in Rio Grande do Norte (RN), 18.46% (12/65) in Sergipe (SE), 15.81% (211/1335) in Bahia (BA), 27.43% (469/1,710) in Espírito Santo (ES), 5.96% (9/151) in Rio de Janeiro (RJ), 10.73% (371/3456) in São Paulo (SP) and 3.45% (2/58) in Santa Catarina (SC) (Baptistotte 2007).

The objective of the present study was to detect and quantify ChHV5 in fibropapillomas in green turtles *C. mydas* from the Brazilian coast and to determine the association between viral load, tumor characteristics and individual health condition. The viral variants detected in FP samples were characterized and their frequencies evaluated to expand the knowledge of this disease on the Brazilian coast.

MATERIALS AND METHODS

Study sites and sampling period

Samples were selected according to collection area based on FP occurrence data from 2000 to 2005. Tumors collected in the states of CE and ES were chosen because of the high prevalence of FP, while samples collected in the states of SP and BA were selected becuase a large number of turtles were monitored in these regions, in the same period. In total, tumors were collected from 169 turtles: 37 in the state of ES, 38 in BA, 40 in SP and 60 in CE. At least 1 tumor was collected per animal. In BA, 2 animals presented 2 tumors and 1 presented 3. In SP, 2 turtles presented 2 tumors each.

The turtles sampled in the present study were inspected by Projeto TAMAR during monitoring of turtles on beaches, but animals captured intentionally for research or trapped in fishing nets were also included. A few sick turtles were rescued and sent for rehabilitation, while others were found dead. In February 2011, 45 skin samples were collected from green turtles during the nesting season on Trindade Island and used as negative control, since there is no record of the disease on the island.

General turtle body parameters

Curved carapace length (CCL) was measured as the distance between the anterior edge, defined as the nuchal (precentral) scute, along the central carapace line, and the posterior edge, defined as the middle of the line uniting the supracaudal scutes. All measurements were made using a flexible measuring tape placed directly on the carapace, as described in the standard methods adopted by Projeto TAMAR, a sea turtle conservation initiative.

General body condition (normal, underweight, or emaciated) was determined according to Walsh (1999). Turtles were classified as 'normal' when presenting a convex plastron, normal eyes, neck muscles with adipose tissue, and protuberant axillar and inguinal regions. 'Underweight' status criteria included poorly concave plastron, normal or slightly sunken eyes, presence of adipose tissue in the neck, and slightly sunken axillar and inguinal regions. 'Emaciated' status criteria were a considerably concave plastron, sunken eyes, salient neck muscles with little or no surrounding adipose tissue, and thin axillar and inguinal regions. For data analysis, turtles were grouped according to body condition as healthy

(normal), debilitated (underweight and emaciated), and dead (animals affected by FP and found dead due to different causes). Underweight and emaciated turtles were grouped together.

Tumor samples

The samples used were 175 fibropapillomas collected from 139 green turtles on the Brazilian coast in 2009 and 2010 during routine monitoring of sea turtles by Projeto TAMAR. The presence of external tumors was observed by visual inspection. Tumor samples were evaluated for pigmentation (presence or absence), size (largest diameter in cm), morphology (smooth or papillary), and quantified based on the number of external tumors in each animal. Tumor score was calculated considering the size and number of tumors (Work & Balazs 1999). These scores reflect the severity of FP, from non-affected (score 0) to strongly affected (score 3).

Tumors and skin samples were excised using sterilized surgical instruments, placed in plastic bags appropriate for sample collection, and stored at –80°C until processing. Samples of healthy skin measuring 1 cm^2 or the whole tumor were collected. Samples were collected with permission of the Biodiversity Permits and Information System, SISBIO, authorization number 19116-1 by the Chico Mendes Institute for Biodiversity Conservation, Ministry of Agriculture, Livestock and Food Supply (Brazil).

Total DNA extraction

A tumor section (0.05 g) was macerated in 5 ml phosphate buffered saline (PBS) 10 mM (pH 7.4). The suspension was centrifuged at 350 × *g* for 10 min, and DNA was extracted from 200 µl of the supernatant using guanidine isothiocyanate and phenol (Chomkcynski 1993).

