UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL
FACULDADE DE VETERINÁRIA
PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS VETERINÁRIAS

DETECÇÃO E CARACTERIZAÇÃO DO HERPESVIRUS ASSOCIADO À FIBROPAPILOMATOSE EM TARTARUGAS-VERDES (*Chelonia mydas*) NA COSTA BRASILEIRA

Carla Rosane Rodenbusch

Porto Alegre, 2012.

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Tese apresentada como requisito parcial para obtenção do grau de Doutor em Ciências Veterinárias na área de Virologia
Orientador: Cláudio Wageck Canal

PORTO ALEGRE
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Carla Rosane Rodenbusch

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Aprovada em 09 MAR 2012.

APROVADO POR:

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Orientador e Presidente da Comissão

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Dra. Cecília Baptistotte
Membro da Comissão

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Prof. Dr. Paulo Michel Roehe
Membro da Comissão

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Prof. Dr. Sandro L. Bonato
Membro da Comissão

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RESUMO

A fibropapilomatose (FP) é uma doença emergente com alta prevalência em tartarugas, caracterizada por múltiplos papilomas, fibromas e fibropapilomas cutâneos ou viscerais. Essa doença foi denominada inicialmente de "green turtle fibropapillomatosis" (GTFP) por ter sido registrada pela primeira vez em tartarugas-verdes (*Chelonia mydas*). Na costa brasileira, o primeiro registro de FP foi em 1986 no Estado do Espírito Santo (ES) e, durante o período de 2000 a 2004, das 4.471 tartarugas-verdes examinadas, 14,96% apresentavam tumores. O agente etiológico da FP nas tartarugas-verdes ainda é incerto, mas acredita-se que seja de caráter multifatorial uma vez que poluição, temperatura da água e outros fatores interferem na ocorrência dos tumores. Um herpesvírus tem sido isolado de fibropapilomas e está presente em 95% das infecções naturais e 100% dos tumores induzidos experimentalmente. O objetivo deste estudo foi investigar a presença e caracterizar o vírus associado à FP em tartarugas-verdes, determinar a carga viral, pigmentação e escore de tumores e verificar a condição corporal das tartarugas; além de fazer a associação entre essas características. Este estudo foi dividido em três manuscritos. No primeiro, documentamos a ocorrência da FP em uma tartaruga-verde no Estado do Rio Grande do Sul (RS). O segundo e o terceiro trabalhos foram realizados com amostras de fibropapilomas de tartarugas-verdes encontradas nos estados do Ceará (CE), Bahia (BA), Espírito Santo (ES) e São Paulo (SP) porque esses estados apresentam uma frequência alta de FP e/ou um grande número de animais capturados em anos anteriores. O segundo estudo relata o sequenciamento de um fragmento de DNA do gene da DNA polimerase do herpesvírus em 38 amostras de fibropapilomas que revelam a ocorrência de seis variantes do vírus no Brasil. O último estudo relata a analise, por PCR e PCR em tempo real, de 175 amostras de fibropapilomas coletados nos estados de CE, BA, ES e SP e faz a associação da carga viral com o tipo de superfície dos tumores (lisa ou verrucosa), presença de pigmentação, condição corporal e escore de tumores nas tartarugas. Quarenta e três amostras foram submetidas à análise histopatológica para determinar o tipo de lesão. Quarente e cinco amostras de pele de tartarugas coletadas na Ilha de Trindade (livre de FP) foram analisadas em busca da presença do vírus. Todas as amostras de Trindade foram negativas para a presença do vírus e 87% das amostras foram positivas na PCR em tempo real. A maioria das amostras (75%) foi coletada de tartarugas sadias, 33% tinham escore de tumores 1, 28% escore 2 e 39% escore 3. Setenta por cento dos tumores eram verrucosos e 41% eram pigmentados. A carga viral máxima foi de 889.674,98 cópias/mg de tumor e animais com tumores de escore 3 apresentaram as mais altas cargas virais. Altas cargas virais também estavam associadas de forma significativa aos animais com fibropapilomatose encontrados mortos em SP e BA e a superfície verrugosa dos tumores nas tartarugas amostradas no CE.

ABSTRACT

*The fibropapillomatosis (FP) is an emerging disease with high prevalence in turtles, characterized by multiple cutaneous or visceral papillomas, fibromas and fibropapillomas. The disease was initially called green turtle fibropapillomatosis (GTFP) because it was first recorded in green turtles (*Chelonia mydas*). In the Brazilian coast, the FP was first recorded in 1986 in the State of Espirito Santo, and during the period of 2000 until 2004, 14.96% of the 4471 green turtles examined had tumors. The etiologic agent of FP in green turtles is still uncertain, but is believed to be multifactorial, as pollution, water temperature and other factors. A herpesvirus has been isolated from fibropapillomas and is present in 95% of the natural FP and 100% of the experimentally induced tumors. The objective of the present study was to investigate the presence of the virus associated with FP in green turtles and characterize FP lesions, as viral load, pigmentation, body condition and score of tumors, in addition to making the association between these traits. The study was divided into three manuscripts. The first documented the occurrence of FP in green turtles in RS. The second and third study was performed with samples of fibropapillomas of green turtles from the states of Ceará (CE), Bahia (BA), Espírito Santo (ES) and São Paulo (SP) because these states have a high incidence of FP and/or a large number of animals captured in previous years. The second study reports the sequence of a DNA fragment of the herpesvirus from 38 samples of FP, which revealed the occurrence of six virus variants in Brazil. The final study reports the analysis thought PCR and real time PCR of 175 fibropapillomas collected from turtles in the states of CE, BA, ES and SP, and makes the association of viral load with the surface of tumors (smooth or warth), pigmentation, body condition and score of the tumors. Forty-three samples were subjected to histopathological analysis to determine the type of injury and skin samples from 45 turtles collected in the Ilha de Trindade (free FP) were analyzed for the presence of the virus. All samples of the Ilha de Trindade were negative for the virus and 87% of samples were positive in real-time PCR. The majority of the samples (75%) was collected from healthy turtles, 33% had tumors with score 1, 28% score 2 and 39% score 3. Seventy percent of the tumors were warty and 41% were pigmented. The maximum viral load was 889,674.98 copies/mg of tumor and tumors with score 3 had the highest viral loads. High viral loads were also significantly associated with FP from the animals dying in SP and BA, and to tumors with warty surface from the CE.*

SUMÁRIO

1. **INTRODUÇÃO**

A fibropapilomatose (FP) é uma doença neoplásica emergente que está associada a infecção por um herpesvírus. A primeira descrição de sua ocorrência na costa brasileira foi em 1986, no Estado do Espírito Santo (BAPTISTOTTE et al., 2001). Segundo Baptistotte et al. (2005), a frequência média de tumores em tartarugas-verdes (*Chelonia mydas*) na costa brasileira entre os anos de 2000 e 2004 foi de 14,96%.

Atualmente, os únicos dados publicados no Brasil, relatam a ocorrência da doença por identificação visual ou histopatológica. Matushima et al. (2001) realizaram um estudo morfológico, imunoistoquímico e ultra-estrutural de fibropapilomas de tartarugas-verdes no Estado de São Paulo, porém não conseguiram isolar e caracterizar o vírus.

O aumento da prevalência somado ao fato de que a FP é fatal em muitos casos, tem aumentado o interesse no potencial impacto desta doença sobre o longo período de vida das populações de tartarugas-verdes. Esse interesse tem se refletido em esforços na busca de identificar o agente etiológico (Herbst et al., 1995).

Neste contexto, o objetivo do presente trabalho foi detectar o herpesvírus associado a fibropapilomatose e caracterizar os tumores em tartarugas-verdes da costa brasileira. A caracterização foi baseada no aspecto macroscópico (aparência dos tumores), microscópico (exame histopatológico das lesões) e virológico (detecção do vírus por PCR e determinação da carga viral por PCR em tempo real).

Num primeiro estudo, descrevemos a primeira documentação da FP e detecção do herpesvírus em tartarugas-verdes no RS. Neste estudo, foram descritas as características macroscópicas e microscópicas da lesão, a detecção do vírus por PCR e confirmação por sequenciamento além da quantificação viral. Num segundo estudo, foram sequenciadas amostras de fibropapilomas de tartarugas-verdes encontradas nos estados do Ceará (CE), Bahia (BA), Espírito Santo (ES) e São Paulo (SP) e verificou-se a ocorrência de 6 variantes virais circulante no Brasil. Finalmente no terceiro estudo, foram analisadas 175 amostras de fibropapilomas de tartarugas-verdes nos estados do CE, BA, ES e SP e mais 45 amostras de pele de tartarugas da Ilha de Trindade (livre de FP). Fez-se a descrição do estado corporal dos indivíduos; do escore e das características macro e microscópicas dos tumores e a determinação a carga viral, além de associações entre a carga viral e os fatores analisados.

2. REVISÃO BIBLIOGRÁFICA

2.1 Tartarugas marinhas

Dividem-se em duas famílias: *Cheloniidae* e *Dermochelyidae*. A família *Cheloniidae* é representada por seis espécies: *Caretta caretta* (tartaruga-cabeçuda), *Chelonia mydas* (tartaruga-verde ou aruanã), *Eretmochelys imbicata* (tartaruga-de-pente), *Lepidochelys olivacea* (tartaruga-oliva), *Lepidochelys kempii* (tartaruga-lora) e *Natator depressus* (tartaruga flatback). A família *Dermochelyidae* é composta pela espécie *Dermochelys coriacea* (tartaruga-de-couro ou gigante) (IUCN, 2011).

No Brasil, são encontradas cinco espécies de tartarugas marinhas: tartaruga-cabeçuda, tartaruga-de-pente, tartaruga-oliva, tartaruga-verde e tartaruga-de-couro (TAMAR, 2009). Todas elas estão incluidas na lista nacional das espécies da fauna brasileira ameaçadas de extinção (BRASIL, 2003) e todos os estágios de vida, incluindo ovos e filhotes, são amplamente protegidos por lei.

A Portaria do IBAMA, nº. 1.522, de 19/12/89, é o instrumento legal que declara as tartarugas marinhas ameaçadas de extinção. A portaria foi redigida com base na lista mundial de espécies ameaçadas da União Internacional para Conservação da Natureza (IUCN), da qual fazem parte as sete espécies de tartarugas marinhas.

Uma das principais instituições responsáveis pela conservação e pesquisa das tartarugas marinhas no Brasil é o Projeto TAMAR. Essa instituição foi criada em 1980 e desde então, passou a designar o Programa Brasileiro de Conservação das Tartarugas Marinhas, que é executado pelo ICMBio (Instituto Chico Mendes de Conservação da Biodiversidade), através do Centro Brasileiro de Proteção e Pesquisa das Tartarugas Marinhas (Centro TAMAR-ICMBio), órgão governamental; e pela Fundação Centro Brasileiro de Proteção e Pesquisas das Tartarugas Marinhas (Fundação Pró-TAMAR), instituição não governamental, de utilidade pública federal (TAMAR, 2009). Os principais sítios de nidificação no Brasil são protegidos desde 1982 pelo TAMAR e o trabalho nas áreas de alimentação iniciou somente em 1991 para combater os altos níveis de captura incidental pelos pescadores locais (MARCOVALDI & MARCOVALDI, 1999)

As tartarugas marinhas são excelentes indicadores da saúde ambiental, uma vez que são animais de vida longa, respiram ar e são vertebrados marinhos que ocupam a interface ar-água, recebendo o impacto da carga ambiental, tanto na sua alimentação marinha como na inspiração de voláteis tóxicos (AGUIRRE & LUTZ, 2004).

2.1.1 Tartarugas-verdes (*Chelonia mydas*)

São encontradas em mares tropicais e subtropicais entre 40°N e 40°S de latitude. Ocorrem em pelo menos 139 países, em águas costeiras ou praias de desova (HIRT, 1997).

Segundo Plotkin (1997), as tartarugas verdes habitam a zona nerítica, ocorrendo em águas próximas a costa e costeira onde se alimentam principalmente de gramíneas marinhas e algas, e, temporariamente, habitam a zona oceânica durante as migrações das áreas de forrageamento às áreas de reprodução na costa. Algumas dessas migrações reprodutivas de longa distância são feitos espetaculares, com tartarugas nadando milhares de quilômetros pelo oceano aberto diretamente para as praias localizadas em pequenas ilhas oceânicas isoladas. As fêmeas de tartarugas verdes migram das áreas de forrageamento às suas praias natais a cada 2-4 anos e mostram um alto grau de fidelidade local do ninho. As fêmeas fazem em média três oviposições em intervalos de 10 a 17 dias e permanecer perto da praia de nidificação durante o período entre as posturas. Após a postura dos ovos as fêmeas migram centenas de milhares de quilômetros de sua praia de nidificação para residir em zonas costeiras de alimentação. Após o acasalamento, os machos também migram longas distâncias a partir das áreas de reprodução às áreas de forrageamento no final da época de acasalamento ou podem permanecer nas imediações da praia de nidificação.

O conhecimento do estado de saúde das populações é considerado um imperativo para o desenvolvimento de programas de conservação (DEEM et al., 2001). O desenvolvimento das regiões costeiras está exercendo crescente impacto sobre os ecossistemas costeiros, levando por vezes a exclusão ou a extinção de espécies (DASZAK, et al. 2000; WORM, et al. 2006), sendo que as tartarugas-verdes fazem uso dessa região. Além de ameaças antropogênicas, doenças como a fibropapilomatose (FP) podem representar ameaças adicionais para as tartarugas-verdes (HERBST 1994, AGUIRRE et al. 1998).

2.2 Fibropapilomatose

2.2.1 Histórico e prevalência

A FP foi descrita pela primeira vez por Smith e Coates em 1938 no Aquário de New York em uma tartaruga-verde, que havia sido capturada dois anos antes em Key West, Flórida (SMITH; COATES 1938 *apud* HERBST, 1994).

Inicialmente, a FP foi descrita somente em tartarugas-verdes, mas estudos recentes têm descrito a doença em outras espécies, incluindo a tartaruga-cabeçuda, a tartaruga-oliva, a tartaruga-lora e a tartaruga-de-couro. Os tumores têm sido documentados histologicamente em tartarugas-de-pente nascidas e mantidas em cativeiro. Em tartarugas "flatback", os tumores têm sido observados macroscópicamente, mas não confirmados histologicamente (AGUIRRE & LUTZ, 2004).

A FP em tartarugas-verdes foi observada em vários locais por todo o mundo incluindo o oeste do Atlântico e o Golfo do México, o Caribe, o Pacífico e o Oceano Índico. Em alguns locais bem monitorados, a prevalência tem aumentado marcadamente desde 1980, chegando, por exemplo, a 92% em 1991 no Havaí (HERBST et al., 1995). Entre 1998 e 2006, a prevalência em Corisco, oeste da África, foi de 17% (FORMIA et al., 2007). Na Flórida, a prevalência variou entre 1975 e 1981 de 0 a 72,5% (EHRHART, 1991); no Havaí, entre 1983 e 1990, variou de 1% a 92% (BALAZS, 1991); na Austrália, em 1998 variou de 0 a 70% (AGUIRRE, et al., 2000); na Indonésia a média de prevalência em 1994 foi 21,5% (ADNYANA et al., 1997).

Esse aumento da prevalência somado ao fato de que a FP é fatal em muitos casos, tem aumentado o interesse no impacto desta doença sobre o longo período de vida das populações de tartarugas-verdes. Esse interesse tem refletido em esforços de identificar o agente etiológico (HERBST et al., 1995).

O aumento da prevalência de FPs combinado com o inicio de surtos em novas áreas geográficas tem levantado algumas hipóteses: 1) essa emergência é fruto da introdução do herpesvírus associado a FP em populações de tartarugas; 2) o herpesvírus associado a FP já estava estabelecido nas populações de tartarugas marinhas quando a FP chegou como resultado de uma interação entre o vírus e fatores ambientais; e a terceira é baseada em observações de que a doença é mais prevalente em habitats que são próximos a áreas de desenvolvimento urbano e agrícola (GREENBLASTT et al., 2005).

