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Characterization of novel papillomavirus from free-ranging Antillean manatee *Trichechus manatus manatus* with genital papillomatosis

Antonio A. Mignucci-Giannoni[1,2,*], Lesly J. Cabrias-Contreras[1], Michelle M. Dennis[2,3], Sylma M. Escobar-Torres[1], Shin-je Ghim[4], Elizabeth W. Howerth[5], Nelmarie Landrau-Giovannetti[1], Antonio L. Rivera-Guzmán[1], Carla I. Rivera-Pérez[1,2], Joongho J. Joh[4]

[1]Caribbean Manatee Conservation Center, Inter American University of Puerto Rico, Bayamón, PR 00957, USA
[2]Center for Conservation Medicine and Ecosystem Health, Ross University School of Veterinary Medicine, Basseterre, St. Kitts
[3]Biomedical and Diagnostic Sciences, College of Veterinary Medicine, University of Tennessee, Knoxville, Tennessee 37996, USA
[4]James Graham Brown Cancer Center, University of Louisville, Louisville, Kentucky 40202, USA
[5]Department of Pathology, College of Veterinary Medicine, The University of Georgia, Athens, Georgia 30602, USA

ABSTRACT: The Antillean manatee *Trichechus manatus manatus* is an Endangered subspecies living along the Atlantic coasts of the Americas from Mexico, throughout the Caribbean, to Brazil. In July 2020, a manatee with multiple wounds due to boat-inflicted trauma was rescued from the coast east of Cayo Mata, Salinas, Puerto Rico. This manatee had neutropenia, leukopenia, and monocytosis associated with immunosuppression and nutritional deficiency anemia, as well as bacteria and fungi within the lesions. The manatee had genital lesions which included papules and linear plaques, microscopically characterized by mucosal hyperplasia with cytopathic changes typical of papillomavirus infection. Superficial epithelial cells had strong nuclear immunolabeling when examined using a monoclonal antibody specific to papillomavirus. The sequencing data of PCR products with papillomavirus-specific degenerative primers indicated that these lesions contained a novel manatee papillomavirus (*Trichechus manatus* papillomavirus, TmPV). The genomic DNA was amplified using a rolling circle amplification, and fully sequenced to be 7586 bp (GenBank accession no. OK073977). Other TmPVs were previously isolated from Florida manatees *T. manatus latirostris*. This novel virus was designated TmPV type 5 (TmPV5) based on its genomic characterization and sequence comparison. The TmPV5 genome shared 50.7, 48.9, 69.4, and 62.1% similarities with TmPV1, TmPV2, TmPV3, and TmPV4, respectively. TmPV5 is classified in the genus *Rhopapillomavirus* together with other manatee papillomaviruses. After 2.5 mo of veterinary treatment and rehabilitation, the manatee recovered and was released. This is the first report of papillomatosis in a free-ranging Antillean manatee.

KEY WORDS: Papilloma · Genetics · *Trichechus manatus papillomavirus* · TmPV · Virus

1. INTRODUCTION

The West Indian manatee *Trichechus manatus* is a large, polyphagous herbivore mammal that lives in freshwater or marine ecosystems along the Atlantic coasts of the Americas from Florida (USA), throughout the Caribbean, to Brazil (Self-Sullivan & Mignucci-Giannoni 2012). It is threatened with extinction in the USA but is endangered from anthropic activities in most of its distribution. This species comprises 2 subspecies; the Florida manatee *T. manatus latirostris*, found in the southeastern USA, and the Antillean ma-

*Corresponding author: mignucci@manatipr.org

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natee *T. manatus manatus* found in Mexico, Central America, the Caribbean, and the northeastern coast of South America (Domning & Hayek 1986). While manatees uncommonly succumb to infectious diseases unless they are immunocompromised (Bossart 1999, 2001, 2011, Bonde et al. 2012), they suffer from high mortality due to human-related causes such as hunting, watercraft collisions, and entanglement (Mignucci-Giannoni et al. 2000, Bonde et al. 2012).