ChHV5 detection and quantification

A 438 bp fragment of the DNA polymerase (UL 30) of the herpesvirus was used to qualitatively detect ChHV5 in tumor and skin samples by PCR using the pair of primers GTHV 2/GTHV 3 (Quackenbush et al. 2001). Conventional PCR was carried out in Tris-HCl 10 mM (pH 8.3), $MgCl_2$ 2 mM, KCl 50 mM, DMSO 2.5%, 0.2 mM each dNTP, 10 pmol each primer, 2.5 U *Taq* DNA polymerase (Ludwig Biotecnologia) and 100 ng DNA to a 50 µl final volume. All samples were denatured at 94°C for 5 min, followed by amplification (35 cycles; 40°C for 30 s, 62°C for 30 s, and 72°C for 30 s) and 1 cycle at 72°C for 10 min in a thermal cycler (Veriti™, Applied Biosytems). Then, a 5 µl aliquot from each amplification reaction was electrophoresed in agarose gel 2% with Tris-borate-EDTA (TBE).

Viral DNA was quantified by real-time PCR (qPCR) using previously described primers and probes (Quackenbush et al. 2001) to amplify an 86 bp fragment of the DNA polymerase (UL 30) of the herpesvirus. The reaction was carried out using 5 pmol of each primer, 10 pmol of the probe in 12.5 µl Platinum Quantitative PCR Supermix UDG (Invitrogen), and 100 ng DNA to a 25 µl final volume. Reactions were heated to 50°C for 2 min and 95°C for 10 min to activate *Taq* polymerase, followed by 40 cycles, 15 s at 95°C and 1 min at 62°C, in a thermal cycler (StepOne™ Real Time PCR, Applied Biosystems).

As DNA markers, standard curves were prepared using serial dilutions of the plasmid DNA GTHV pol, constructed by inserting a 483 bp fragment of the ChHV5 genome in a plasmid kit (TOPO TA Cloning™, Invitrogen), according to the manufacturer's instructions. All assays contained 100 ng DNA, which corresponds to approximately 20 000 cells, assuming a value of 6 pg of DNA per cell (Lo et al. 1998). For the sake of a reference point, a copy number of 2×10^4 ChHV5 genomes would be equivalent to 1 copy of ChHV5 per tumor cell under these assay conditions; however, no assumption can be made regarding the distribution of ChHV5 genomes within the tumor.

Sequencing and phylogenetic analysis

Conventional PCR-amplified products were sequenced after purification using a purification kit (GFX, GE Healthcare). The 483 bp fragment of the DNA polymerase was chosen, since it has been demonstrated that samples distribute in the same clades of the phylogenetic tree, whether a 6801 bp fragment of the viral genome, a 2486 bp fragment of glycoprotein B or a 483 bp DNA polymerase fragment are used (Herbst et al. 2004, Patrício et al. 2012). Amplicons of expected sizes were sequenced twice in both directions in an automatic sequencer (ABI Prism 3100 Genetic Analyzer, Applied Biosystems) in the ACTGene Laboratory (Centro de Biotecnologia, UFRGS), using the primers GTHV2

and GTHV3. Afterwards, consensus nucleotide sequences were processed using the BioEdit software and aligned using the Clustal W software (Thompson et al. 1994). The resulting sequences were submitted to GenBank (www.ncbi.nlm.nih.gov). Paired distances using the Kimura-2 parameters (Kimura 1980), calculated for the different nucleotide sequences detected in samples collected in Brazil as well as samples deposited in GenBank, were used to construct a non-rooted phylogenetic tree by the neighbor-joining method in the Molecular Evolutionary Genetics Analysis software (MEGA 5.1) (Tamura et al. 2011). The following parameters were used for multiple alignment: gap opening penalty = 3, gap extension penalty = 1.8, keeping the other default parameters (Patrício et al. 2012). The statistical confidence of the 3 topologies was performed by 1000 bootstrap replications using the same software.

Histological analysis

Tumor samples were fixed in formalin 10%. After 5 d, samples were dehydrated, clarified, paraffin-embedded, sectioned to 5 µm specimens and stained using hematoxylin-eosin.

Statistical analyses

The Mann-Whitney *U*-test was used to assess the differences between viral load and tumor score in turtles or health status. Differences between viral load and tumor pigmentation, viral load and tumor size, and viral load and morphology were evaluated using the Kruskal-Wallis test. Analyses were carried out in the SPSS version 18 software using only qPCR positive samples. The chi-square test was used to compare morphology and pigmentation and the tumor score and body condition of turtles.