O primeiro registro de FP na costa brasileira é de 1986 (BAPTISTOTTE et al., 2001). Entre 2000 e 2004, de 4.471 tartarugas-verdes examinadas 14,96% apresentavam tumores. A prevalência de tumores por ano foi de 12,91% (2000, n=604), 14,96% (2001, n=809), 14,79% (2002, n=818), 19,95% (2003, n=842) e 12,95% (2004, n=1398) (BAPTISTOTTE et al., 2005).

No Estado de Espírito Santo, entre 2000 e 2006, a prevalência de FP foi de 20,9% e no Estado do Ceará, Rio Grande do Norte, Sergipe, Bahia, Rio de Janeiro, São Paulo e Santa Catarina em 2000-2005 variou entre 3,5% e 36,9%, enquanto nenhum tumor foi observado nas ilhas oceânicas de Fernando de Noronha e Atol das Rocas (BAPTISTOTTE, 2007; TOREZANI, 2009).

2.2.2 Caracterização

A FP é uma doença neoplásica caracterizada por um único ou múltiplos crescimentos fibroepiteliais cutâneos, com uma superfície verrucosa ou plana, mas aparecendo como fibromas nas vísceras (KANG et al., 2008). Os tumores podem ainda ser pigmentados ou não (Figura 1), dependendo da pigmentação da pele no local de surgimento da lesão (HERBST, 1994). Os tumores se localizam ao redor dos olhos, na conjuntiva, cavidade oral, pescoço, nadadeiras, cauda, áreas axilares e inguinais e também em órgãos viscerais. O tamanho varia de poucos milímetros até 30 centímetros de diâmetro e podem interferir na visão, locomoção, alimentação e flutuabilidade. Os tumores viscerais podem ser invasivos e afetar a função do órgão ocasionando a morte do animal (YU et al., 2000; GEORGE, 1997).

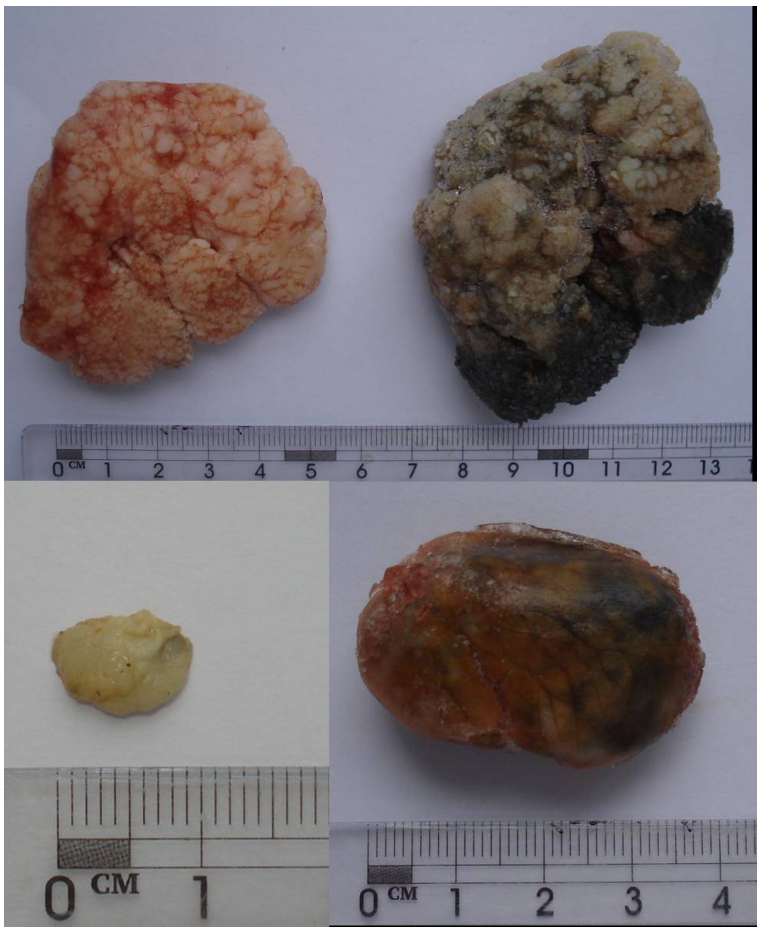

Figura 1: Fibropapilomas, coletados de tartarugas-verdes, mostrando as diferentes características externas.

Aproximadamente 25% a 30% das tartarugas que apresentam tumores externos apresentam tumores internos (AGUIRRE & LUTZ, 2004). WILLIAM et al, (1994) encontraram nódulos no fígado e pulmões de 41% das tartarugas-verdes examinadas em Porto Rico. Os nódulos normalmente são firmes e brancos, mas em alguns casos podem ser gelatinosos e translucentes. Os órgãos comumente afetados em uma amostragem de 13 tartarugas-verdes com FP cutânea e visceral foram pulmões (77%), rins (69%), coração (38%), trato gastrointestinal (31%) e fígado (23%) Nove tartarugas (69%) tinham nódulos em mais de um órgão (HERBST, 1994). Os tumores viscerais tendem a aparecer na fase final da doença e são detectados na necrópsia. Muitos fibromas viscerais consistem em fibroblastos bem diferenciados, similares aos fibromas cutâneos (KANG et al., 2008).

Na Flórida, de 1980 a 2002, entre as 280 tartarugas-verdes encontradas na costa com FP e necropsiadas, uma tartaruga (0,4%) tinha tumor oral, e de 1994 a 2002, entre as 127 tartarugas-verdes capturadas no canal de entrada de uma usina e que tinham FP,

uma tartaruga (0,8%) tinha tumor oral (BRESETTE et al., 2003). No Hawaí, altas taxas de tumores orais foram observadas: de 1989 a 1997, das tartarugas-verdes capturadas em mergulho, 40% apresentavam tumores orais (BALAZS et al., 2000) e entre 254 tartarugas-verdes costeiras inspecionadas de 1993 a 2003, 203 (80%) apresentavam tumores orais (WORK et al., 2004).

Segundo Work e Balazs (1999) pode-se estabelecer um escore dos tumores através da associação entre o tamanho e a quantidade de tumores (Tabela 1). Para isso os tumores devem ser medidos e classificados de A a D, sendo A tumores menores de 1 cm, B de 1 a 4 cm, C maior que 4 a 10 cm e D maior de 10 cm. O Escore obtido reflete a severidade da FP nas tartarugas, variando de não afetada (escore 0) a severamente afetada (escore 3).

No Brasil, em um estudo realizado entre os anos de 2000 e 2006 com tartarugas-verdes no canal de descarga de efluentes de uma indústria siderúrgica de Vitória (Espírito Santo – ES), a prevalência de FP foi de 34,4%, enquanto que a prevalência no mesmo período na costa do ES foi de 21,2%. Das tartarugas com fibropapilomas capturadas no canal, 35,2% tinham um grau leve de acometimento, 52,5% tinham grau moderado e 12,3% tinham grau severo. As tartarugas com FP tinham um comprimento curvilíneo de carapaça (CCC) maior que as sem tumores, sendo que as com acometimento severo apresentam o maior CCC que as moderadas e leves (TOREZANI, et al., 2009).

Tabela 1: Escores de severidade tumoral baseados no tamanho e quantidade de tumores. (Work a Balazs, 1999)

	Escore dos tumores			
	0	1	2	3
Tamanho do tumor				
(A) <1 cm	0	1 - 5	>5	>5
(B) 1 – 4 cm	0	1 - 5	>5	>5
(C) >4 – 10 cm	0	0	1 - 3	>4
(D) >10 cm	0	0	0	>1

Baptistotte (2007) caracterizou a ocorrência da FP em tartarugas na costa brasileira analisando 10.170 tartarugas marinhas durante os anos de 2000 a 2005. Das

1.243 tartarugas-de-pente analisadas, 2 apresentavam tumores; das 250 tartarugas-cabeçudas, 5 apresentavam tumores; das 288 tartarugas-oliva, 3 apresentavam tumores e nenhuma das tartarugas-gigantes apresentava tumores. O maior número de tumores foi registrado nas tartarugas-verdes, das quais 1.288 das 8.359 analisadas apresentavam tumores. A média de prevalência nacional das tartarugas-verdes foi de 15,41% no período. O CCC mínimo dos animais afetados foi 30 cm e o máximo de 112 cm, sendo que a prevalência de fibropapilomas aumentou com o CCC até 80 cm e decresceu abruptamente. De 202 tartarugas analisadas no ES neste período, o número de tumores variou de 1 a 179 em um mesmo indivíduo, tendo como média 21 tumores. A maioria dos tumores (72,5%) estavam localizados na região anterior corpórea do animal, 25,2% na região posterior e 2,3% na carapaça e plastrão, sendo que nenhum tumor foi observado na cavidade oral. Quanto ao escore de tumores, 40,61% das tartarugas tinham escore 1, 51,27% tinham escore 2 e apenas 8,12% tinham o escore 3.

Monezi et al. (2006) confirmaram a circulação do vírus na corrente sanguínea de tartarugas ao analisarem o sangue e tumores de 16 tartarugas da Base de Ubatuba do Projeto TAMAR. Eles realizaram uma PCR a partir de sangue extraído do seio venoso cervical e de tumores extraídos cirurgicamente e detectaram o vírus em pelo menos um tipo de amostra em 11 animais, sendo que 8 amostras de tumores e 4 de sangue foram positivas.

Microscopicamente, os tumores associados com a FP são divididos em três categorias baseadas nas características predominantes: papiloma, fibropapiloma e fibroma. Os papilomas representam a fase inicial de desenvolvimento dos tumores e consistem, principalmente, em proliferação da epiderme. Os fibromas são caracterizados por proliferação da matriz dermal, freqüentemente envolvendo tecido colagenoso relativamente maduro e representam a fase crônica das três categorias. Os fibropapilomas mostram ambas as mudanças, sugerindo que sejam uma fase intermediária (KANG et al., 2008).

As células epiteliais nas lesões proliferativas podem apresentar degenerações com corpúsculos de inclusão intranuclear eosinofílicos, enquanto que o estroma fibrovascular tem fibroblastos bem diferenciados e frequentemente apresenta infiltrado perivascular de células mononucleares (GREENBLASTT et al, 2005).

2.2.3 Curso clínico, morbidade e mortalidade

Os fibropapilomas cutâneos podem se tornar grande o suficiente para interferir na locomoção e são facilmente enredados em linhas de pesca. Os fibropapilomas oculares podem ocluir a visão e aqueles que invadem a córnea podem causar a destruição do globo ocular (BROOKS et al, 1994).

Os fibromas viscerais podem se expandir para o estroma do órgão afetado e interromper a função normal do órgão. Disfunção cardíaca, problemas de flutuabilidade e comprometimento respiratório, hidronefrose e obstrução gastrointestinal são observados ou causas suspeitas de morte tartarugas afetadas (HERBST, 1994).

Tartarugas afetadas por FP apresentam anemia e hipoproteinemia, além de leucopenia, linfopenia e eosinopenia quando comparados com animais sem tumores. Esses parâmetros hematológicos tendem a ser mais severos em tartarugas que apresentam tumores maiores (WORK e BALAZS, 1999). A caquexia pode ser causada pela incapacidade de locomoção, ingestão ou digestão de alimento, demanda energética excessiva pelo crescimento dos tumores, aumento energético para locomoção, efeito fisiológico de certas citocinas como o fator de necrose tumoral, mediado pelo sistema imune e/ou por doenças concomitantes (HERBST, 1994).

A regressão dos tumores em tartarugas-verdes é relatada por vários autores. Jacobson et al. (1989) mantiveram 6 tartarugas juvenis com FP por vários meses em cativeiro. Alguns tumores no mesmo animal diminuíram de tamanho enquanto outros aumentaram num intervalo de 4 meses entre a captura e recaptura. Ehrhart et al. (1991) mantiveram 3 tartarugas-verdes em cativeiro por aproximadamente 3 meses. Durante esse período, uma tartaruga perdeu os tumores, outra desenvolveu 8 novos tumores e a terceira se manteve inalterada. No estudo desenvolvido por Hirama e Ehrhart (2007) de 25 tartarugas-verdes com FP na captura inicial, 22 (88%) apresentaram regressão na segunda captura. Vinte e uma das tartarugas mostraram uma redução de 50% no número de tumores e uma das tartarugas mostrou ausência completa da doença após 1560 dias. No Brasil, um estudo realizado no Espírito Santo entre 2007 e 2008, de 37 tartarugas, três tartarugas decresceram do escore 3 para o escore 2 (SANTOS et al, 2010)

2.2.4 Etiologia

O agente etiológico do FP nas tartarugas-verdes ainda é incerto. Histologicamente, os FPs são caracterizados por hiperplasia epidermal papilar benigna apoiado numa ampla haste de estroma fibrovascular proliferativo. Agentes conhecidos

que causam lesões cutâneas proliferativas semelhantes em outras espécies incluem carcinógenos químicos, luz ultravioleta, vírus oncogênicos e parasitas metazoários (HERBST et al., 1995).

Os herpesvírus são associados com várias doenças de tartarugas marinhas incluindo a doença dos pulmões-olhos-traquéias (LETD – lung-eye-trachea disease), a "gray patch disease" (GPD) e a fibropapilomatose (FP) (CURRY et al., 2000; STACY et al., 2008).

O padrão de disseminação da doença durante surtos em tartarugas-verdes de cativeiro consiste com uma etiologia contagiosa. Um herpesvírus tem sido identificado em fibropapilomas, mas o vírus não foi isolado e, desta forma, os postulados de Koch não foram preenchidos (HERBST et al., 1995). O herpesvírus associado ao fibropapiloma em tartarugas marinhas está presente em 100% dos tumores induzidos por inoculação de filtrados de células tumorais (ENE et al., 2005) e em 95% das infecções naturais, sendo que em 79% dos fibropapilomas e fibromas analisados por PCR em tempo-real quantitativo, o vírus estava presente em níveis que excediam 10^4 cópias por 100 ng de DNA total de tumores (QUACKENBUSH et al., 2001). A análise de pele normal de tartarugas com FP revela níveis significativamente baixos do vírus (0,0012 a 0,0017 cópias por células)

As sanguessugas marinhas da espécie *Ozobranchus* são um potencial vetor de transmissão do herpesvírus uma vez que podem carrear o DNA viral. Alguns exemplares podem apresentar até 10 milhões de cópias do DNA viral por sanguessuga (GREENBLATT et al., 2004).

Seqüências de herpesvírus tem sido detectadas em fibropapilomas de tartarugas-verdes, tartarugas-cabeçudas e tartarugas-oliva no Havaí, Flórida e Costa Rica usando primers que reconhecem regiões conservadas do gene da DNA polimerase de herpesvírus e partículas semelhantes a herpesvírus tem sido visualizadas por microscopia eletrônica (QUACKENBUSH et al., 2001). A expressão do gene da DNA polimerase não tem sido detectada em fibropapilomas ou fibromas, o que sugere que a infecção é predominantemente latente nos tumores, semelhante ao que ocorre com o herpesvírus humano 8 nas lesões de sarcoma de Kaposi ou do gallid herpesvírus 2 causador da doença de Marek das galinhas (GREENBLATT et al., 2004; ROIZMAN & KAIPE, 2001; ROIZMAN & PELLET, 2001; WHITLEY, 2001).

A análise de uma seqüência de 23.055 pares de bases do genoma dos herpesvírus associados a fibropapilomatose demonstra que o vírus está organizado da mesma forma

que o gênero *Alphaherpesvirinae* e que mais de 96% das seqüências de nucleotídeos são conservadas entre os vírus encontrados em *C. mydas* da Flórida, Havaí, Austrália, Porto Rico, Barbados e Califórnia; *C. careta* da Austrália e *L. olivacea* da Costa Rica (GREENBLATT et al., 2005).

Herbst et al. (2004) detectaram cinco variantes virais em 25 tartarugas de três espécies (*C. mydas, C. caretta* e *L. kempii*). Essas variantes foram determinadas através da comparação de 6.801 pb do genoma viral e são denominadas de Flórida A, B, C e D e a Havaina (HA). As variantes da Flórida A, B e C são semelhantes, mas a variante D difere das anteriores em 5,6% enquanto que a variante HA difere em 2,2% das mesmas. Esse mesmo padrão de variantes é mantido quando se analisa a seqüência de 483 pb da DNA polimerase. Ene et al. (2005) descreveram a co-infecção de uma tartaruga-verde pelas variantes A e B.