One of the reported diseases in manatees is cutaneous papillomatosis (Bossart 2011), caused by papillomaviruses (PVs). PVs are species- and tissue-specific, contain circular double-stranded DNA, are small (7.2 to 8.1 kb), and are found in many domestic and wild vertebrates and in humans (Nicholls & Stanley 2000). PVs infect the epithelial cells, at times causing neoplasia, or at other times just persisting subclinically (Bernard et al. 2010). Four types of *Trichechus manatus* papillomavirus (TmPV) have been described in the Florida manatee; 2 are from dermal lesions (Bossart et al. 2002a, Rector et al. 2004, Woodruff et al. 2005, Donà et al. 2011), and 2 are from genital mucosal lesions (Ghim et al. 2014, Zahin et al. 2015, 2018). These identified manatee PVs are TmPV1 (GenBank accession no. NC006563), TmPV2 (NC016898), TmPV3 (NC038526), and TmPV4 (NC028267). To date, however, no PV lesions have been identified from Antillean manatees. We present a novel PV from an Antillean manatee with immunocompromise-induced mucosotropic genital papillomatosis.

2. MATERIALS AND METHODS

2.1. Animal and veterinary examination

A male Antillean manatee was rescued on 15 July 2020 east of Cayo Mata, Salinas, Puerto Rico (17.95269° N, 66.28849° W). Upon admittance to the Caribbean Manatee Conservation Center (CMCC) aquatic veterinary facility on the same day and prior to placement in a medical tank, the animal was physically examined to assess its health condition, and to collect blood, urine, and fecal samples. The manatee measured 265.2 cm curvilinear total length (258.4 cm straight length), weighed 280 kg, and had multiple external wounds located in the caudal region of the body due to a recent boat strike. During this initial examination, dozens of light gray 1–3 mm diameter macules and papules and 5–15 mm long linear plaques were observed and photographed in the tissues surrounding the preputial mucosa (Fig. 1A). Multiple biopsies were collected representing the lesion and adjacent grossly normal mucosa. Half of each of the bisected lesion biopsy samples was saved for histological evaluation, and the other half was shipped to the University of Louisville (Louisville, Kentucky, USA) for molecular analysis.

2.2. Histopathology and immunohistochemistry

Biopsy samples were fixed in 10% neutral-buffered formalin, embedded in paraffin wax, sectioned 5 µm thick, and stained with hematoxylin and eosin for histopathologic evaluation (Slaoui et al. 2017). For PV immunohistochemistry, 4 µm unstained paraffin sections were prepared on charged slides, deparaffinized and rehydrated through xylene and graded alcohol washes, and subjected to heat-induced epitope retrieval at 110°C for 15 min in a commercial buffer (Reveal Decloaker, Biocare Medical). Immunohistochemical labeling and counterstaining with hematoxylin were performed on an automated staining system at room temperature using a streptavidin alkaline phosphatase detection system (4Plus streptavidin AP label, Biocare Medical) with Warp Red chromagen (Biocare Medical) and mouse anti-PV (CM177, Biocare Medical) at 1:80 dilution. This antibody is a cocktail of a monoclonal antibody that targets the major capsid protein (L1) of multiple human PVs (HPVs), and a monoclonal antibody that targets the L1 open reading frame (ORF) of bovine papillomavirus type 1 and is cross-reactive for multiple human subtypes. Positive control tissue was a cutaneous papilloma from a dog, and the negative control consisted of unimmunized mouse control serum (Cancer Diagnostics).

2.3. PCR and genomic analysis

DNA was extracted from frozen tissues using a kit (DNeasy, Qiagen). Biopsied tissues were finely chopped, treated in Proteinase K digestion buffer in the kit, and incubated in a thermomixer overnight at 55°C. DNAs were column purified according to the manufacturer's instructions. The concentrations of purified DNAs were determined using a NanoDrop spectrophotometer. PCR and rolling circle amplification (RCA) were employed to identify potential PV DNAs in the DNA extracts. First, potential PV DNAs were amplified with 3 degenerate primers specific to PVs (AR-L1F1/AR-L1R5, AR-L1F1/AR-L1R3, AR-E1F2/AR-E1R3) (Rector et al. 2004) using a commercialized PCR mix (Q5 high-fidelity 2× master mix, New Eng-