RESULTS

In 2009 and 2010, mean FP apparent prevalence values in green turtles recorded by Projeto TAMAR in the states CE, BA, SP and ES were 31.38% (220/701), 23.98% (301/1255), 4.54% (90/1971) and 12.69% (287/2262). All green turtles monitored, which included animals trapped in fishing nets and delivered by fishermen as well as beached individuals (alive or dead), were included in the analyses. Samples collected on the coast of the 4 states surveyed represent 19.48% of all turtles with fibropapilloma collected during this period in the 9 states where there are research and conservation bases of Projeto Tamar.

The CCLs of nesting females sampled at Trindade Island were between 97 and 130 cm. Skin samples of these animals were negative for ChHV5 both in conventional and qPCR.

Turtles sampled on the continental Brazilian coast were juveniles, with CCL between 33 and 76 cm, of unknown gender. Of these, 75% (131/175) were healthy, 15% were dead, and 10% were debilitated (emaciated or underweight). A total of 33, 28, and 39% of turtles had tumor scores 1, 2 and 3, respectively. Seventy percent (122/175) of the tumors were papillary and 41% were pigmented. No association between pigmentation and morphology of tumors was observed (p-value = 0.031), though there was an association between dead and debilitated animals and tumor score (p-value <0.001). Tumor diameter ranged from 0.3 to 7 cm (mean: 1.6 ± 1.4 cm). Seventy-three percent of tumors were positive for ChHV5 in conventional PCR whereas 87% (153/175) of samples were positive by qPCR. All samples testing positive in conventional PCR were also positive in qPCR. Fourteen percent (26/175) of the samples analyzed were positive in the qPCR and negative in the conventional PCR analyses, and all negative samples in the qPCR were also negative in the conventional PCR analyses, resulting in a kappa value of 0.56. Products obtained from conventional PCR were sequenced. qPCR was carried out to quantify viral load in tumor tissues (ChHV5 copies cell^{-1}). No sample used as negative control was positive in PCR analyses. Fig. 1 shows that

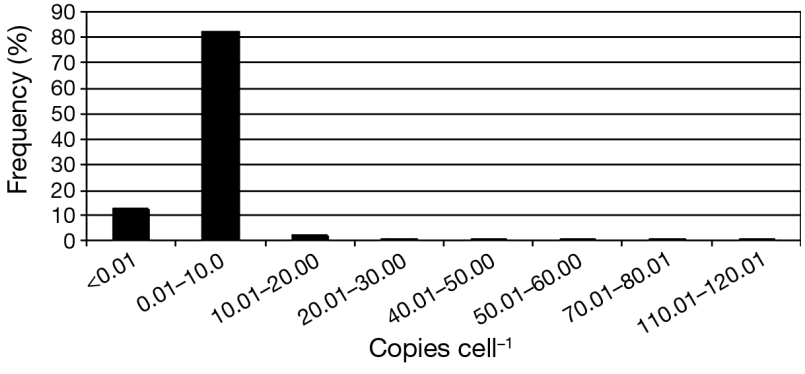

Fig. 1. Frequency distribution of ChHV5 viral loads (copies cell^{-1}) from tumors of green turtles *Chelonia mydas* in Brazil

12.6% of the samples were negative or presented viral load <0.01 copy cell^{-1}, and 82% of samples presented viral loads between 0.01 and 10.0 copies cell^{-1}. Maximum viral load was 118.62 copies cell^{-1}, with mean 2.79 ± 12.12 copies cell^{-1}.

Median values were used in the analyses because data did not fit assumptions of normality. Table 1 shows that there is a significant association between tumor score and viral load ($p < 0.05$). The turtles presenting tumor score 3 exhibited high viral loads. Table 1 also shows that there is a significant association between viral load and outside aspect of tumors. Papillary tumors had higher viral load per cell. To determine tumor score, tumors were classified as small (<4 cm), intermediate (between 4.1 and 10 cm), and large (10 cm), as in Work & Balazs (1999). To evaluate the association between viral load and tumor size, tumors were classified as small (<2 cm), intermediary (between 2.1 and 4.0 cm), and large (>4.0 cm), and the p-value obtained was 0.097, which indicates that there is no such association.

The association between tumor score and viral load was also evaluated for samples considering the Brazilian states in which they were collected (CE, BA, ES and SP). No significant association ($p > 0.05$) was observed between viral load and aspect, viral load and pigmentation, viral load and tumor score, and viral load and body condition of turtles in the states of BA and ES. In turn, a significant association ($p = 0.022$) between viral load and tumor score was observed in samples collected in SP, where animals with tumor score 3 (severe fibropapillomatosis) presented high viral loads. Papillary tumors presented high viral load in CE ($p = 0.042$). In SP, all turtles were considered healthy. Data of the analysis for each Brazilian state surveyed are given in Table S1 in the Supplement; www.int-res.com/articles/suppl/d111p207_supp.pdf.