Greenblatt et al. (2005) descreveram quatro grupos de variantes: o Atlântico (formado por seqüências da Flórida e Barbados), o Pacífico Médio (Havaí), o Pacífico Oeste (Austrália) e o Pacífico Leste (Costa Rica e Califórnia). O grupo Atlântico apresenta 0,98% de divergência dos grupos Pacífico Oeste e Médio. O Pacífico Oeste e o Médio divergem em 0,42%, mas as seqüências do Pacífico Médio são distintas por apresentarem uma deleção de 6 pb na posição 1164-1169 da glicoproteina B. O Atlântico, o Pacífico Oeste e o Pacífico Médio diferem do Pacífico Leste em 2,8 a 3,1%. O Pacífico Leste é o grupo com maior variabilidade de nucleotídeos.

Stacy et al. (2008) propuseram um novo gênero, o *Chelonivirus* na subfamília α-*herpesvirinae* baseado em seqüências da DNA polimerase viral detectadas por PCR em tartarugas marinhas.

Existem 6 herpesvírus que acometem as tartarugas, todos pertencem a família *Herpesviridae*. Dois deles pertencem a subfamília *Alphaherpesvirinae*. São eles, o Chelonid herpesvirus 5 (ChHV 5) que é o vírus associado a FP e o Chelonid herpesvirus 6 (ChHV 6) associado a LETD. Os outros quatro pertencem a subfamília *Gammaherpesvirinae* e são chamados de Chelonid herpesvirus 1, 2, 3, e 4, sendo que o primeiro está associado a GPD (DAVIDSON et al., 2009).

Através de seqüenciamentos de segmentos do genoma viral de diferentes tartarugas de vida-livre, foi determinada a existência de 5,6% de diversidade em seqüências de nucleotídeos e em função de taxas de mutações previstas, conclui-se que pelo menos quatro linhagens conduzem a uma variante do ChHV 5 que divergiu milhões de anos atrás. Portanto, o aparecimento de surtos de GTFP em vários locais ao

redor do mundo durante as últimas duas décadas é pouco provável que seja devido a mutações do vírus, porque é altamente improvável que essas mutações tenham ocorrido nas quatro linhagens. É muito mais provável que as mudanças no ambiente ou fatores ecológicos que afetam a transmissão do vírus ou da expressão da doença expliquem o recente aumento da prevalência da doença (ENE et al., 2005; GREENBLASTT et al, 2005).

A ingestão de *Lyngbya majuscula*, uma cianobactéria que produz um complexo promotor de tumor, foi associada a FP em Queensland, Austrália e no Havaí. A ingestão desta cianobactéria por tartarugas-verdes mostrou uma correlação positiva com tartarugas que tinham lesões de fibropapilomatose, mas não foi uma correlação de causa e efeito demonstrando apenas que de alguma forma ela contribui para a doença (ARTHUR et al., 2008).

Acredita-se que a FP esteja relacionada com a poluição, porém Aguirre et al. (1994) não encontraram associação entre a doença e inseticidas carbamatos, organofosforados, organoclorados, selênio e metais pesados, uma vez que tartarugas-verdes que apresentavam FP não apresentaram ou apresentaram níveis baixos de detecção desses contaminantes. Os tumores parecem manifestar-se mais freqüentemente em ambientes de condições estressantes, assim como regiões com baixa qualidade de água e na presença de contaminantes e toxinas (FORMIA et al., 2007).

A temperatura da água, como um fator estressante que pudesse desencadear a FP, foi estudada por Haines e Kleese (1977). Neste estudo eles comprovaram que tartarugas submetidas a um aumento gradual de temperatura de 25°C a 30°C e subseqüente permanência a 30°C; e tartarugas submetidas a um aumento abrupto de temperatura de 25°C a 30°C apresentaram uma diminuição do tempo de apresentação dos sinais clínicos e aumento da severidade das lesões tumorais, enquanto que tartarugas que foram submetidas a um aumento gradual de temperatura de 25°C para 30°C e subseqüente diminuição gradual de temperatura a 25°C mostraram sinais e lesões semelhantes aos controles.

Em um estudo realizado no Hawaií foram encontrados elevados índices de fibropapilomatose em regiões hidrográficas com alto nível de nitrogênio (N) (HOUTAN et al, 2010). Quando o N é abundante, as plantas armazenam o excesso de N ambiental como arginina (Arg). Um estudo no Havaí identificou duas algas invasoras consumidas pelas tartarugas, *Hypnea musciformis* e *Ulva fasciata*, como tendo elevados níveis de Arg (McDERMID et al, 2007). Análises posteriores de isótopos revelaram que até 43%

do N armazenado nessas espécies era originário de despejo de esgoto (DAILER et al, 2010). Algas não-nativas, portanto, parecem sequestrar o N antropogênico, armazenando-o como Arg e passando-o como forrageira para as tartarugas. (HOUTAN et al, 2010). Em muitas doenças crônicas, a Arg está envolvida na inflamação das células, na disfunção imune (PERANZONI et al, 2008) e na promoção de tumores virais (MANNICK et al, 1994), mas a Arg é especialmente importante para os herpesvírus que estão associados aos tumores de FP. Experiências mostram que o herpesvírus não cresce sem Arg (INGLIS 1968; MIKAMI et al, 1974; OLSHEVSKY e BECKER, 1970) e ela é como um alicerce fundamental do envelope viral que facilita a fusão e entrada no núcleo das células (KLYACHKIN e GERAGHTY, 2008). A Arg também parece promover a replicação do herpesvírus associados a tumores de córnea (MISTRY et al, 2001) e foi encontrada altamente concentrada em lágrimas de coelhos com lesão de herpesvírus na córnea (KAHAN et al, 1979). Isto é particularmente relevante, pois 93% das tartarugas verdes do Havaí com FP tem tumor ocular (WORK et al, 2004). Não se sabe como o herpesvírus pode promover o crescimento do tumor, mas estudos em morcego mostram que o herpesvírus pode inibir a apoptose e manipular o crescimento celular (IRMLER et al, 1997).

Outros vírus também já foram pesquisados em fibropapilomas. O papilomavírus é encontrado em tartarugas marinhas das espécies *Chelonia mydas* e *Caretta caretta* em lesões de dermatite proliferativa (MANIRE, 2008). Lackovich et al. (1998) confirmaram a ausência de papilomavírus em FPs usando 3 pares de iniciadores degenerados e Casey et al. (1997) confirmam a presença de retrovírus em tartarugas com FP, porém não estavam associados aos tumores.

2.2.5 Transmissão

A GTFP é transmitida experimentalmente através da inoculação ou escarificação de um extrato de fibropapiloma livre de células (obtido por clarificação e filtração) e os primeiros tumores aparecem 15 a 43 semanas após. Esses tumores são histologicamente indistinguíveis dos tumores da infecção natural (HERBST et al., 1995). O tratamento deste filtrado com clorofórmio, antes da inoculação, diminui a atividade carcinogênica do vírus, já a ultracentrifugação não causa o mesmo efeito (HERBST et al., 1996).

Evidencias indicam que, após deixarem sua praia natal, as tartarugas passam muitos anos na zona pelágica (oceano aberto) antes de voltarem para as áreas de alimentação e desenvolvimento, na zona nerítica (próximo a costa). A FP é detectada,

principalmente, em tartarugas jovens e imaturas nessas áreas costeiras (HERBST et al, 2004). Segundo Ene et al. (2005) existem várias hipóteses sobre quando as tartarugas se infectam com o ChHV 5. Elas poderiam adquirir o vírus na fase inicial das suas vidas, incluindo o período pré-natal, nascimento (eclosão) ou na zona pelágica. Elas também poderiam ser infectadas nas fases mais tardias das suas vidas, após emigrarem do oceano aberto para os habitats neríticos. Cada hipótese gera previsões sobre a distribuição espacial das variantes do ChHV 5 entre os locais costeiros dentro de uma região. Neste estudo, realizado com tartarugas de três regiões diferentes (Indian River Lagoon, Florida Keys & Bay e West Central Coast) da Flórida, eles concluem que as tartarugas se infectam com variantes específicas do vírus após chegarem no habitat nerítico, ainda jovens, pois eles encontraram diferentes variantes virais (A, B, C e D), sendo que a variante A e a C estavam presentes em mais de uma espécie de tartaruga, o que comprova a transmissão interespécies. Fora de Laguna Indian River, a variante A foi a variante mais detectada e foi encontrada em mais de 94% das tartarugas doentes e 70% das tartarugas-cabeçudas na Baía da Flórida/Florida Keys. No entanto, na Laguna Indian River, a variante B foi encontrada em mais de 94% das tartarugas infectadas e essa variante não foi encontrada fora dessa região. Baseando-se no fato de que as tartarugas das três áreas estudadas são oriundas de uma população pelágica mista, os autores sugerem que a distribuição das variantes virais nas tartarugas seria relativamente homogênea entre os três locais se isso correspondesse a uma infecção nas fases iniciais do seu ciclo de vida. Porém a distribuição heterogênea das variantes virais nos tumores das tartarugas-verdes a partir de diferentes locais da Florida reforça a hipótese de que as tartarugas são infectadas depois de chegarem ainda jovens nos habitats neríticos.

2.2.6 Sorologia

Sorologicamente é possível distinguir a infecção pelo herpesvírus associado a doença dos pulmões-olhos-traquéias (LETD) e o herpesvírus associado a fibropapilomatose, o que demonstra que as duas doenças são eventos independentes (COBERLEY et al., 2001).

2.2.7 Diagnóstico e tratamento

O diagnóstico de FP pode ser feito através da visualização dos tumores, contudo exames histopatológicos devem ser realizados para confirmação (BAPTISTOTTE, 2007).

Alguns fibropapilomas regridem e podem ser mantidos sem causarem maiores problemas às tartarugas. Nos casos em que os tumores afetam a sobrevida dos animais ou são muito grandes, o tratamento inclui a remoção cirúrgica do tumor e terapia de suporte para diminuir a debilidade (FORMIA et al., 2007; TOREZANI et al., 2009).

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3. AMOSTRAGEM

Para a realização da presente tese foram coletadas amostras de fibropapilomas de tartarugas-verdes (*Chelonia mydas*) em cinco Estados do Brasil: Ceará (CE), Bahia (BA), Espírito Santo (ES), São Paulo (SP) e Rio Grande do Sul (RS). Também foram coletadas amostras de pele de tartarugas-verdes na Ilha de Trindade e de tartarugas-verdes e tartarugas-cabeçudas (*Caretta caretta*) no RS. Todas as amostras de pele foram coletadas de animais sem tumor.

A escolha dos Estados do CE, BA, ES e SP foi baseada nos dados publicados por Baptistotte (2007) que mostram que entre 2000 and 2005, a prevalência média de fibropapilomatose em tartarugas verdes foi de 15,41% (1288/8359), com 36,94% (181/490) no CE, 31,43% (33/105) no Rio Grande do Norte (RN), 18,46% (12/65) em Sergipe (SE), 15,81% (211/1335) na BA, 27,43% (469/1710) no ES, 5,96% (9/151) no Rio de Janeiro (RJ), 10,73% (371/3456) em SP e 3,45% (2/58) em Santa Catarina (SC). Os Estados do CE, BA e ES foram escolhidos pois mostraram uma prevalência alta de FP durante esse período (36,94%, 15,81% e 27,43%, respectivamente) e o Estado de SP foi escolhido por ter um número grande de animais capturados por ano.

As amostras nestes quatro Estados foram coletadas pelo Projeto Tamar. A base do Tamar no CE está localizada em Almofala (02°50'S, 40°09'W), Municipio de Itarema, litoral oeste do Estado, a 242 km de Fortaleza. A área de atuação totaliza cerca de 40 km de praias. No Estado da BA, são monitorados aproximadamente 200 km de praias do litoral norte, numa área que se estende da foz do Rio Real (11°27'S, 37°21'W), divisa com o Estado de SE rumo ao sul de Salvador (13°00'S, 38°27'W). No Estado do ES são monitorados descontinuamente 224 km de litoral, situados entre Itaúnas (18°24'S, 39°42'W) ao norte, na divisa com a BA, e Anchieta (20°48'S, 40°38'W) ao sul. A maior parte da área monitorada está localizada na região do extremo norte, uma parte na região costeira central, localizada na região da grande Vitória e outra área no litoral sul. No Estado de SP, a área monitorada pela base de Ubatuba, no Município de Ubatuba (23°26'S, 45°05'W) vai da divisa de Paraty (RJ) ao norte, até o Municipio de Caraguatatuba (SP), ao sul, totalizando 106 km de extensão, totalizando 73 praias (BAPTISTOTTE, 2007).

No Estado do CE foram coletadas 60 amostras de fibropapiloma, na BA foram, 38; no ES, 37 e em SP, 40. Todas coletadas de tartarugas-verdes.

No RS, uma amostra de fibropapiloma foi coletada de uma tartaruga-verde na praia de Tavares (31º 19'S, 50º 59'W) e as amostras de pele (11 de tartaruga-verde e 3 de tartaruga-cabeçuda) foram coletadas no litoral norte do RS, que compreende 120 km de praias e estende-se desde Torres (29°20'S, 49°43'W) ao norte até Balneário Pinhal (30°14'S, 50°13'W) ao sul.

A Ilha de Trindade (20°30'S, 29°20'W), que é o principal sitio de desova das tartarugas-verdes, está localizada a 1140 km da costa do ES em direção a África (ALMEIDA et al, 2011). Nela, foram coletadas 45 amostras de pele de tartarugas-verdes durante a temporada reprodutiva de 2011.

Foi criado um protocolo padrão de coleta para que todas as amostras fossem coletadas e acondicionadas da mesma forma. O número total de amostras coletadas foi 176 amostras de fibropapilomas e 58 de pele. As coletas foram realizadas durante os anos de 2009 a 2011.

4. ARTIGOS CIENTÍFICOS

4.1 Detection and characterization of fibropapilloma associated herpesvirus of marine turtles in Rio Grande do Sul – Brasil*

*Manuscrito submetido à revista Pesquisa Veterinária Brasileira na forma de artigo original.

Detection and characterization of fibropapilloma associated herpesvirus of marine turtles in Rio Grande do Sul – Brasil[1]

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Fibropapillomatosis (FP) is a benign tumoral disease that affects sea turtles, hampering movement, sight and feeding, ultimately leading to death. In Brazil, the disease was described for the first time in 1986. Research suggests the involvement of a herpesvirus in association with environmental and genetic factors as causal agents of FP. The present study investigates and characterizes this herpesvirus in sea turtles living in the coast of state Rio Grande do Sul (RS), Brazil. From October 2008 to July 2010, 14 turtles were observed between the beaches of Torres and Tavares, of which 11 were green turtles (*Chelonia mydas*) and 3 were loggerhead turtles (*Caretta caretta*). All turtles were young and mean curved carapace length was 37.71 ± 7.82 cm, and varied from 31 to 55 cm. Only one green turtle presented a 1-cm, papillary, pigmented fibropapilloma. Skin and fibropapilloma samples were analyzed by convencional and real time PCR assays to detect and quantify herpesvirus. All skin samples were negative, though the fibropapilloma specimen was positive in both tests. Viral load was 9,917.04 copies of viral genome per milligram of tissue. The DNA

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fragment amplified from specimen of fibropapilloma was sequenced and presented the same amino acid sequence as a sample detected in a loggerhead turtle in Australia. This study reports the first molecular characterization of herpesvirus associated with fibropapilloma in turtles from the coast of RS.
INDEX TERMS: Fibropapilloma, sea turtle, herpesvirus, Rio Grande do Sul.