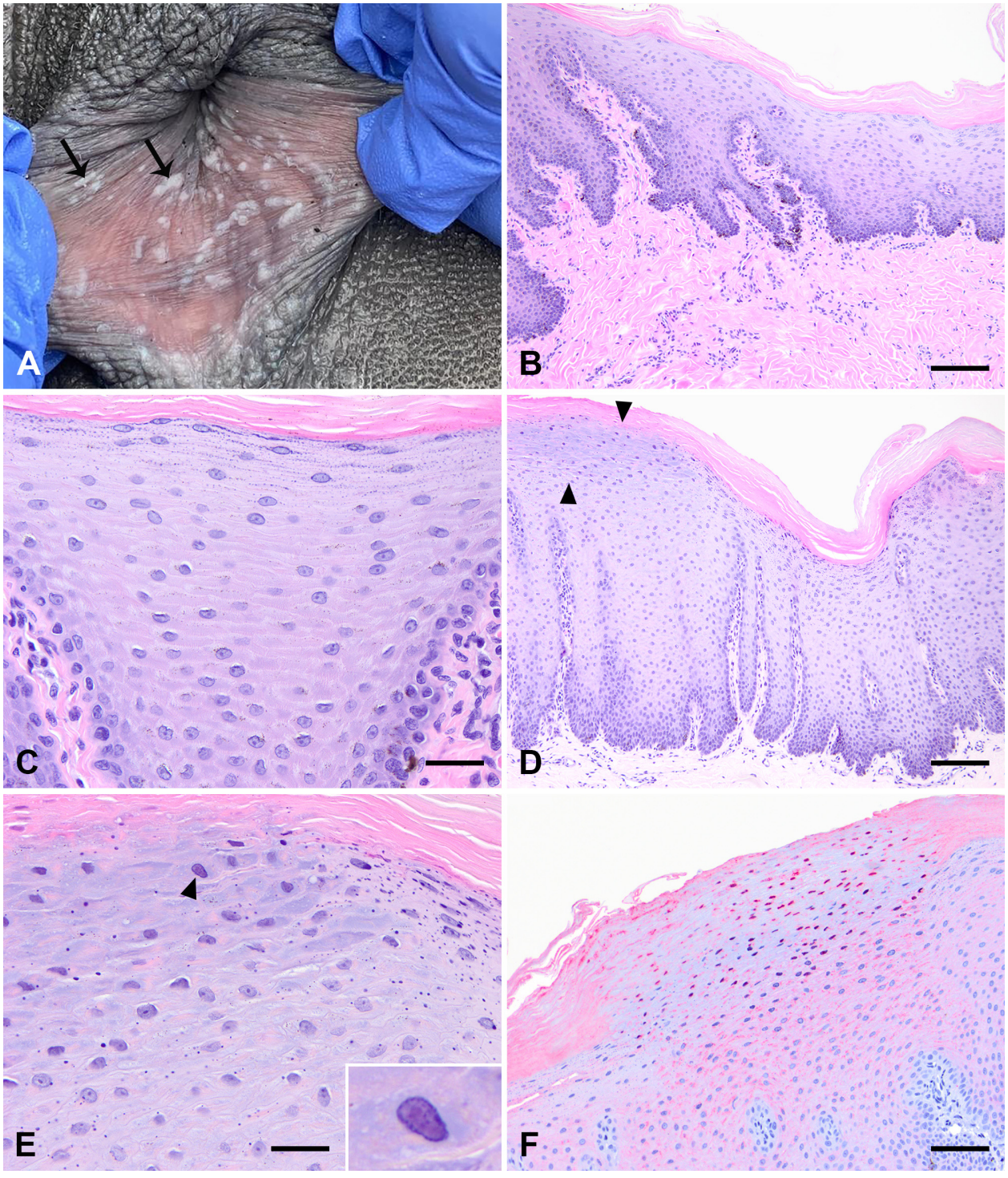

Fig. 1. Genital papillomatosis, preputial ostium in an Antillean manatee *Trichechus manatus manatus*. (A) Multiple gray to white papules and linear plaques (arrows) within the preputial mucosa. (B) Control (grossly normal) mucosa from the preputial ostium. Scale bar = 100 µm. (C) Higher magnification of control mucosa from the preputial ostium. Scale bar = 40 µm. (D) The lesion consists of a plaque of acanthosis (same magnification as B), and superficial epithelial cells have blue-tinged cytoplasm (arrowheads). Scale bar = 100 µm. (E) Higher magnification of (D) showing epithelial features of viral cytopathic effect including pale blue-gray cytoplasm, hyperchromatic nuclei, enlarged keratohyalin granules (as compared to C), and a granule with putative intranuclear inclusion body (arrowhead; magnified view inset). Scale bar = 40 µm. (F) Immunohistochemistry, anti-papillomavirus (red chromagen). Nuclei in the stratum granulosum and corneum have strong nuclear immunolabeling (red staining) for papillomavirus antigen. Scale bar = 100 µm