Amplification products of 32 fibropapilloma samples from 27 turtles were used to sequence ChHV5 (Table 2). These samples were chosen because they generated a large number of amplification products in conventional PCR, and because they represented the 4 Brazilian states, as follows: (1) 8 fibropapillomas (1 per turtle) were from CE; (2) 6 tumors (1 per turtle) from ES; (3) 10 tumors (1 from each of 5 turtles, 2 tumors from 1 turtle, and 3 from 1 turtle) from BA; and (4) 8 tumors (1 tumor from each of 4 turtles, 2 tumors from each of 2 turtles) were from SP. Six genetic variants (var) of ChHV5 were detected (GenBank ID JN938584 to JN938589), of which var 1–4 were most closely related to Atlantic variants, and var 5 and 6 were most closely related to western Atlantic/eastern Caribbean variants (Fig. 2).

Var 1 and 2 were detected only in SP samples, while var 3 was detected only in ES tumors (Fig. 3). Var 4 was observed in 59.4% (19/32) of samples and was the most commonly detected in tumor samples collected in SP, BA and ES (Table 2). Var 5 and 6 were detected only in BA and CE, respectively. Except for var 5, all other variants were detected in

Table 1. Viral load, tumor characteristics (aspect, pigmentation and score) and body condition of *Chelonia mydas* turtles sampled in 4 Brazilian states from 2009 to 2010. Differences between viral load and tumor score in turtles or body condition were evaluated using the Mann-Whitney *U*-test, and differences between viral load and tumor pigmentation or morphology were evaluated using the Kruskall-Wallis test. n: number of fibropapilloma samples with viral loads >1 copy cell^{-1}. Median: different letters indicate significant difference in median value

	n	Viral load			p-value
		Mean	SD	Median	
Aspect					
Smooth	42	0.6484	1.6044	0.0235	0.031
Papillary	111	4.1480	15.0415	0.2917	
Pigment					
Absent	91	2.4901	9.7467	0.2440	0.259
Present	62	4.2107	16.5495	0.2888	
Tumor score					
1	46	2.9796	12.3066	0.070[a]	0.001
2	45	3.5836	17.6850	0.078[a]	
3	62	3.0539	8.8513	0.582[b]	
Body condition					
Healthy	115	3.2099	14.0329	0.2662	0.401
Debilitated	15	2.0516	4.8735	0.3741	
Dead	23	3.8115	10.7784	0.8377	

Table 2. Distribution of ChHV5 variants found in tumors from green turtles *Chelonia mydas* in the 4 Brazilian states surveyed, from 2009 to 2010, and respective viral loads expressed as copies cell^{-1}. BA: Bahia; CE: Ceará; ES: Espírito Santo; SP: São Paulo

Variant	State	Tumors (n)	Turtles (n)	Viral load (mean ± SD)
1	SP	2	2	0.685 ± 0.589
2	SP	2	1	0.885 ± 0.424
3	ES	2	2	1.135 ± 0.171
4	ES, SP, BA, CE	4, 4, 9, 2	4, 4, 7, 2	4.310 ± 11.743
5	BA	1	1	0.837
6	CE	6	6	0.790 ± 0.708