RESUMO.- [Detecção e caracterização do herpesvírus associado ao fibropapiloma de tartarugas marinhas no Rio Grande do Sul – Brasil.] A fibropapilomatose (FP) é uma doença tumoral benigna que pode causar a morte das tartarugas marinhas por dificultar a sua locomoção, visão e alimentação. Pesquisas sugerem o envolvimento de um herpesvirus em associação com fatores ambientais e genéticos como agentes causais da FP. No Brasil, foi descrita pela primeira vez em 1986. O objetivo do presente trabalho foi pesquisar e caracterizar esse herpesvirus em tartarugas marinhas do litoral do estado do Rio Grande do Sul (RS). De outubro de 2008 a julho de 2010, foram encontradas 14 tartarugas marinhas entre as praias de Torres e Tavares, das quais 11 eram tartarugas-verdes (*Chelonia mydas*) e 3 eram tartarugas cabeçudas (*Caretta caretta*). Todas as tartarugas eram jovens e o comprimento curvilinio de carapaça médio foi de 37,71 ± 7,82 cm, variando de 31 a 55 cm. Apenas uma tartaruga-verde apresentou um fibropapiloma de 1 cm, pigmentado e de superfície papilar. Amostras de pele e do fibropapiloma foram submetidas a PCR convencional e PCR em tempo real para detecção e quantificação do herpesvirus. Todas as amostras de pele foram negativas e o fibropapiloma foi positivo em ambas as técnicas, apresentando uma carga viral de 9.917,04 cópias de genoma viral/mg de tecido. O fragmento de DNA amplificado na amostra de fibropapiloma foi seqüenciada e revelou ter a mesma seqüência de aminoácidos de uma amostra detectada em uma tartaruga cabeçuda da Austrália. Essa é a primeira caracterização molecular do herpesvirus associado ao fibropapiloma em tartarugas do litoral do RS.
TERMOS DE INDEXAÇÃO: fibropapiloma, tartaruga marinha, herpesvirus, Rio Grande do Sul

INTRODUCTION

Fibropapillomatosis (FP) in sea turtles is an emerging disease, with high prevalence figures being reported from the 1980's on (Herbst et al. 2004). It is characterized by one or multiple fibroepithelial growths whose surface is either smooth or rough, apart from the presence of fibromas in viscera (Kang et al. 2008). Tumors are benign, but interfere in movement, feeding and operation of organs, weakening the animal and

even leading to death (Herbst 1994).

The first record of FP in the Brazilian coastline was made in 1986 (Baptistotte et al. 2005). In the state of Espírito Santo, between 2000 and 2006, the prevalence of fibropapillomas was 20.9%, while in the states of Ceará, Rio Grande do Norte, Sergipe, Bahia, Rio de Janeiro, São Paulo and Santa Catarina values varied between 3.5 and 36.9%, between 2000 and 2005. In the same period, no tumor was recorded in turtles living in the oceanic islands of Fernando de Noronha and Atol das Rocas (Baptistotte 2007, Torezani et al. 2010).

The pattern of spreading of FP is that of an infectious disease (Herbst et al. 1995). A herpesvirus has been identified in 100% of tumors induced by inoculation of tumor cell infiltrates (Ene et al. 2005) and 95% of natural infections (Quackenbush et al. 2001). However, the disease seems have a multifactor character, since the presence of ectoparasites (Greenblatt et al. 2004), environmental pollution (Torezani et al. 2010, Santos et al. 2010), ingestion of macroalgae (Van Houtan et al. 2010) as well as water temperature (Haines & Kleese 1977) seem to influence FP occurrence. The herpesvirus detected in fibropapillomas belongs to the family *Herpesviridae*, subfamily *Alphaherpesvirinae*, genus *Iltovirus*, and was called chelonid herpesvirus 5 (ChHV-5) (Davison et al. 2009).

The present study investigates and characterizes ChHV-5 in turtles living in the coast of the state of Rio Grande do Sul (RS).

MATERIALS AND METHODS

Turtles

The turtles used in this study were found beached or dead, between October 2008 and July 2010, between the beaches of Torres (31°17'S) and Tavares (29°20'S) on the coast of RS. Beached turtles were sent to the Centro de Estudos Costeiros, Limnológicos e Marinhos (CECLIMAR) for diagnosis and rehabilitation. Samples were collected under authorization number 19116-11, given by Instituto Chico Mendes para a Conservação da Biodiversidade, an organ of the Ministério do Meio Ambiente. When fibropapillomas were present, samples were collected from growths. In animals with no fibropapilloma, skin specimens were collected for analysis. Collections were carried out using standard surgical instruments. Specimens were frozen at -80°C upon processing.

PCR amplification and sequencing

Tissues collected were macerated in a 10 mM phosphate buffered saline (PBS) pH 7.4 (0.05 g/ 5 mL). The suspension was clarified at 350 *g* for 10 min. DNA extraction was

carried out using a 200-µL aliquot of the supernatant, according to the method by Chomksinsky (1993).

A 2-µL DNA aliquot was submitted to PCR in a final 50-µL volume using the specific primers for DNA polymerase of turtle herpesvirus, GTHV 2 and GTHV 3, described by Quackenbusch et al. (2001). The convencional PCR reaction was conducted in 10 mM Tris-HCl (pH 8.3), 2 mM $MgCl_2$, 50 mM KCl, 2.5% DMSO, 0.2 Mm each dNTP, 10 ρmol each primer, and 2.5 U *Taq* DNA polymerase (Ludwig Biotechnology Ltda.) All samples were denaturated at 94°C for 5 min and then were amplified with 35 cycles (94°C for 30 s, 62°C for 30 s, and 72°C for 30 s) and then a 10-min cycle at 72°C in a Veriti™ thermal cycler (Applied Biosystems). From each amplification reaction, 5 µL was electrophoresed in agarose gel 2%. The PCR products were 483-bp fragments and were purified using the kit GFX Purification (GE Healthcare, UK). Automated sequencing was carried out in an ABI-PRISM 3100 Genetic Analyzer automatic sequencer (Applied Biosystems) in the Laboratório ACTGene (Centro de Biotecnologia, UFRGS, Porto Alegre, Brazil).

Real time quantitative PCR

Samples were also submitted to real time PCR to determine the number of copies of viral DNA. The primers and probes used were previously described by Quackenbusch et al. (2001), which amplify an 86-bp fragment of the gene of the viral DNA polymerase. The reaction was conducted in a 25-µL final volume formed by 2 µL DNA, 5 ρmol each primer and 10 ρmol probe in 12.5 µL Platinum Quantitative PCR Supermix UDG (Invitrogen). Reaction mixtures were heated to 50°C for 2 min and to 95°C for 10 min to activate *Taq* polymerase followed by 40 cycles of 15 s at 95°C and 1 min at 62°C in a thermal cycler StepOne™ Real-Time PCR (Applied Biosystems). The standard curves used in reactions were log-transformed serial dilutions of the GTHV DNA pol, constructed by inserting a 483-bp fragment of the DNA polymerase gene of the herpesvirus in a vector, following the instructions provided by manufacturer of the kit TOPO TA Cloning ™ (Invitrogen).

Histological analysis

Sections of tumors were fixed in formalin 10%. After 5 days, specimens were dehydrated, clarified, embedded in paraffin, cut into 5-µm slices and stained according to the hematoxylin-eosin method.

RESULTS

Between October 2008 and July 2010, 14 turtles were observed, of which 11 were green turtles (*Chelonia mydas*) and 3 were loggerhead turtles (*Caretta caretta*) (Table 1). Only one green turtle presented a fibropapilloma, which measured 1 cm, in the pelvic region (Figure 1). The tumor surface had a papillary aspect, and was pigmented. All turtles were young, of undetermined sex, and curved carapace length (CCL) varying between 31 and 55 cm (mean: 37.71 ± 7.82 cm).

All skin specimens were negative for the herpesvirus in both conventional and real time PCR. However, the fibropapilloma sample was positive for the herpesvirus in PCR, and detected a viral load of 9,917.04 copies of viral genome per milligram of tissue. The number of copies of the genome was obtained by multiplying the value obtained by 7.5, the number of dilutions used in the DNA extraction routine. The correlation coefficient of the standard curve was > 0.9.

The histopatological analysis revealed a papillary pattern, with the presence of melanocytes, epithelial hyperplasia, hyperkeratosis and a nuclear halo, apart from moderate hyperplasia of estroma and dyskaryosis (Figure 2).

The amino acid sequence of the sample of fibropapilloma was confirmed to be a fragment of the gene of DNA polymerase of ChHV-5 by alignment with sequences deposited in GenBank using the BLAST (Basic Local Alignment Search Tool).

DISCUSSION

This is the first molecular characterization of ChHV-5 in turtles in RS. In Brazil, DNA of herpesvirus had been detected by PCR in samples of fibropapillomas and blood collected in sea turtles in the state of São Paulo (Monezi et al. 2006).

All five sea turtle species that occur in Brazil are observed in the country's southernmost seawaters: *Chelonia mydas*, *Caretta caretta*, *Dermochelys coriacea*, *Lepidochelys olivacea*, and *Eretmochelys imbricata* (Marcovaldi & Marcovaldi 1999, Pinedo et al. 1996). The first three species mentioned are often observed beached on the shores of RS (Bugoni, Krause & Petry 2001) and two were found during the study period.

Juvenile sea turtles use the coast of RS for feeding and developing, especially in summer and spring in the southern hemisphere (Soto & Beheregaray 1997). Sequencing of the fibropapilloma specimens collected in RS had the same amino acid sequence of sample AF299107, detected in a loggerhead turtle in Australia, when aligned with samples described by Quackenbusch et al. (1998) and Quackenbusch et

al. (2001) (Figure 3). There is no record of turtle migration between Brazil and Australia, but telemetry (Hays et al. 2001) and population genetics (Naro-Maciel et al. 2007), have revealed a connection between turtles observed in Brazil and those sampled in Ascencion Island. The study conducted by Naro-Maciel et al. (2007) also indicates the occurrence of haplotypes of turtles from Atol das Rocas, Trindade Island, Ascencion Island, Africa, Mexico, Costa Rica and Surinam in turtles observed in two sites of the Brazilian coast (Almofala, Ceará, and Ubatuba, São Paulo).

It is believed that the similarity between the herpesvirus strains detected in RS and the Australian strains may be explained in the light of the high conservation of the viral genus *Alphaherpesvirinae*. The analysis of the sequence of 23,055 base pairs of the genome of herpesvirus associated to the fibropapillomatosis demonstrated that over 96% of nucleotide sequences were conserved across the virus detected in *C. mydas* in Florida, Hawaii, Australia, Porto Rico and California, in *C. caretta* living in Australia and *L. olivacea* in Costa Rica (Greenblatt et al. 2005).

Histopathology showed the presence of epithelial hyperplasia and nuclear halos as described by Matushima et al. (2001) in fibropapillomas from state São Paulo. The proliferative cutaneo lesion of the green turtle from this report was similar to the previously described in fibropapillomas of green turtles from Florida (Lucke 1938, Smith & Coates 1938).

There is no record of the prevalence of fibropapillomatosis on sea turtles in the RS and few of anecdotal information circulates specially among marine biologists and veterinarians of wild animal regarding the presence of these disease in turtles found off the coast of the RS. The result of this study serve as a warning to the disease in the RS, since the high viral load was found.

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Fig. 1. Fibropapilloma with papillary, pigmented aspect.

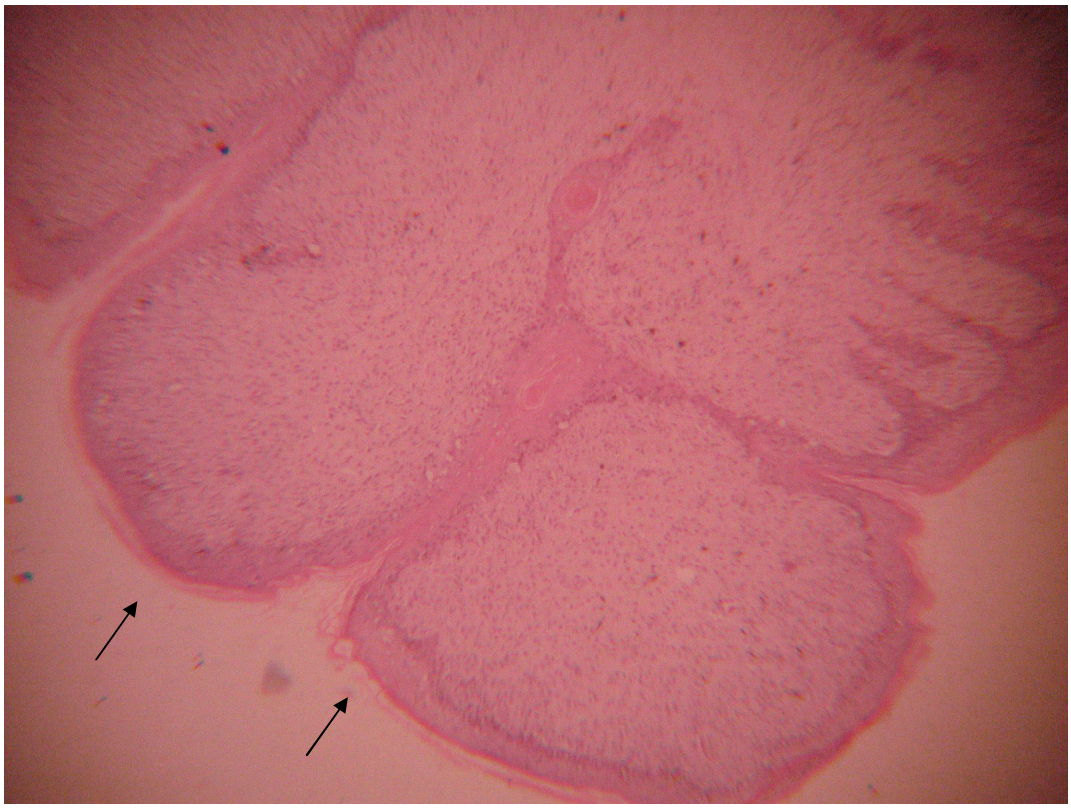

Fig. 2. Histopathological section of fibropapilloma showing a papillary pattern (Stained with hematoxylin-eosin, 5 x).

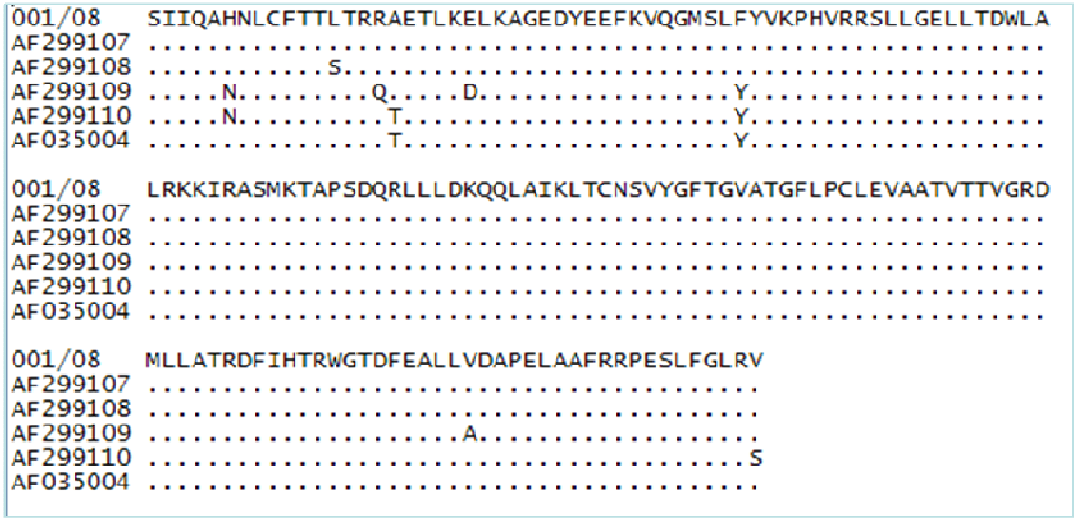

Fig. 3. Sequence alignment of amino acids of the DNA polymerase gene of ChHV-5. Samples AF299107 (*C. caretta*, Australia), AF299108 (*C. mydas*, Australia), AF299109 (*L. olivacea*, Mexico), AF299110 (*C. mydas*, Barbados) and AF35004 (*C. mydas*, Florida) were obtained in GenBank.

Table 1. Detection of herpesvirus in sea turtles. * Copies of viral genome/mg.