land BioLabs). PCR conditions were an initial denaturation of 30 s at 98°C, followed by 40 cycles of 10 s at 98°C, 10 s at 45°C, and 1 min at 72°C. Next, the presence of the predicted DNA amplicons was examined on 4% agarose (NuSieve 3:1 Agarose, Lonza), followed by ethidium bromide staining. The amplicons with the correct sizes were purified from the gel using a gel extraction kit (Qiaquick, Qiagen), processed for DNA sequencing with the primers employed for its amplification at the Center for Genetics and Molecular Medicine Sequencing Service at the University of Louisville, and blasted for their identifications as PV DNA (https://blast.ncbi.nlm.nih.gov/Blast.cgi). RCA was carried out using the TempliPhi amplification kit (Cytiva) to amplify the whole viral genome. Approximately 200 ng of amplified DNAs were digested with multiple restriction enzymes.

For the genomic DNA sequences, the RCA product was purified on the columns of the DNeasy kit (Qiagen), and its sequencing was outsourced (Functional Biosciences). The primers for the initial sequencing of the RCA product were designed based on the initial DNA sequence data of PCR amplicon, and the entire sequencing was done by primer walking. Both strands were then sequenced to confirm the sequencing data, and the sequencing data were assembled using the SeqMan program (Dnastar).

The ORFs of the viral genome were searched with EditSeq and converted to their encoded proteins using the MapDraw program (Dnastar). The genomic DNA sequence was imported into the MegAlign program (Dnastar) to align with other available PV genomic DNA sequences. The entire genome and the L1 (major capsid protein) sequence were compared using BLAST (version 2.2.8) and MegAlign programs. In addition, genomic and peptide sequences of a few other PVs were retrieved from the GenBank database and aligned with TmPV5 using the ClustalW algorithm in MegAlign to analyze their similarities. Phylogenetic trees were generated using genomic DNA and L1 peptide sequences (Bernard et al. 2010), and bootstrap support values were calculated from 1000 replicates.

3. RESULTS

3.1. Antillean manatee with multiple wounds due to a boat strike

The rescued manatee was in a weak state of health with fresh skin lesions from a recent boat strike. It was identified as a 15 yr old male, a previous patient of the CMCC initially rescued as a week-old orphan in 2005, and released back to the wild in 2010. From 2010 to his rescue date in 2020, the manatee had thrived in the wild. In opportunistic post-release monitoring veterinary examinations in 2011 and 2012, the manatee was clinically healthy with no signs of disease or adverse conditions, and particularly, genital papillomatosis was absent.

During the initial veterinary examination when rescued, the manatee had an ideal body condition (3/5) (Castelblanco-Martínez et al. 2021) and normal vital signs (2 breaths per 5 min respiratory rate, 67 bpm heart rate, 34.5°C body temperature, and <2 s capillary refill time). However, he was found to be lethargic, dehydrated (5%), with congested mucous membranes, and with abrasions and lacerations on the dorsal peduncle and lumbar area. The latter were predominantly parallel curvilinear lacerations typical of propeller injury, with a single perpendicular laceration consistent with a skeg, rudder, or hull strike. The presence of a bacterial biofilm and raised lesion margins suggested that the skin wounds were 7–10 d old. Bacterial and fungal culture of the wounds isolated *Acinetobacter baumannii/haemolyticus*, and *Mucor* spp., the latter disseminated on the body's dorsal surface, an additional indicator of immunosuppression. The preputial mucosa had multifocal macules, papules, and linear plaques with a velvety texture (Fig. 1A). Hematologic findings included neutropenia, leukopenia, and monocytosis associated with immunosuppression and nutritional deficiency anemia. Blood chemistry findings included a significant increase in creatine phosphokinase (780 U l^{-1}, normal range 34–240 U l^{-1}, Alsina-Guerrero 2011), fibrinogen (411 mg dl^{-1}, normal $\bar{x}$ 369 mg dl^{-1}; Barratclough et al. 2016), and Alpha 1 globulin (0.54+ g dl^{-1}, normal range 0.10–0.40 g dl^{-1}), values commonly observed in inflammatory processes and trauma. An elevated anion gap indicated metabolic acidosis. Urinalysis revealed relatively low urine pH (6.5, normal 7.1–8.9; Cabrias-Contreras et al. 2021). Immunoglobulin analysis revealed activated IgM and an IgG initiation phase, consistent with an infectious process. No significant findings were made with coprology, acute phase proteins through electrophoresis, or serology.