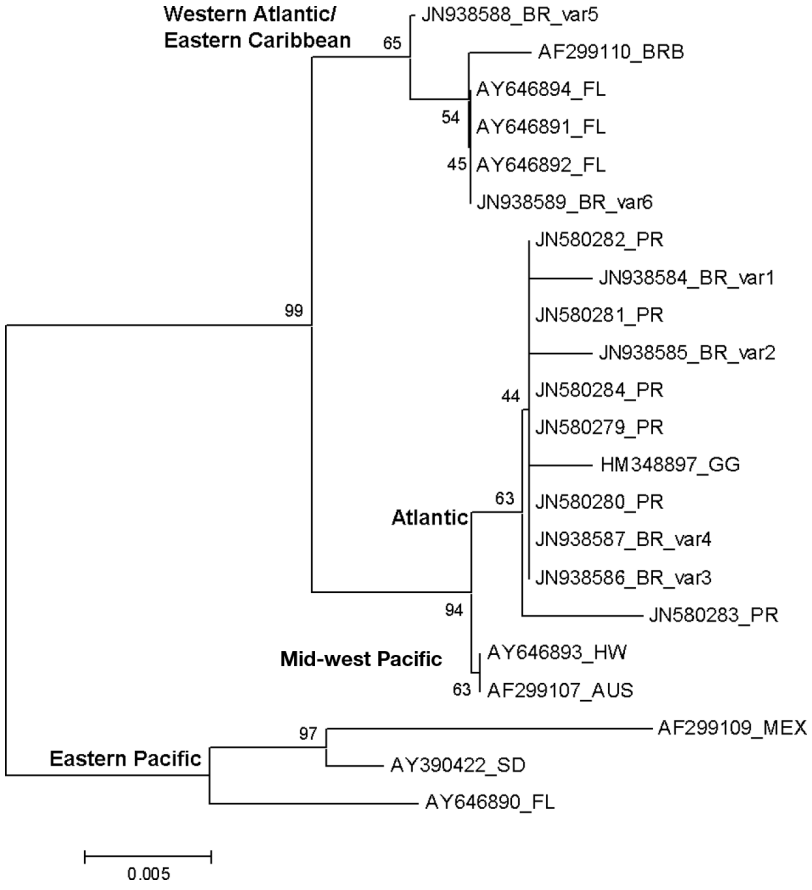

Fig. 2. Nucleotide phylogenetic tree of DNA polymerase gene of the ChHV5 using the neighbor joining distances method, with 1000 bootstrap replicas. Sequences were generated in this study or retrieved from GenBank. BR: Brazil; FL: Florida, USA; HW: Hawaii, USA; AUS: Australia (Pacific Coast); BRB: Barbados; MEX: Mexico (Pacific Coast); PR: Puerto Rico; GG: Gulf of Guinea; SD: San Diego, California, USA

multiple tumors, and multiple variants were observed in a single turtle from BA (var 4 and 5) and SP (var 1 and 2). Table 2 shows the viral loads of samples according to viral variants. Mean viral loads of var 1, 2, 3, 4 and 6 were 0.68, 0.88, 1.13, 4.31 and 0.79 copies cell^{-1}, respectively, while the viral load of var 5 (present in 1 sample only) was 0.83 copies cell^{-1}.

FP diagnosis was confirmed by histopathology. In total, 43 tumors were analyzed. Thirty-three were classified as fibropapilloma, and 10 as fibroma. Forty-two tumors presented stroma hyperplasia, 30 of which also exhibited epithelial hyperplasia. The main histological findings are shown in Table 3.

DISCUSSION

FP mainly affects immature turtles and the disease is rare (0–12%) in adult females going through the nesting period, normally with mild and focal lesions (Baptistotte 2007). In feeding sites such as Hawaii, USA, mid-sized turtles (CCL between 40 and 90 cm) have been shown to be more commonly and severely affected (Balazs 1991). In the present study, the