Number	Species	CCL (cm)	Sample	PCR	Real time PCR *
001/08	*C. mydas*	54.0	Fibropapilloma	Positive	9,917.04
002/08	*C. caretta*	35.0	Skin	Negative	Negative
001/09	*C. caretta*	32.5	Skin	Negative	Negative
001/10	*C. mydas*	31.5	Skin	Negative	Negative
002/10	*C. mydas*	55.0	Skin	Negative	Negative
003/10	*C. caretta*	32.0	Skin	Negative	Negative
004/10	*C. mydas*	42.0	Skin	Negative	Negative
005/10	*C. mydas*	31.0	Skin	Negative	Negative
006/10	*C. mydas*	35.0	Skin	Negative	Negative
007/10	*C. mydas*	32.0	Skin	Negative	Negative
008/10	*C. mydas*	36.0	Skin	Negative	Negative
009/10	*C. mydas*	38.0	Skin	Negative	Negative
111/10	*C. mydas*	40.0	Skin	Negative	Negative
112/10	*C. mydas*	34.0	Skin	Negative	Negative

4.2 Viral variants of the Chelonid herpesvirus 5 (ChHV 5) in fibropapillomas of green turtles (*Chelonia mydas*) in Brazil*

* Manuscrito submetido à revista Virus Genes na forma de artigo original.

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Viral variants of the Chelonid herpesvirus 5 (CHhv 5) in fibropapillomas of green turtles (*Chelonia mydas*) in Brazil

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Abstract

Fibropapillomatosis is a benign neoplastic disease that affects sea turtles worldwide. Herpesvirus (Chelonid herpesvirus 5) has been associated with the presence of tumors. Thirty-two tumors were collected from 27 juvenile green turtles in 4 Brazilian states (Ceará - CE, Bahia - BA, Espírito Santo - ES and São Paulo - SP). The virus was detected by PCR, and the amplification product of the DNA polymerase gene was sequenced. Six viral variants (var) were identified in Brazil. Two variants (var 1 and var 2) were found only in the SP samples, and another variant (var 3) was found only in ES. Var 4 was present in 19 samples collected in all states. Var 5 and var 6 were present only in samples from BA and CE, respectively. Var 4 was prevalent in SP, BA and ES. Coinfection by more than one variant in one animal was verified in the samples from BA and SP. These findings are similar to those described in the literature, and the heterogeneity of these viral variants support the hypothesis that turtles become infected after their recruitment to nearshore developmental habitats.

Keywords

Chelonid herpesvirus 5; green turtle; fibropapilloma; *Chelonia mydas*

INTRODUCTION

Over the past 20 years, fibropapillomatosis (FP) has emerged as an epidemic in sea turtles. The disease was first recorded in 1930 in the Florida Keys in a few green

turtles (*Chelonia mydas*), and its prevalence increased in the 1960s. It is currently considered a pandemic, with infection rates greater than 70% in some habitats [1]. FP is a debilitating neoplastic disease that is known to cause morbidity and mortality in marine turtles in various locations around the world. Tumors located on the flippers, the axillary or inguinal regions, the neck, the mouth, or the head can disrupt locomotion, feeding or vision. In some cases, tumors occur as visceral fibromas that can lead to organ failure and death [2]. The precise etiology of FP is still under investigation. It is believed that the disease is multifactorial [3], and studies show that FP has been associated with the presence of ectoparasites [4], environmental pollution [5-7], ingestion of microalgae [8] and water temperature [9]. There is convincing evidence that a virus is the causative factor of the disease, and recent studies show a strong correlation between herpesvirus and FP, which has been supported by molecular investigations (polymerase chain reaction) [10, 11]. The virus detected in FP belongs to the family *Herpesviridae*, subfamily *Alphaherpesvirinae*, genus *Iltovirus*, and was called Chelonid herpesvirus 5 (ChHV 5) [12]. The first record of FP in Brazil was from the state of Espírito Santo in 1986. Since then, outbreaks have been frequently observed in feeding areas. Records indicate an increase in FP incidence from 3.2% in 1997, 10.8% in 1998, and 10.9% in 1999 to 12.4% in 2000 [13]. Between 2000 and 2005, the average prevalence of FP in green turtles in Brazil was 15.41% (1288/8359), with 36.94% (181/490) in 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% (469/1710) 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) [13]. Green turtles (*Chelonia mydas*) have a circumglobal distribution in tropical and subtropical seas generally between latitudes 40ºN and 40ºS [14]. This species is currently classified as endangered by the Red List of Threatened Species of the Internacional Union for Conservation of Nature [15]. Although Brazil has a coastline of 8000 km, green turtles nest almost exclusively on oceanic islands, primarily on Trindade Island [16], but also on Atol das Rocas [17] and Fernando de Noronha [18]; scant green turtle nestings have been observed on the mainland [19]. The states of CE, BA, ES and SP are protected feeding areas for green turtles and were chosen because they had a high prevalence of FP, except for SP, which had a lower prevalence but a large number of turtles caught in previous years.

The objective of the present study was to characterize the viral variants present

in FP samples from green turtles and determine the relative frequency of each variant in four different coastal areas of Brazil (CE, BA, ES and SP).

MATERIALS AND METHODS

Tumor samples

Between June 2009 and June 2010, 32 tumors were collected from 27 juvenile green turtles (*Chelonia mydas*) on the beaches of CE, BA, ES and SP by Projeto TAMAR – ICMBio (TAMAR), which is the Brazilian Sea Turtle Conservation Programme. In CE and ES, 8 and 6 fibropapillomas were collected, respectively, with one tumor per animal. In BA, ten tumors were collected, with 2 tumors from one animal and 3 from another animal. In SP, 8 were collected, with 2 tumors from each of 2 animals. Skin fragment of a green turtle without fibropapillomatosis was used as negative control in all analyzes. The tumors and skin were collected with surgical instruments and kept at -80°C until processing. The tumor samples were collected with authorization 19116-1 issued by the Chico Mendes Institute for Biodiversity Conservation of the Ministry of Agriculture.

Extraction of viral DNA

The tumors and skin collected were macerated in 10 mM phosphate buffered saline (PBS) at a pH of 7.4 (0.05 g/5 mL). The suspension was clarified at 350 x *g* for 10 min. The DNA extraction was performed using a 200-µL aliquot of the supernatant according to the method by Chomkczynsk [20] using guanidine isothiocyanate and phenol.

ChHV 5 detection by PCR and genetic typing

A single PCR employing GTHV 2/GTHV 3 primers [11] flanking a 483-bp fragment in DNA polymerase of the turtle herpesvirus was used to detect ChHV 5 in the tumor samples. The conventional PCR reaction was conducted in 10 mM Tris-HCl (pH 8.3), 2 mM $MgCl_2$, 50 mM KCl, 2.5% DMSO, 0.2 Mm of each dNTP, 10 ρmol of each primer, 2.5 U of *Taq* DNA polymerase (Ludwig Biotechnology Ltda.) and 2 µL of DNA

in a final volume of 50 µL. All samples were denatured at 94°C for 5 min and then amplified with 35 cycles (94°C for 30 s, 62°C for 30 s, and 72°C for 30 s), followed by a 10-min cycle at 72°C in a Veriti™ thermal cycler (Applied Biosystems). The PCR products were electrophoresed in 2% agarose gels and visualized under UV light and compared with a 100-bp molecular weight ladder (Fermentas, USA).

The amplification products were purified using the GFX Purification kit (GE Healthcare, UK). Amplicons of the expected size were sequenced in both directions on an ABI-PRISM 3100 Genetic Analyzer automatic sequencer (Applied Biosystems) in the Laboratório ACTGene (Centro de Biotecnologia, UFRGS, Porto Alegre, Brazil) using the GTHV2/GTHV3 primers.

Subsequently, the nucleotide sequences were processed using the BioEdit program and aligned with Clustal W [21], and the resulting sequences were submitted to GenBank (http://www.ncbi.nlm.nih.gov). Kimura 2-parameter pairwise distances [22], calculated for the different nucleotide sequences detected in Brazil, were used to construct a phylogenetic unrooted tree by using the neighbor joining distance methods in the Molecular Evolutionary Genetics Analysis software MEGA 4 [23]. The statistical confidence of the tree topologies was performed by 1000 bootstrap replications using the same software.

RESULTS

A PCR method already described in the literature was used to detect a DNA polymerase gene fragment from ChHV 5, and 100% of the samples analyzed displayed amplification products of the expected sizes. No amplification was detected in the negative controls. The sequencing of the amplification products showed that there are six variants, (var) 1, 2, 3, 4, 5 and 6 (GenBank accession numbers JN938584 to JN938589), of the virus in CE, BA, ES and SP in Brazil (Figure 1). Two variants (var 1 and var 2) were found only in SP, and another (var 3) was found only in ES. Var 4 was present in 19 (59.4%) samples collected in all states. Var 5 and var 6 were present only in BA and CE, respectively. Var 4 was prevalent in the samples from SP, BA and ES (Table 1). In BA, var 4 was present in both of the tumor samples collected from the same animal, and the turtle with three tumors had two tumors from var 4 and one tumor from var 5. In SP, two tumors were collected from each of two turtles. The results showed that one turtle had var 2 in both samples, and the other turtle had var 1 in one

sample and var 4 in the other sample.

DISCUSSION

ChHV 5 DNA was found in 100% of the Brazilian turtle samples analyzed. Herpesvirus DNA polymerase sequences have been previously detected by PCR from every tested fibropapilloma and fibroma reported [10, 11, 24].

Various authors have described the viral variants of ChHV 5 by analyzing different genes of the viral DNA. Analyzing glycoprotein B, four groups of variants were described: the Atlantic (formed by sequences from Florida and Barbados), the Middle Pacific (Hawaii), the Western Pacific (Australia) and the Eastern Pacific (Costa Rica and California). The Atlantic group has 0.98% of divergence from the Middle Pacific and Western Pacific groups. The Western Pacific and the Middle groups differ by 0.42%, but the sequences are distinct from the Middle Pacific, with a 6-bp deletion at position 1164-1169. The Atlantic, Western Pacific, Middle Pacific and Eastern Pacific groups vary from 2.8% to 3.1%. The Eastern Pacific group has the greatest nucleotide variability [25].

Comparing 6801 bp of the viral genome, 5 viral variants were found in 25 turtles of 3 species (*C. mydas*, *C. caretta* and *L. kempii*) and were named Florida A, B, C and D and Hawaiian (HA). Variants Florida A, B and C are nearly identical. Variant D, which was isolated from the loggerhead turtle (*Caretta caretta*), differs by 5.6% from the other variants, whereas the HA variant differs from the Florida variants A, B and C by only 2.2% [26]. The same pattern of variants is maintained within the 483-bp sequence of the DNA polymerase [26] that was analyzed in the present study, and we detected 6 viral variants. When the 6 Brazilian variants were compared with those described by Herbst et al. [26], var 1, 2, 3 and 4 were closer to var HA, while var 5 and 6 resembled var Florida A, B and C (Figure 1). Var 1, 2, 3 and 4 differed from var HA in 4 nucleotides that resulted in the substitution of 2 amino acids. Var 5 differed from var Florida A, B and C by 1 nucleotide, resulting in the substitution of one amino acid, while var 6 differed by 2 nucleotides, resulting in 2 amino acid substitutions (Figure 2).

Evidence indicates that after dispersing from their natal beaches, post-hatchling sea turtles spend several years in the pelagic (open ocean) environment before recruiting to neritic (nearshore) developmental feeding habitats [27]. FP is detected primarily in juvenile and immature sea turtles in these coastal habitats [26]. Infection with ChHV 5

could occur either in the natal beach environment, the post-hatchling pelagic environment, or after the recruitment of juveniles to neritic environments [2]. The observation that postpelagic stage juveniles develop the disease in nearshore feeding grounds can be explained by two hypotheses. One hypothesis is that exposure to the etiologic agent occurs in the pelagic zone, where the disease has a long latent period that results in the clinical disease only developing in older juveniles after they have migrated to the neritic feeding grounds. The second hypothesis is that exposure to the etiologic agent occurs after the juveniles have been recruited to near-shore feeding grounds. The wide variation in the prevalence of disease among size/age matched populations of juvenile green turtles from different nearshore sites lends support to the second hypothesis [28].

Similar to what has been reported in Florida [2], var 4 prevailed in three out of the four regions analyzed, and var 6 prevailed in another region (Table 1). Var 1 and var 2 were only found in SP, and var 3 was only found in ES. The heterogeneous distribution of the viral variants in green turtle tumors from different Brazilian coastal locations is not consistent with post-hatchling green turtles being exposed to ChHV 5 in the pelagic environment. These results are consistent with the hypothesis that infection occurs after juveniles recruit to specific nearshore localities, where the relative frequency of viruses present can vary extensively from site to site depending on turtle movements and hydrologic conditions [2]. The fact that large differences in prevalence can be found at short distances (<1 km), as seen, for example, by comparing the prevalence of FP in the Indian River (50%) with that from the nearshore Sabellariid reefs on the ocean side of the barrier island at Wabasso Beach (0%), reinforces the hypothesis that turtles are infected after they are recruited to nearshore developmental habitats [28]. Variant 4 was found in the four states surveyed, and this finding can be explained by the genetic heterogeneity of the turtles or their migration along the Brazilian coast. Genetic studies on mitochondrial DNA (mtDNA) indicate that the juvenile green turtle population in feeding grounds can be formed from genetically distinct stocks, with contributions from several different nesting populations [29, 30]. Naro-Maciel et al.[31] showed through genetic analyses based on mtDNA that juvenile green turtles found in CE and SP originate largely from Ascencion Island (United Kingdom), although they may also come from several other rookeries in the Atlantic, mainly Tortuguero (Costa Rica), Matapica (Suriname), Aves Island (Venezuela) and Trindade Island (Brazil). There is no known pattern of migration for green turtles in the

waters of the Brazilian coast. A study in SP showed that green turtles captured in Ubatuba were recaptured in Mucuri 596 days later in the southernmost part of BA, approximately 900 km (along the coastline) from Ubatuba. The farthest recapture to the south occurred near Rio Grande, in Rio Grande do Sul, which is approximately 1200 km (along the coastline) from Ubatuba, and the recapture interval was 483 days [32].

All variants were found in at least two tumor samples, with the exception of var 5. To confirm its identity, the DNA of the tumor was analyzed again, resulting in the same DNA sequence. Coinfection of the same turtle by different variants has been described in green turtles from Florida [2], and we found co-infection of turtles by two virus variants in BA (var 4 and 5) and SP (var 1 and 4) in different tumors. The analysis of the DNA sequencing chromatograms did not show a mixture of nucleotides, indicating that these variants could be not found in the same tumor.

FP has been associated with the presence of ectoparasites [4], environmental pollution [5-7], ingestion of microalgae [8] and water temperature [9]. The four states analyzed in this work have a large amount of human activity (agriculture, industry or urban development) in the coastal region, in addition to having different sea surface temperatures. According to the Center of Hydrography of the Navy of Brazil, CE and BA (northeast) have an average temperature of 25°C, while ES and SP (southeast) have an average sea surface temperature of 20°C. These factors may contribute to the high prevalence in these states as well as to the diversity of viral variants found.

This is the first detection and characterization of ChHV 5 in green turtles conducted in Brazil. Only *C. mydas* was sampled in this work because this is the most populous species on the Brazilian coast. Six viral variant were characterized, and some of the variants showed a heterogeneous distribution along the coast line. We also found the coinfection of the same turtle with different variants. These findings are consistent with those described in the literature [2] and reinforce the hypothesis that the ChHV 5 is acquired after turtles recruit to nearshore developmental habitats and allow us to conclude that ChHV 5 circulates in green turtles from the Brazilian coast.

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Table 1: Viral variant of Brazilian fibropapilloma samples, with the number of samples assigned.

Turtle	State	n	Variant (n)
1	ES	1	4
2	ES	1	4
3	ES	1	4
4	ES	1	4
5	ES	1	3
6	ES	1	3
7	BA	1	4
8	BA	1	4
9	BA	2	4 (2)
10	BA	3	4 (2), 5 (1)
11	BA	1	4
12	BA	1	4
13	BA	1	4
14	CE	1	4
15	CE	1	6
16	CE	1	6
17	CE	1	6
18	CE	1	6
19	CE	1	6
20	CE	1	6
21	CE	1	4
22	SP	2	2 (2)
23	SP	2	1 (1), 4 (1)
24	SP	1	4
25	SP	1	1
26	SP	1	4
27	SP	1	4
Total		32	

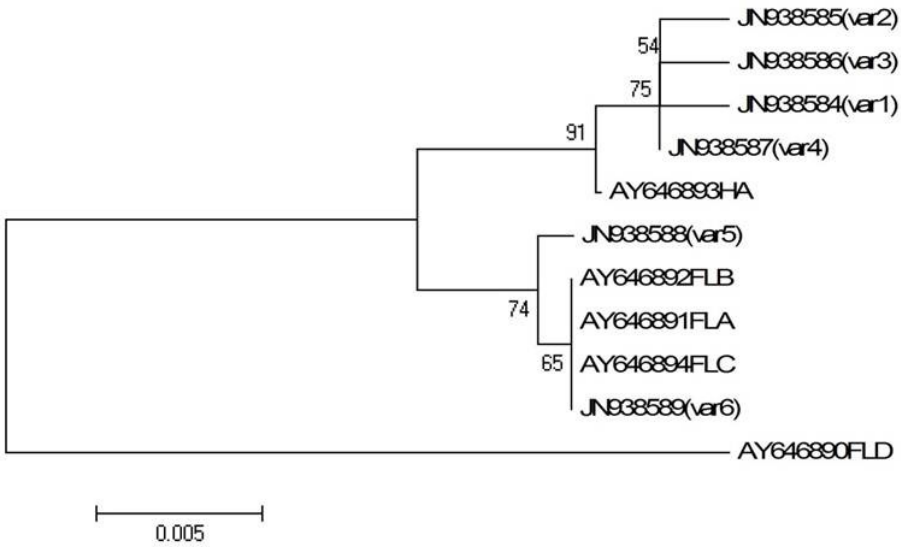

Fig 1: Nucleotide phylogenetic tree showing the genetic relationship among six Brazilian variants (var) for ChHV 5 and other sequences previously described [2] using the neighbor joining distance method, with 1000 bootstrap replications.

```
JN938584(var1)  SIIQAHNLCFTTLTRRAETLKELKAGEDYEEFKVQGMSLFYVKPHVRRSLLGELLTDWLA
JN938587(var4)  ........................................................
JN938585(var2)  ........................................................
JN938586(var3)  ........................................................
JN938588(var5)  ................T.......................................
JN938589(var6)  ................T......................Y.................
AY646893HA      ........................................................
AY646894FLC     ................T......................Y.................
AY646890FLD     .....................................Y..................
AY646891FLA     ................T......................Y.................
AY646892FLB     ................T......................Y.................

JN938584(var1)  LRKKIRASMKTAPSDQRLLLDKQQLAIKLTCNSVYGFTGVATGFLPCLEVAATVTTVGRD
JN938587(var4)  ........................................................
JN938585(var2)  ........................................................
JN938586(var3)  ........................................................
JN938588(var5)  ........................................................
JN938589(var6)  ........................................................
AY646893HA      ........................................................
AY646894FLC     ........................................................
AY646890FLD     ........................................................
AY646891FLA     ........................................................
AY646892FLB     ........................................................

JN938584(var1)  MLLATRDFIHTRWGTDFEALLVDAPELAAFRRPESLFGLRV
JN938587(var4)  ...................................
JN938585(var2)  ......N............................
JN938586(var3)  ............................Q......
JN938588(var5)  ...................................
JN938589(var6)  ...................................
AY646893HA      ...................................
AY646894FLC     ...................................
AY646890FLD     ..................T................
AY646891FLA     ...................................
AY646892FLB     ...................................
```

Fig 2: Alignment of the deduced amino acid sequences of the 483 bp fragment of DNA polymerase gene from Brazil samples and those described by Ene et. al, 2005. The first amino acid (S) corresponds to position 649 and the last (V) at position 809 of the complete DNA polymerase gene.

4.3 Chelonid herpesvirus 5 in fibropapillomas of green turtles (*Chelonia mydas*) in Brazil*

* Manuscrito em fase de redação, a ser submetido a revista científica.

Chelonid herpesvirus 5 in fibropapillomas of green turtles (*Chelonia mydas*) in Brazil

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INTRODUCTION

Green turtles (*Chelonia mydas*) have a circumglobal distribution in tropical and subtropical seas generally between latitudes 40ºN and 40ºS (Hirth, 1997). This species is currently classified as endangered by the Red List of Threatened Species of the Internacional Union for Conservation of Nature (IUCN, 2011). Although Brazil has a coastline of 8000 km, green turtles nest almost exclusively on oceanic islands, mainly on Ilha de Trindade (Moreira et al, 1995), but also on Atol das Rocas (Bellini et al, 1996) and Fernando de Noronha (Bellini and Sanches, 1996); scant green turtle nesting has been observed on the mainland (Marcovaldi e Marcovaldi, 1999). The development of coastal regions is exerting increasing adverse impact on coastal ecosystems, sometimes leading to the exclusion or extinction of species (Daszak et al, 2000; Worm et al, 2006). In addition to anthropogenic threats, diseases such as fibropapillomatosis (FP) may pose additional threat to *C. mydas* (Herbst 1994, Aguirre et al, 1998).

Cutaneous FP in green turtles was first reported over 50 years ago. In the last decade, FP has emerged as a significant worldwide epizootic with prevalences as high as 92% in some green turtle populations (Balazs, 1991; Herbst, 1994). Tumors located on the flippers, axillary or inguinal regions, neck, mouth, or head can disrupt locomotion, feeding or vision. In some cases tumors occur as visceral fibromas that can lead to organ failure and death (Ene et al, 2005). The precise etiology of FP is still under investigation but the disease has been associated with the presence of ectoparasites (Greenblatt et al, 2004), environmental pollution (Torezani et al 2010, Santos et al 2010), ingestion of microalgae (Van Houtan et al 2010) and water temperature (Haines and Klee 1977). There is convincing evidence that a virus is the causative factor of the

disease, and recent studies show a strong correlation between the identification of Chelonid herpesvirus 5 (ChHV 5) and FP, which has been supported by molecular investigations (polymerase chain reaction) (Lu et al, 2000, Quackenbush et al 2001). The first record of FP in Brazil was the state of Espirito Santo in 1986 and since then have been frequently observed in feeding areas. Records indicate an increase in FP incidence: 3.2% in 1997, 10.8% in 1998, 10.9 in 1999 and 12.4% in 2000 (Baptistotte, 2007). Between 2000 and 2005, the average prevalence of FP in green turtles in Brazil was 15.4% (1288/8359) with 36.9% (181/490) in Ceará (CE), 31.4% (33/105) in Rio Grande do Norte (RN), 18.5% (12/65) in Sergipe (SE), 15.8% (211/1335) in Bahia (BA), 27.4% (469/1710) in Espírito Santo (ES), 6% (9/151) in Rio de Janeiro (RJ), 10.7% (371/3456) in São Paulo (SP) and 3,4% (2/58) in Santa Catarina (SC) (Baptistotte, 2007)

The aim of the present study was to detect and quantify the ChHV 5 DNA in fibropapillomas of green turtles from the Brazilian coast and to determine associations between viral load and characteristics of tumors.

MATERIALS AND METHODS

Tumor samples

One hundred and seventy-five fibropapilloma samples were collected on beaches of the states of CE, BA, ES and SP between June 2009 and August 2010. During the month of February 2011 skin samples were collected from 45 green turtles nesting on the Ilha de Trindade. Thirty seven, 38, 40 and 60 tumors samples were collected in the states of ES, BA, SP and CE, respectively. The states of CE, BA and ES were chosen because a high prevalence of FP was detected, apart from the SP state, which despite having a lower prevalence, had a large number of turtles caught. In some turtles from BA two or more tumours were collected. The Ilha de Trindade was chosen because is the principal site of the green turtle nesting in Brazil and is free of fibropapillomatosis.

The collected tumors were evaluated for pigmentation (presence or not), size (cm), number and shape of the outer surface (smooth or warty). The score of tumors was determined considering the size and number of tumors and

reflects the severity of fibropapillomatosis in green turtles, ranging from unaffected (score 0) to strongly affected (score 3) (Work et al., 1999). Tumors and skin were collected with surgical instruments and kept frozen (-80°C) until processing.

Curved carapace length (CCL) was measured to the nearest 0.1 cm with a flexible plastic tape, from the anterior point at midline (nucal scute) to the midpoint of the line segment connecting the posterior tips of the supracaudal scutes. Overall body condition and presence or absence of external tumours was determined visually through physical examination of the turtle. Overall body condition (normal, underweight or emaciated) was determinated following Walsh (1999). A turtle was classified as “normal” if the plastron was convex, eyes were normal, the muscles of the neck area had fatty tissues and axillary and inguinal areas were protuberant; “underweight” if the plastron was little concave, eyes were either normal or sunken, the muscles of the neck area had surrounding fatty tissues and axillary and inguinal area were slightly sunken; or “emaciated” if the plastron was very concave, the eyes were sunken, the muscles of the neck area were more obvious with little or no surrounding fatty tissues, and axillary and inguinal areas were very thin. In this work we had put together the body condition of turtles as follows: healthy (normal body condition), weak (underweight and emaciated) and killed.

DNA extraction

Tumors were macerated in 10 mM phosphate buffered saline (PBS) pH 7.4 (0.05 g/5 mL). The suspension was clarified at 350 *g* for 10 min. DNA extraction was carried out using a 200 µL aliquot of the supernatant, using guanidine isothiocyanate and phenol (Chomksinsky, 1993).

ChHV 5 detection

A single PCR employing GTHV 2/GTHV 3 primers (Quackenbusch et al. 2001) flanking a 483 bp fragment in DNA polymerase of ChHV 5 was used to detect turtle herpesvirus in tumor samples. The conventional PCR reaction was conducted in 10 mM Tris-HCl (pH 8.3), 2 mM $MgCl_2$, 50 mM KCl, 2.5% DMSO, 0.2 Mm of each dNTP, 10 ρmol of each primer, 2.5 U of *Taq* DNA polymerase (Ludwig Biotechnology Ltda.) and 2 µL of DNA in a final volume of 50 µL. All

samples were denatured at 94°C for 5 min and then amplified with 35 cycles (94°C for 30 s, 62°C for 30 s, and 72°C for 30 s), followed by a 10-min cycle at 72°C in a Veriti™ thermal cycler (Applied Biosystems). The PCR products were electrophoresed in 2% agarose gels and visualized under UV light and compared with a 100-bp molecular weight ladder (Fermentas, USA).

ChHV 5 quantification

Samples were also submitted to real time PCR to determine the number of copies of viral DNA. The primers and probes used were previously described (Quackenbusch et al. 2001), which amplify an 86-bp fragment of the DNA polymerase gene. The reaction was conducted in a 25-µL final volume formed by 2 µL DNA, 5 ρmol each primer and 10 ρmol probe in 12.5 µL Platinum Quantitative PCR Supermix UDG (Invitrogen). Reaction mixtures were heated to 50°C for 2 min and to 95°C for 10 min to activate *Taq* polymerase followed by 40 cycles of 15 s at 95°C and 1 min at 62°C in a thermal cycler StepOne™ Real-Time PCR (Applied Biosystems). The standard curves used in reactions were log-transformed serial dilutions of the GTHV DNA pol, constructed by inserting a 483-bp fragment of the DNA polymerase gene of the herpesvirus in a vector, following the instructions provided by manufacturer of the kit TOPO TA Cloning ™ (Invitrogen).

Histological analysis

Tumor sections were fixed in formalin 10%. After 5 days, specimens were dehydrated, clarified, embedded in paraffin, cut into 5-µm slices and stained using the hematoxylin-eosin method.

Statistical analysis

The U of Mann-Whitney test was used to analyze the difference between viral load and score of tumors. The Kruskal-Wallis test was used to evaluate the differences between viral load and the pigmentation or shape. Analyses were performed in SPSS version 18 software and only samples that were positive in real-time PCR were included.

RESULTS

All samples collected at the Ilha de Trindade were from healthy adult females that had curved carapace length (CCL) between 97 and 130 cm. The PCR result was negative. In the coastal region turtles were young (between 33 and 76 cm) and indeterminate sex. The turtles sampled on the coast, 75% (131/175) were considered healthy, 15% were dead and 10% weak (emaciated or underweight). With respect to the score of the tumors, 33% had a score of 1, 28% had score 2 and 39% had a score of 3. These tumors had a warty surface in 70% of the samples, smooth in 30% and 41% were pigmented. The size of collected tumors ranged from 0.3 to 7.0 cm (mean 1.6 ± 1.4 cm). The conventional PCR used as screening test was positive in 73% of the tumors samples. The real-time PCR to quantify viral load in tumors resulted in 87% (153/175) of samples positive, and all samples that were positive in the conventional PCR were also positive in the real-time PCR. According to Figure 1, 15.4% of the samples were negative or had a viral load lower than 1 copy/mg and 62.9% of tumor samples had viral load between 1 and 10,000 copies/mg tumor. The maximum viral load was 889,674.98 copies/mg of tissue, and the median was 977 copies.

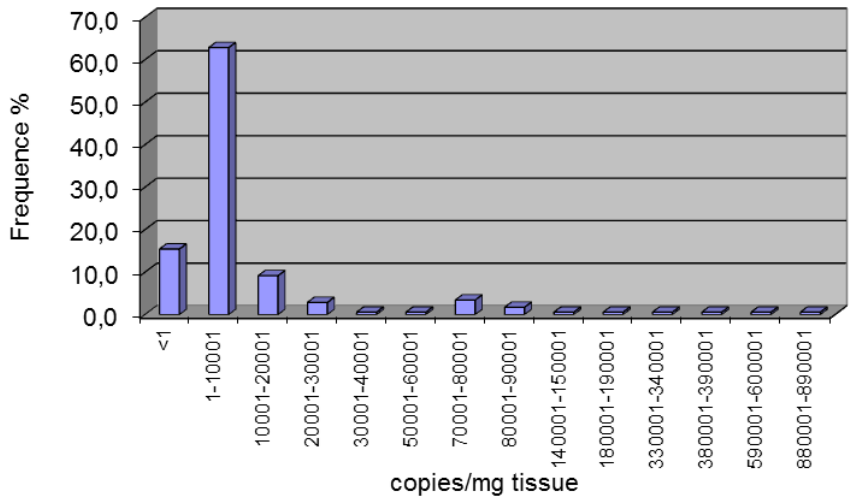

Fig 1: Number of ChHV 5 DNA copies in fibropapillomas of green turtles from Brazil.

To investigate the association between viral load and tumor characteristics (pigmentation and external appearance) was performed U of Mann-Whitney test and for assess the association between viral load and score of tumors, health status of turtles and states was performed Kruskal-Wallis test. There was no significant association (p=0,360) between viral load and the four states analyzed. Table 1 shows that there was only a significant association between the tumor score ($p < 0.05$) and viral load. Animals showing tumor score 3 had a higher viral load per tumor.

Table 1: Associations between viral load and characteristics of the 153 fibropapilloma samples that showed viral load greater than 1 copy / mg of tissue.

	n	Mean	SD	Median*	p valor
SURFACE					
Smooth	42	648.42	1604.37	23.45	0.390
Wartyo	111	4126.50	15046.42	291.56	
PIGMENT					
No	91	2463.88	9751.36	217.73	0.200
Yes	62	4210.68	16549.51	288.77	
TUMOR SCORE					
1	46	2979.61	12306.62	70.025[a]	0.001
2	45	3530.45	17693.55	70.29[a]	
3	62	3053.92	8851.37	582.02[b]	
HEALTH STATUS					
Healthy	115	3209.86	14032.95	266.22	0.612
Weak	15	2051.65	4873.54	374.06	
Dead	23	3711.55	10808.44	310.00	

* Different letters indicate significant difference in median value

The same association was performed in CE, BA, ES and SP states. Table 2 shows that there was no association ($p > 0.05$) between the data in the state of ES. In the state of BA and SP was a significant association ($p < 0.05$) between the health status of turtles and viral load where the dead animals showed a higher viral load. In the state of the CE there was association between viral load and external appearance of tumors (p= 0.042). Tumors with a warty surface displayed higher viral loads.

Table 2: Associations, shown among the four states, between viral load and characteristics of the 153 fibropapilloma samples with viral loads greater than 1 copy / mg of tissue.