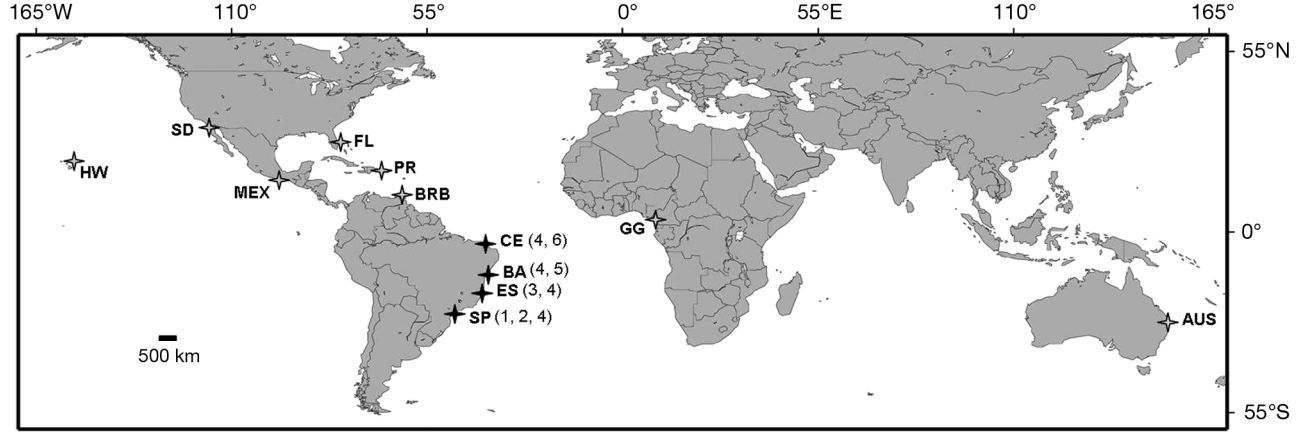

Fig. 3. Sites where ChHV5 samples were collected. Grey stars represent samples retrieved from GenBank; black stars indicate the samples collected in Brazil. Numbers in parentheses are the variants found in the Brazilian states. HW: Hawaii, USA; MEX: Mexico; FL: Florida, USA; PR: Puerto Rico; BRB: Barbados; AUS: Australia; SD: San Diego, CA, USA; GG: Gulf of Guinea; CE: Ceará; BA: Bahia; ES: Espírito Santo; SP: São Paulo (map constructed using www.seaturtle.org/maptool/)

Table 3. Main histopathological findings in 43 tumors sampled from green turtles *Chelonia mydas* in Brazil between 2009 and 2010

Histopathological findings	Absent	Present
Papillary feature	10	33
Hyperplastic stroma	1	42
Hyperplastic epithelia	13	30
Hiperqueratosis	16	27
Prominent nucleolus	13	30
Diskariosis	11	32
Nuclear halo	7	36
Nuclear inclusion	38	5

CCL of turtles presenting tumors sampled in the 4 Brazilian states varied between 33 and 67 cm. In Brazil, the highest frequency of tumors is observed in turtles with a CCL between 30 and 80 cm (Baptistotte 2007). The observation of turtles with a CCL below 30 cm is rare, since this is the size turtles have when they recruit to the coast. Two reasons may explain the low FP prevalence in animals with a CCL above 80 cm: (1) the disease may be self-limiting and, at the time a CCL > 80 cm is reached, animals may have been cured due to increased resistance, and/or (2) the turtles may die due to the disease before they reach larger sizes (Foley et al. 2005). This combination may also explain the absence of FP in turtles with a CCL >80 cm during the reproduction period in Trindade Island. There is no record of FP on the island, and the negative PCR results for the skin samples collected there confirm that ChHV5 does not circulate in that environment. Quackenbush et al. (2001) described the presence of the virus in turtles without tumors, though these animals lived in regions where the disease had been recorded.

Most turtles (75%) were classified as healthy, and 67% presented tumor scores 2 or 3. In a study that examined 87 turtles in the state of ES in 2008, 94% of the animals presented tumor scores 2 or 3, with no significant statistical difference in the relationship between body condition and tumor score (Santos et al. 2010), similarly to what was observed in the present study.

In green turtles, cutaneous fibropapillomas may develop as 1 single tumoral mass or multiple masses varying from 0.1 to >30 cm in diameter (Herbst & Klein 1995). These tumoral masses may be papillary or smooth, sessile or pedunculated (Herbst 1994). In the present study, 70% of tumors were smooth and varied from 0.3 to 7.0 cm (mean: 1.6 ± 1.4 cm).

The microscopic histopathology findings obtained in the present study showed that 98% of samples presented stroma hyperplasia and that, of these, 70% also exhibited epithelial hyperplasia, a value that is considerably high when compared with the value of 55% for stroma hyperplasia and of 28% for epithelial hyperplasia reported by Matushima et al. (2001). The authors also observed that 77% of the tumors were classified as fibropapillomas. The remaining tumors were fibromas. The epidermal and dermal proliferation rate varies across lesions. Lesions formed mainly by epidermal proliferation with little or no underlying dermal involvement are suitably called papillomas, while lesions formed predominantly by dermal constituents with relatively normal epidermal proliferation are called fibromas. The masses in which both tissues are hyperplastic are called fibropapillomas (Herbst 1994). Forty-one percent of the tumors were pigmented upon macroscopic inspection, though 53% presented melanocytes in the histopathological analyses. In general, tumor pigmentation is associated with skin pigmentation in the site of origin. Differences in tumor pigmentation result from the distribution of melanophores inside tissues. Strongly pigmented masses contain melanophores that are diffusely distributed in the dermis and between epidermis cells (Herbst 1994). Rarely, intranuclear inclusion bodies, compatible with herpesvirus, are noted in the epidermal layers of tumors (Jacobson et al. 1989). We observed intranuclear inclusion corpuscles in 11% dos tumors, and Herbst et al. (1999) report the occurrence of 8% of such corpuscles in tumors of naturally infected animals and of 42% in experimental infections. Here, stromal hyperplasia, epithelial hyperplasia and nuclear findings (inclusion corpuscles, dyskaryosis and nuclear halo), typical of viral infections, confirm the diagnosis of FP.