	BA					CE					ES					SP				
SURFACE	n	Mean	SD	Median*	p valor	n	Mean	SD	Median*	p valor	n	Mean	SD	Median*	p valor	n	Mean	SD	Median*	p valor
Smooth	1	1.69	-	-	0.317	15	789.62	2457.12	7.19[a]	0.042	13	400.83	629.41	19.97	0.260	20	782.83	1099.53	388.48	0.882
Warty	25	2961.47	10341.95	310		41	6321.1066	19597.54	330.1[b]		22	577.57	877.08	126.215		16	4875.31	17104.51	312.02	
PIGMENT																				
No	13	1269.57	2131.96	310.00	0.858	35	3026.18	8226.42	16.99	0.068	23	474.83	824.89	105.89	0.404	20	4543.54	17741.51	419.02	0.679
Yes	13	4425.71	14306.40	145.67		21	7861.59	25666.42	354.67		12	583.04	744.81	208.08		16	1964.88	6057.81	279.99	
TUMOR SCORE																				
1	3	1404.39	656.65	1365.9	0.284	50	4674.55	17796.13	123.23	0.154	26	341.72	558.31	103.12	0.235	36	3397.47	13721.75	358.81	**
2	5	173.79	222.21	24.14		4	6135.28	8793	2842.15		6	894.1	1264.58	479.45		0				
3	18	3830.92	12162.88	135.83		2	6370.47	4469.23	6370.47		3	122.74	1055.98	1740.02		0				
HEALTH STATUS																				
Healthy	3	929.04	1576.95	37.11[ab]	0.042	17	1422.96	3522.92	16.99	0.184	18	260.75	460.27	103.12	0.256	8	13173.89	28258.93	141.81[ab]	0.022
Weak	8	81.94	109.82	35.99[a]		22	6866.2	25154.66	136.62		6	944.71	931.35	774.88		9	165.53	233.34	37.36[ab]	
Dead	15	4706.4	13218.62	767.84[b]		17	5633.11	11172.28	675.18		11	686.89	1030.99	122.17		19	812.01	881.24	510.69[b]	

* Different letters indicate significant difference in median value

** Test not performed because only one of scores was observed.

The majority of the tumors displayed hyperplasia of the stroma (98%), 53% had melanocytes, and 70% epithelial hyperplasia (Figure 2). The majority of the samples that showed epithelial hyperplasia also showed nuclear features suggestive of viral infection (Figure 3).

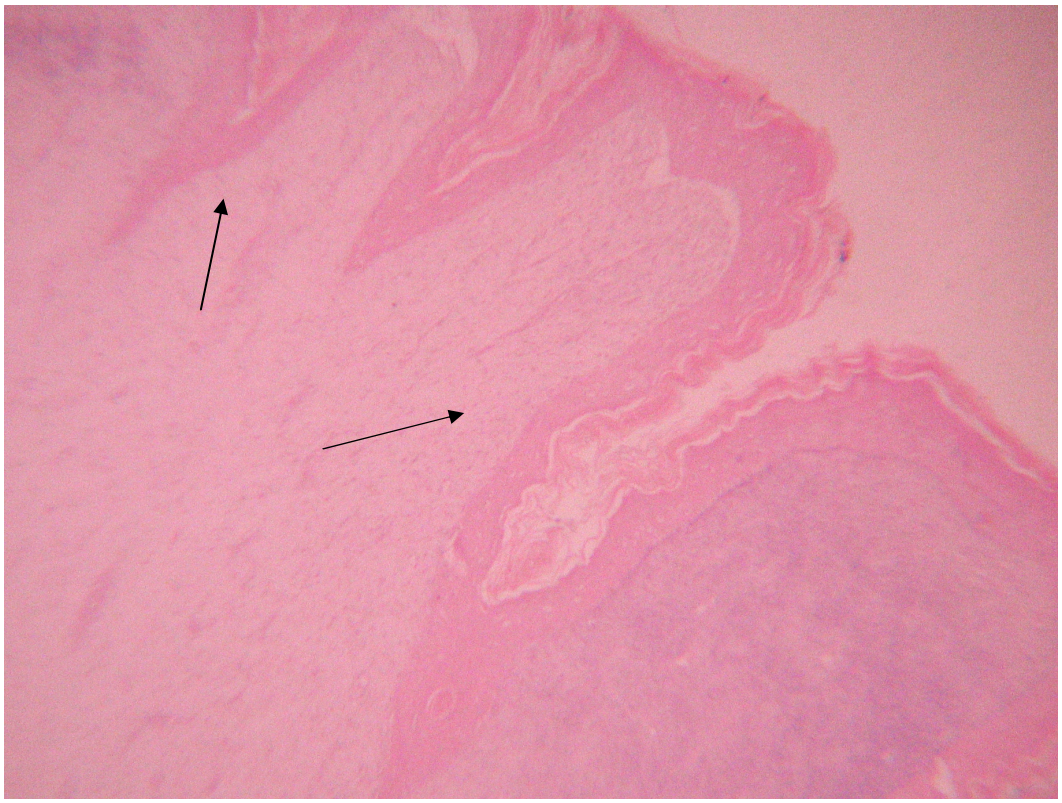

Figure 2: Fibropapilloma from a green turtle, *Chelonia mydas*, showing typical arborizing pattern of papillary epidermal hyperplasia supported by fibrovascular stroma.

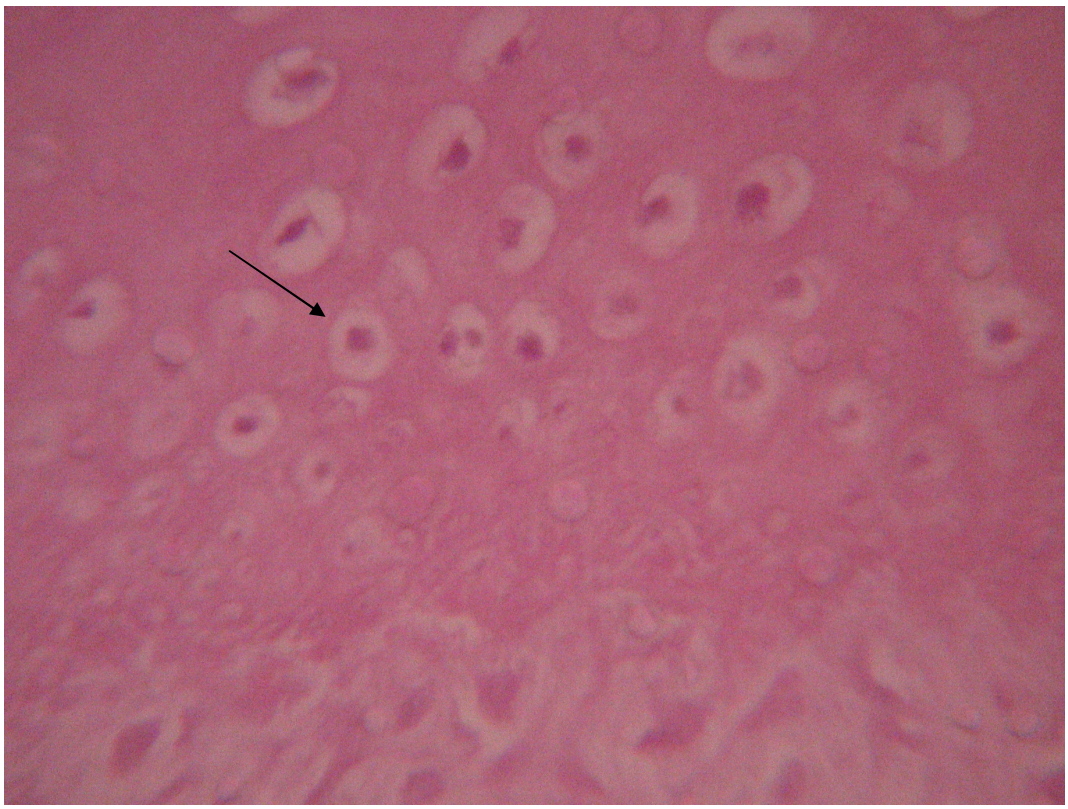

Figure 3: Nuclear halo and nuclear feature discariotic.

DISCUSSION

FP appears to affect certain age and size classes of turtles more than others. FP is rare (0-12%) among nesting adult females and lesions tend to be focal and mild, although these are underestimates of the true prevalence in the adult population (Herbst, 1994). In Hawaiian feeding ground sites, intermediate-sized turtles (40 – 90 cm carapace length) were most commonly and most severely affected (Balazs, 1991). Turtles with tumors sampled in four states were between 33 and 76 cm in carapace length. In Brazil, the higher frequency of tumors in green turtles is displayed between 30 and 80 cm carapace length (Baptistotte, 2007). The absence of the FP in animals less than 30 cm could be explained by lack of sufficient time for the onset of the disease. Low prevalence above 80 cm could be explained in two ways: 1) the disease would be self-limiting and at this stage of life individuals would have been cured due to an increased resistance conferred by age, 2) the prevalence would result in mortality turtles before they reach a larger size. Foley et al (2005) suggest a

combination of both explanations. This same combination may explain the absence of FP in turtles in reproduction period of the island of Trinidad, as they had a carapace length greater than 80 cm. There is no record of fibropapillomatosis on the Ilha de Trindade and negative PCR result confirms the absence of virus circulation in the skin of green turtles on the island in the reproductive period. Quackenbush et al (2001) described the presence of the virus in the skin of tumor free turtles, but these were in regions with a history of the disease.

The turtles used in this study were captured by the TAMAR project to biometric study and identification. Some sick individuals were captured for treatment and rehabilitation and other found dead. Most turtles (75%) were considered healthy and 67% had score of tumors 2 or 3. In a study in Espirito Santo between 2007 and 2008, 87 green turtles with tumor examined 94% had a tumour score of 2 or 3 and had no significant difference between the different bodily conditions and the tumor scores (Santos et al, 2010).

Cutaneous fibropapillomas of green turtles are single to multiple raised masses ranging from 0.1cm to greater than 30 cm in diameter. Individual masses may be either verrucous or smooth and either sessile or pedunculated (Herbst, 1994). Our findings of stromal hyperplasia, epithelial hyperplasia and nuclear findings characteristic of viral infection confirm the diagnosis of fibropapillomatosis. Tumors had a surface smooth in 70% and 41% were pigmented. The size of collected tumors ranged from 0.3 to 7.0 cm (mean 1.6 ± 1.4 cm). Tumor pigmentation is usually related to the pigmentation of the skin at the site of origin. Differences in tumor pigmentation are due to the distribution of melanophores within the tissues. Highly pigmented masses contained diffusely distributed melanophores within the dermis and between epidermal cells (Herbst, 1994).

The characteristic description of cutaneous FP is that of papillary epidermal hyperplasia supported on broad fibrovascular stromal stalks (Fig 2). The ratio of epidermal to dermal proliferation varies among lesions. Lesions that are comprised primarily of proliferating epidermis with little or no underlying dermal involvement are properly called papillomas while those lesions predominantly comprised of proliferating dermal components with relatively normal epidermis are called fribromas. Those masses in which both tissues are

hyperplastics are termed fibropapillomas (Herbst, 1994). Authors have postulated that there is a developmental progression from papilloma (early lesions) through fibropapilloma to fibroma (chronic lesions) (Jacobson et al, 1989). Hyperkeratosis is not always noted if tumors arise from noncornified epidermal sites, including the cloaca and conjunctiva. The epidermis is often thrown into papillary projections, with anastomosing rete ridges extending into the dermis. The basal cells are frequently vacuolated, with individual cell necrosis, and there can be dermal-epidermal cleft formation, with epidermal necrosis and ulceration. Single-cell or more extensive vacuolation in the stratum spinosum may be associated with acantholysis and epidermal pustule formation. Areas of epidermal ballooning degeneration, with eosinophilic intranuclear (herpesviral) inclusions, were identified in some cases (Herbst, 1994).

FP occurs in turtles throughout the world; however, only animals in Hawaii, Florida, Costa Rica, Barbados, Austrália e México have previously been positively identified as harboring ChHV 5 (Quackenbush et al, 1998; Quackenbush et al, 2001). We now extend these data to include turtles from Brasil. Eighty-seven percent of tumors from these animals were positive for ChHV5 DNA *pol* sequences.

A mean of 977 copies per mg tumor of ChHV 5 DNA pol sequences were detected in tumor collected from turtles from four different locations from Brazil. This value would suggest that ChHV 5 plays a dominant role in maintenance of the tumor state. The viral DNA load in individual tumors sampled from Hawaii and Florida were relatively homogeneous, ranging from 1.7 to 25 copies of ChHV 5 per tumor cell. In contrast, real-time PCR revealed that the copy numbers of viral DNA in tumors from Costa Rica and Australia varied by as much 5 logs (Quackenbush, et al, 2001), the same way as occurs in Brazil. This copy number variation may reflect the stage of tumor development. Spontaneous regression of fibropapillomas has been documented and may also result in viral copy number variation (Bennett et al. 2000). ChHV 5 DNA and RNA loads were measured in the superficial (dermis and epidermis), medial (center), and deep (stalk) sections of eight fibropapillomas. Differences in viral DNA loads were observed among the tumor sections, but no consistent pattern of ChHV 5 genome distribution emerged. The expression levels found in these

sections were small, indicating that the majority of ChHV 5 genomes in a visible fibropapilloma is not replicating (Greenblatt et al, 2004).

Once the pigmentation of tumors depends on the pigmentation of the skin where it develops (Herbst, 1994), we did not observe a significant association between viral load and pigmentation of tumors, but found that animals with tumor score 3 (strongly affected) had a higher viral load, probably due to the advanced state of development of the disease.

Of the 153 positive samples in qPCR, were 68% of tumors with a warty surface. Only in CE there was an association between viral load and type of surface, being that tumors with the surface warty presented viral load greater.

SP and BA showed a significant association between viral load and health status of turtles where the dead animals had higher viral load. High viral loads were also observed in moribund turtles in Australia, Costa Rica and Mexico, while low viral loads were found in healthy freshly dead turtles in Hawaii, Florida and Barbados (Quackenbush et al, 1998; Quackenbush et al, 2001).

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5. DISCUSSÃO

Neste trabalho foi analisado um total de 176 amostras de tumores e 58 amostras de pele. Esses resultados deram origem a três manuscritos que apresentam suas respectivas discussões. Cabe neste momento a discussão do agrupamento dos resultados destes manuscritos. No primeiro manuscrito (Detection and characterization of fibropapilloma associated herpesvirus of marine turtles in Rio Grande do Sul – Brasil) a amostra de fibropapiloma foi sequenciada e posteriormente comparada com as variantes virais descritas no segundo manuscrito (Viral variants of the Chelonid herpesvirus 5 (CHhv 5) in fibropapillomas of green turtles (*Chelonia mydas*) in Brazil). A amostra do RS mostrou pertencer a variante 4 e com isso demonstramos que esta variante prevalece no Brasil e está presente desde o CE até o RS.

As 32 amostras do segundo manuscrito foram escolhidas para o sequenciamento, pois apresentavam quantidade suficiente de DNA para a análise. Essas amostras foram analisadas quanto as características do tumor, assim como no terceiro manuscrito (Chelonid herpesvirus 5 in fibropapillomas of green turtles (*Chelonia mydas*) in Brazil), e revelaram que as variantes 2, 3 e 5 estão presentes apenas em tumores com a superfície verrucosa, enquanto as outras (1, 4 e 6) estão presentes também em tumores de superfície lisa. Todas as variantes, com exceção da 5 que foi detectada em apenas uma amostras, foram encontradas em tumores com e sem pigmentação, demonstrando que a pigmentação do tumor realmente é decorrente da pigmentação da pele onde ele se origina e que isso é ao acaso (HERBST, 1994). Quanto ao escore dos tumores, as variantes 1, 2 e 5 foram encontradas somente em tartarugas de escore tumoral 3 (severo), a variante 3 em escores 1 (leve) e 2 (moderado) e as variantes 4 e 6 em todos os escores. Na tabela 1 do terceiro manuscrito vimos que o escore 3 está associado a alta carga viral, o que sugere que as variantes 1, 2 e 5 apresentem uma alta carga viral, uma vez que elas só foram encontradas neste escore.

As demais associações entre cargas, características e variantes não são significativas devido ao reduzido número de amostras analisadas no manuscrito 2.

6. CONCLUSÕES

1. O Chelonid herpesvirus 5 foi encontrado em uma amostra de fibropapiloma de tartaruga-verde e estava ausente da pele de tartarugas sem fibropapilomatose em animais amostrados no RS.
2. Seis variantes virais do Chelonid herpesvirus 5 foram encontradas em amostras de fibropapiloma de tartarugas-verdes amostradas nos estados de CE, BA, ES e SP.
3. Duas variantes (variantes 1 e 2) foram encontradas somente em SP e a variante 3 foi encontrada somente no ES. A variante 4 estava presente em todos os estados e foi a mais prevalente nos estados de SP, BA e ES. A variante 5 foi encontrada somente na BA e a variante 6 somente no CE, sendo a mais prevalente neste Estado.
4. A coinfecção de tartarugas-verdes por mais de uma variante viral foi verificada em amostras de fibropapilomas coletadas em tartarugas nos estados da BA e SP.
5. O Chelonid herpesvirus 5 não foi detectado nas 45 amostras de pele de tartarugas-verdes coletadas na Ilha de Trindade e 13 amostras de pele de tartarugas-verdes e tartarugas-cabeçudas coletadas no RS.
6. Tumores de escore 3 (severo) apresentaram associação significativa com altas cargas virais.
7. Em SP e na BA, amostras de fibropapilomas de animais encontrados mortos apresentam associação significativa com altas cargas virais.
8. Em amostras do Estado do CE, tumores com a superfície verrugosa apresentaram associação significativa com altas cargas virais.
9. As variantes virais 1, 2 e 5 apresentaram altas cargas virais, pois foram detectadas somente em tartarugas com tumores de escore 3.

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ANEXOS

ANEXO 1 – Marine leech *Ozobrachus margoi* (Annelida: Hirudinea) parasitizing loggerhead turtle (*Caretta caretta*) in Rio Grande do Sul, Brazil.*

*Manuscrito submetido à Revista Brasileira de Parasitologia Veterinária na forma de nota de pesquisa.

RESEARCH NOTE

Marine leech *Ozobrachus margoi* (Annelida: Hirudinea) parasitizing loggerhead turtle (*Caretta caretta*) in Rio Grande do Sul, Brazil.

Sanguessugas *Ozobrachus margoi* (Annelida: Hirudinea) parasitando uma tartaruga cabeçuda (*Caretta caretta*) no Rio Grande do Sul, Brazil

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Abstract

This paper reports the finding of several *Ozobrachus margoi* (Annelida: Hirudinea) parasitizing a loggerhead turtle (*Caretta caretta*) found in the municipality of Tavares, Rio Grande do Sul state, Southern Brazil. Since this parasite is considered a vector of Chelonid herpesvirus 5 (ChHV-5), the collected leeches were tested for the presence of ChHV-5. All specimens were negative by PCR analysis. Although *O. margoi* has been considered a common sea turtle parasite, this is the first official record describing the collection of this parasite on a loggerhead turtle in Southern Brazil, an area in the subtropical zone. This finding draws attention to the presence of this parasite and to the risk for leech-borne infectious diseases in turtles found in Southern Brazil coastland.

Keywords: *Ozobranchus margoi*, Hirudinea, leech, loggerhead turtle, ectoparasite

Resumo

Este artigo relata a descoberta de vários *Ozobranchus margoi* (Annelida Hirudínea) parasitando uma tartaruga cabeçuda (*Caretta caretta*) encontrada no município de Tavares, Rio Grande do Sul, sul do Brasil. Uma vez que esse parasito é considerado vetor do chelonid herpesvirus 5 (ChHV 5), as sanguessugas foram testadas para a

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presença deste vírus. Todas as amostras foram negativas pela analise de PCR. Embora o *O. margoi* seja considerado um parasito comum de tartarugas marinhas, este é o primeiro registro oficial que descreve a coleta deste parasita em uma tartaruga cabeçuda no sul do Brasil, dentro da zona subtropical do país. Este achado chama a atenção para a presença deste parasita e para o risco de sanguessugas transmitirem doenças infecciosas em tartarugas no litoral sul do Brasil.

Palavras-chaves: *Ozobranchus margoi*, Hirudinea, sanguessuga, tartaruga cabeçuda, ectoparasita.

Introduction

Leeches are members of phylum Annelida, class Hirudinida. Most leeches are blood-suckers and can be found as ectoparasites of several terrestrial or aquatic vertebrates (DAVIES; GOVEDICH, 2001). *Ozobranchus* spp. belongs to the family Ozobranchidae, characterized by the presence of typical lateral digitiform branchiae (MacCALLUM; MacCALLUM, 1918). This family comprises two main species, which are permanent and exclusive ectoparasites of sea turtles: *O. branchiatus* and *O. margoi* (CHRISTOFFERSEN, 2008).

Although *O. branchiatus* and *O. margoi* can parasitize several species of sea turtles, these organisms demonstrate some degree of host preference. In general, *O. branchiatus* is commonly found parasitizing the green turtle (*Chelonia mydas*). On the other hand, *O. margoi* is more frequently associated to parasitism on the loggerhead turtle (*Caretta caretta*) (BUNKLEY-WILLIAMS et al., 2008).

A single turtle can carry more than one hundred leeches. The parasitism by Ozobranchus spp. may cause severe skin lesions, deep cutaneous erosion, eyes injury and even the host death (RAJ, 1959; DAVIES; CHAPMAN, 1974; SCHWARTZ, 1974, BUNKLEY-WILLIAMS et al., 2008). Another potential hazard associated to *Ozobranchus* spp. parasitism is the risk of chelonid herpesvirus 5 (ChHV-5) transmission. ChHV-5 is associated with the development of fibropapillomatosis (FP), a pathological condition of sea turtles characterized by the occurrence of debilitating tumors in the skin and internal organs, which can progress to animal death (GREENBLATT et al., 2004).

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Ozobranchus spp. is considered a common ectoparasite of sea turtles, and it has been reported to parasitize turtles in different locations around the world (BUNKLEY-WILLIAMS et al., 2008; CHRISTOFFERSEN, 2008). *O. branchiatus* was reported in *C. mydas* (MacCALLUM; MacCALLUM, 1918; HENDRICKSON, 1958; REME, 1980; CHOY et al., 1989; WILLIAMS et al., 1994; PEREIRA et al., 2006) while *O. margoi* was already reported parasitizing both *C. caretta* and *C. mydas*. Places where *O. margoi* were found in *C. mydas* included Florida and North Carolina (USA), Australia, and Hawaii (RICHARDSON, 1969; SCHWARTZ, 1974; DAVIES; CHAPMAN, 1974; CHOY et al., 1989). *O. margoi* was identified parasitizing *C. caretta* (RAJ, 1959; DAVIES; CHAPMAN, 1974).

In Brazil, a great deal of anecdotal information circulates specially among marine biologists and veterinarians of wild animals regarding the presence of these parasites in turtles found in the coastland of tropical areas of the country. However, no official records reporting the collection and identification of *O. margoi* on sea turtles along Brazilian coastland have been published, particularly concerning the subtropical zone. Here, we report the finding of several *O. margoi* parasitizing a loggerhead turtle (*C. caretta*) found in Rio Grande do Sul state, Brazil. Also, the collected leeches were tested for the presence ChHV-5 by PCR analysis.

Report and Discussion

A loggerhead sea turtle *C. caretta* (Fig. 1A) was found dead on the beach sand in the municipality of Tavares (31º 19’ 4.8’’ S; 50º 59’ 49.2’’ W), Rio Grande do Sul state, Southern Brazil. Forty-eight adult specimens of *O. margoi* were identified attached to the skin of the pelvic region of the loggerhead turtle, around the cloaca (Fig. 1B). Some leeches were collected. The specimens of *O. margoi* were deposited at the parasite collection of Parasitology Laboratory from Instituto de Pesquisas Veterinárias Desidério Finamor (IPVDF), Brazil.

The collected *O. margoi* specimens (Fig. 2) were identified based on morphological characteristics (DAVIES, 1978). *O. margoi* adult specimens were usually 10-15 mm long. They are white/whitesh and often show large dark spots, usually after blood meal. The body is segmented, and divided into trachelosome and urosome. They show five pairs of typical gills. The gills are thin structures, like bristles, which branch out as smaller parts. The first gill pair occurs on segment XIII and is bigger and more complex than the others. From the second to the fifth pair, the gills

become sequentially smaller and less complex. The mouth is terminal-ventral (DAVIES, 1978).

Some of the collected leeches were submitted to PCR analysis to detect ChHV-5. Leeches were macerated in sterile Phosphate Buffered Saline (PBS) solution and DNA extraction was conducted as previously established (CHOMCZYNSKI, 1993). PCR for ChHV-5 detection was carried out according to Quackenbusch et al. (2001). The DNA samples from collected leeches yielded no amplicon through the PCR test, and therefore these samples were considered negative for ChHV-5. Despite the fact that collected leeches were negative for ChHV-5, we cannot dismiss the possibility of *Ozobranchus* spp. as a vector of ChHV-5 in Brazil coastland. Greenblatt et al. (2004) reported that *Ozobranchus* spp. may carry a high viral load, reaching 10 million copies per leech. Moreover, considering the lack of reports on this subject, further studies searching for ChHV-5 and also FP in sea turtle in Southern Brazil should be conducted.

The loggerhead turtle is one of the most common sea turtles in Brazilian coastland, including Brazilian subtropical zone. Nevertheless, this turtle is currently under the risk of extinction, being classified as an endangered species (IUCN, 2011). Wyneken et al. (1988) mentioned that the overall fall in sea turtle populations has been directly or indirectly attributed to the destruction of their habitats, and to anthropic action observed in the nesting beaches. Also, strong evidence suggests that predation of juveniles and eggs, predatory fishing and water pollution contribute to this risk. In order to avoid the decrease in turtle populations, conservation programs are currently in progress in Brazil (MARCOVALDI; MARCOVALDI, 1999). It is important to note that another important conservation procedure should include the identification of diseases that affect sea turtles. The occurrence of parasitic and infectious diseases can impair animal health and also cause death, reducing sea turtle numbers. In this sense, *Ozobranchus* spp. represents a health hazard to sea turtles, since it can cause direct damages and also be vector of infectious agents.

As far as we aware, this is the first official record describing the collection of *O. margoi* on a loggerhead turtle (*C. caretta*) in Southern Brazil, within the subtropical zone of the country. Such finding draws the attention of marine biologists, veterinarians and parasitologists to the presence of this parasite and to the risk of leech-borne infectious diseases in sea turtles in Southern Brazil coastland.

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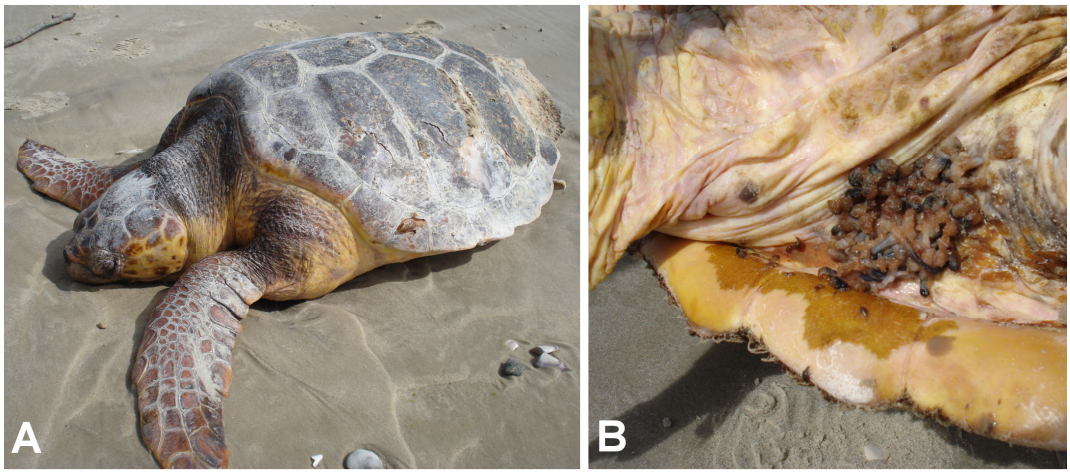

Figure 1. Panel A, Loggerhead turtle (*Caretta caretta*) found dead on the beach sand in the municipality of Tavares, Rio Grande do Sul state, Southern Brazil. Panel B, Several marine leeches (*Ozobranchus margoi*) attached to the groin skin of the loggerhead turtle.

Figure 2. Two specimens of *Ozobranchus margoi* found attached to the loggerhead turtle. Bar 1 cm.

ANEXO 2 - Carta de recebimento do artigo "Marine leech *Ozobrachus margoi* (Annelida: Hirudinea) parasitizing loggerhead turtle (*Caretta caretta*) in Rio Grande do Sul, Brazil" pela Revista Brasileira de Parasitologia Veterinária.

COLÉGIO BRASILEIRO DE PARASITOLOGIA VETERINÁRIA

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Jaboticabal, 6 de setembro de 2011.

Prezados senhores,

A Revista Brasileira de Parasitologia Veterinária confirma o recebimento do artigo a ser submetido para publicação, intitulado: **0299/2011 - Marine leech *Ozobrachus margoi* (Annelida: Hirudinea) parasitizing loggerhead turtle (*Caretta caretta*) in Rio Grande do Sul, Brazil**, de autoria de Carla Rosane Rodenbusch, Fernanda Simone Marks, Cláudio Wageck Canal, José Reck.

Atenciosamente,

Rosangela Z. Machado

Profa. Dra. Rosangela Zacarias Machado
Editora-chefe da RBPV

ANEXO 3 – Email de submissão do artigo **"**Detection and characterization of fibropapilloma associated herpesvirus of marine turtles in Rio Grande do Sul – Brazil" para a revista Pesquisa Veterinária Brasileira.

Prezada Dra. Carla,
O seu artigo foi registrado como **Trabalho 2465 WM**.
Att.
Jürgen Döbereiner
Editor Pesq.Vet.Bras.
----- Original Message -----
From: Carla Rosane Rodenbusch
To: jurgen.dobereiner@terra.com.br
Sent: Friday, September 09, 2011 5:18 PM
Subject: artigo submissão

Boa tarde,
Segue em anexo o artigo, de minha autoria, intitulado "Detection and characterization of fibropapilloma associated herpesvirus of marine turtles in Rio Grande do Sul - Brazil" e três figuras pertencentes ao mesmo para avaliação e publicação na Revista Pesquisa Veterinária Brasileira.
Atenciosamente,
Carla R. Rodenbusch
MSc Med. Vet. CRMV 7944
Doutoranda Laboratório de Virologia
Faculdade de Veterinária - UFRGS
Fone: (51) 91035556

ANEXO 4 – Confirmação da submissão do artigo "Viral variants of the Chelonid herpesvirus 5 (ChHV 5) in fibropapillomas of green turtles (*Chelonia mydas*) in Brazil" pela revista Virus Genes

Virus Genes

Viral variants of the Chelonid herpesvirus 5 (CHhv 5) in fibropapillomas of green turtles (Chelonia mydas) in Brazil

--Manuscript Draft--

Manuscript Number:	VIRU1552
Full Title:	Viral variants of the Chelonid herpesvirus 5 (CHhv 5) in fibropapillomas of green turtles (Chelonia mydas) in Brazil
Article Type:	Original Research
Section/Category:	Animal Virus
Keywords:	Chelonid herpesvirus 5; green turtle; fibropapilloma; Chelonia mydas
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Abstract:	Fibropapillomatosis is a benign neoplastic disease that affects sea turtles worldwide. Herpesvirus (Chelonid herpesvirus 5) has been associated with the presence of tumors. Thirty-two tumors were collected from 27 juvenile green turtles in 4 Brazilian states (Ceará - CE, Bahia - BA, Espírito Santo - ES and São Paulo - SP). The virus was detected by PCR, and the amplification product of the DNA polymerase gene was sequenced. Six viral variants (var) were identified in Brazil. Two variants (var 1 and var 2) were found only in the SP samples, and another variant (var 3) was found only in ES. Var 4 was present in 19 samples collected in all states. Var 5 and var 6 were present only in samples from BA and CE, respectively. Var 4 was prevalent in SP, BA and ES. Coinfection by more than one variant in one animal was verified in the samples from BA and SP. These findings are similar to those described in the literature, and the heterogeneity of these viral variants support the hypothesis that turtles become infected after their recruitment to nearshore developmental habitats.