FP is diagnosed in turtles around the world. However, animals presenting the disease and that were also ChHV5 carriers were found only in Hawaii, USA, Florida, USA, Costa Rica, Barbados, Australia, Mexico (Quackenbush et al. 1998, 2001), Puerto Rico, San Diego, California, USA, and the Gulf of Guinea (Patrício et al. 2012). The results of the present study show that Brazil has to be included in this list, since 87% of the tumors in Brazilian turtles presenting FP were positive in qPCR for ChHV5. Mean ChHV5 viral load in the samples collected in 4 Brazilian states was 2.79 copies cell^{-1} (which varied between 0.01 and 118.62 copies). Viral loads in tumor samples collected in Hawaii and Florida were relatively uniform, varying between 1.7 and 25 copies of ChHV5 per tumor cell. In turn, qPCR showed that ChHV5 loads in tumors collected in Costa Rican and Australian turtles varied within a 100 000 copy range

(Quackenbush et al. 2001), a disparity similar to that observed in tumors collected in Brazil. These differences in viral loads may indicate that samples were collected from tumors at different stages. The literature cites spontaneous regression of fibropapillomas (Bennett et al. 2000, Machado Guimarães et al. 2013. Since the virus is predominantely latent in fibropapillomas (Greenblatt et al. 2004, Kang et al. 2008) and tumor size is not associated with viral load (Work et al. 2009), it is believed that replication is high, which increases viral loads at the onset of tumor development, and that subsequently this load decreases, so that regression may result in lower viral loads. In Marek's disease, a neoplastic syndrome also caused by a herpesvirus, resistance increases gradually within weeks. This 'age-related resilience' manifests as tumor lesion regression and is observed only in genetically resistant chickens (Baaten et al. 2004). The kappa value obtained (0.56) shows that there is no good agreement between the results of the conventional qPCR and of PCR. Indeed, qPCR was more sensitive than PCR as a means of detecting ChHV5 in fibropapillomas in green turtles, detecting 14% more samples, with a detection threshold of 0.01 copies $cell^{-1}$, similarly to what was observed for bovine herpesvirus 1 (Dehkordi et al. 2013). This makes the qPCR the technique of choice to detect ChHV5 in tumor samples collected from green turtles.

Of the 153 PCR-positive samples, 68% were collected from papillary tumors. Only the samples collected in turtles in CE presented a significant association between viral load and morphology, in which papillary tumors presented higher ChHV5 loads. Animals with tumor score 3 (strongly affected) presented higher viral loads, probably due to the advanced stage of the disease. No significant statistical association was observed between body condition and viral load (Table 1); however, body condition and tumor score were correlated ($p < 0.001$). This association is due to the fact that tumor score is a composite parameter, which considers size and number of tumors. Therefore, animals with tumor score 3, for instance, exhibited more tumors and consequently were more debilitated. We also observed an association between viral load and tumor score in samples collected in CE, and in samples collected in the 4 Brazilian states surveyed, when analyzed together (Table 1). Since the results obtained in this work as well as in other studies (Work et al. 2009) show that there is no association between viral load and tumor size, the association between viral load and tumor score is due to the number of tumors detected in turtles. High viral loads were observed in dying turtles in Australia, Costa Rica and Mexico, while low viral loads were detected in healthy turtles that had died shortly before collection of samples in Hawaii, Florida and Barbados (Quackenbush et al. 1998, 2001). In SP, only healthy animals were sampled. Since sampling was random and one of the activities of Projeto TAMAR in the state is performing surgery to remove fibropapillomas, the observation of only healthy animals may be a consequence of tumor removal. After removal, animals remained infected, but buoyancy, locomotion and feeding are not affected, which in turn improves general health condition. Although SP had only healthy animals, it was in this state that most turtles with tumor score 3 were sampled. This is due to the high number of small tumors.

Four phylogeographic ChHV5 groups have been described from tumors found in green turtles: eastern Pacific (with samples from San Diego and Mexico), western Atlantic/eastern Caribbean (which includes most samples collected in Florida and Barbados), midwestern Pacific (samples from Australia and Hawaii), and Atlantic (Gulf of Guinea and Puerto Rico) (Patrício et al. 2012). When the 6 Brazilian variants were compared to these phylogeographic groups, var 1, 2, 3 and 4 (JN938584 to JN938587), occurring in the southeastern Brazilian coast, were closest to the Atlantic group, while var 5 and 6 (JN938588 and JN938589), identified in northeastern Brazil, were more similar to the western Atlantic/eastern Caribbean (Fig. 2). This is the first study to compare green turtle fibropapilloma samples collected in Brazil with samples from other regions in the world.

The number of variants detected in the present study may be explained in light of the fact that ChHV5 evolves at a higher speed, compared with other herpesviruses (Patrício et al. 2012). The regional diversity of ChHV5 variants observed may be due to the complexity of the green turtle lifecycle. Evidence suggests that, after eggs hatch, green turtle juveniles spend several years in the oceanic environment before recruiting to neritic habitats, near the coast, for feeding and other biological functions (Bolten 2003). FP is detected mainly in juvenile and immature sea turtles in these coastal environments (Herbst et al. 2004). The wide variation in FP prevalence in juvenile green turtle populations in different coastal areas supports the hypothesis that juveniles are exposed to ChHV5 after they recruit to feeding sites near the coast (Herbst 1994). Similarly to what was reported in Florida (Ene et al. 2005), 1 variant (var 4) prevailed in samples collected in 3 out of the 4 states, and var 6 prevailed in 1 state only (Table 1).

Var 1 and 2 were detected only in SP, while var 3 was observed solely in samples collected in ES. The heterogeneous distribution of viral variants in tumors collected from green turtles in different locations on the Brazilian coast is not consistent with the hypothesis that these animals are exposed to ChHV5 in the oceanic environment. If it were true, the variants would be uniformly distributed in samples collected in the 4 states, since turtles native from different regions are, at any rate, in the same oceanic environment. These results are consistent with the hypothesis that infection takes place after juveniles recruit to specific coastal areas, where the relative frequency of the virus may vary considerably across locations, depending on how far turtles swim and on hydrological conditions (Ene et al. 2005). Var 4, which was detected in the 4 Brazilian states surveyed, had been detected in the state of Rio Grande do Sul, southern Brazil (Rodenbusch et al. 2012). This finding may be explained by the migration pattern of turtles. However, although a migration pattern of turtles on the Brazilian coast has not yet been characterized, a study carried out in SP showed that green turtles captured in the coastal city of Ubatuba, in that state, were recaptured 596 d later in Mucuri, southern BA, almost 900 km north. In turn, the southernmost point green turtles were recaptured in Brazil was near the city of Rio Grande, state of Rio Grande do Sul, almost 1200 km away from Ubatuba, 483 d later (Gallo et al. 2006). With respect to tumor scores, var 1, 2 and 5 were detected only in turtles presenting tumor score 3 (severe), which was associated with high viral load, while var 3 was isolated in score 1 (mild) and 2 (moderate) tumors, and variants 4 and 6 were detected in tumors of all scores.

Coinfection of 1 turtle with more than 1 variant has been described in green turtles in Florida (Ene et al. 2005). In the present study, coinfection with 2 variants was detected in turtles sampled in BA (var 4 and var 5) and in SP (var 1 and var 4) in different tumors.

Var 2, 3 and 5 were detected only in papillary tumors, var 6 was isolated only in smooth tumors, and var 4 and 5 were present in smooth as well as papillary lesions. Except for var 5, which was detected in 1 sample only, all variants were detected in tumors either with or without pigmentation. This finding suggests that tumor pigmentation is a result of skin pigmentation only, that this relationship is random (Herbst 1994) and that variants may be associated with tumor morphology. Taken together, the samples collected in the 2 states located in northeastern Brazil (BA and CE) presented a mean viral load of 2.84 copies cell^{-1}, while collections made in southeastern Brazil (ES and SP) presented a mean viral load of 2.94 copies cell^{-1}, showing that there is no difference in mean viral load between turtles in these regions. However, when viral loads of variants are analyzed and associated with phylogenetic groups, those in the western Atlantic/eastern Caribbean group (var 5 and 6) presented a mean viral load of 0.80 copies cell^{-1}, while variants in the Atlantic group (var 1, 2, 3 and 4) had a mean viral load of 3.50 copies cell^{-1}. This difference is explained by the presence of var 4, which has higher viral load and prevalence, suggesting that viral load is associated with variant, not geographic region.

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