The manatee was initially treated with enrofloxacin as a bactericide, and dorsal skin lesions were cleaned; the manatee was then placed in the medical tank. In addition to the initial treatment, a topical advance and integral bactericide management of the wounds was initiated with hydrogel cleaning (Solosite wound gel, Smith+Nephew), FIBRACOL™ Plus collagen wound dressing with Alginate (KCI USA), Derma Gran-B hy-

drophilic wound dressing with zinc plus, vitamin A and B_6 (McKesson Brand), Viscopaste PB7 zinc paste bandage (Smith+Nephew), and MediHoney® wound and burn dressing with calcium alginate and active *Leptospermum* honey (Derma Science). In addition, an oral treatment for fungal infection of the wounds was implemented with itraconazole, SHaNa Vet (Animal Necessity), and turmeric.

After 2.5 mo of veterinary treatment and rehabilitation, the boat strike lesions had healed, the fungal infection and the preputial TmPV lesions had resolved, and his health check was unremarkable, with IgG and IgM levels within normal limits; therefore, the animal was released into his habituated wild home range with a satellite monitoring telemetry tag on 29 September 2020 in Mar Blanco, Salinas, Puerto Rico (17.94329° N, 66.26322° W).

3.2. Histologic evaluation of genital lesions

Microscopically, the preputial lesions consisted of hyperplastic plaques. These was multifocal acanthosis composed of well-differentiated squamous epithelial cells with decreased melanin pigment compared to non-affected mucosal epithelial cells (Fig. 1B–E). Superficial epithelial cells often had pale blue-gray cytoplasm, enlarged keratohyalin granules, and rare putative intranuclear basophilic inclusion bodies (Fig. 1E). Koilocytes (i.e. enlarged epithelial cells with blue-gray to clear cytoplasm and pyknotic nuclei), were not observed. Multiple nuclei in the epithelial cells in the outer portion of the mucosa stained strongly immunopositive for PV (Fig. 1F).

3.3. Identification of a novel manatee papillomavirus

All 3 genital biopsies were positive for PV by PCR and contained the same PV DNAs (data not shown). Primer set AR-E1F2 (ATG GTN CAG TGG GCN TAT GA), and AR-E1R3 (TTN CCW STA TTN GGN GGN CC) amplified approximately 350 bp DNA fragments (Fig. 2A), and the DNA sequence had the highest homogeneity with TmPV4. Digestion of RCA products with restriction endonuclease enzymes (REs) indicated that the total size of amplified PV genomic DNA had a typical size (7 to 8 kb) of PV genome with *Hin*dIII as a unique RE site (Fig. 2B). In addition, the genomic DNA had multiple cut sites for *Bgl*II, *Eco*RI, and *Pst*I.

Complete genomic sequencing data revealed that the size of this PV DNA was 7586 bp with 42.54 % GC

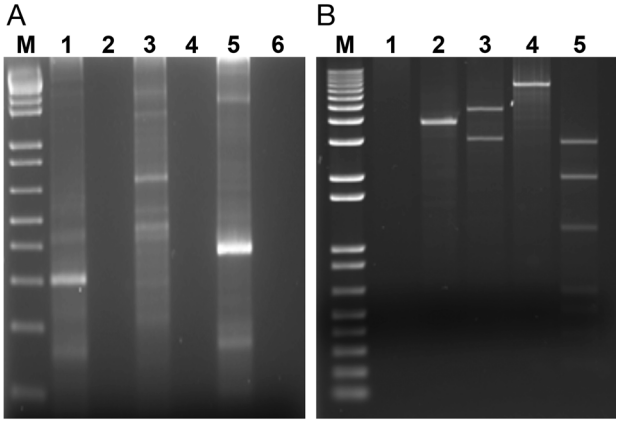

Fig. 2. Presence of papillomavirus (PV) DNA in the papillomatosis of an Antillean manatee. (A) Detection of PV DNA by PCR amplification with PV degenerative primers. DNAs extracted from tissue biopsies were used as templates for PCR. The L1F1/L1R3 (lanes 1, 2), L1F1/L1R5 (lanes 3, 4), and E1F2/E1R3 (lanes 5, 6) primer sets were used, and the E1F2/E1R3 set produced the correct amplicons of approximately 350 bp, potentially indicating the presence of PV DNA. Water was used as a negative control (lanes 2, 4, 6). A 1 kb plus ladder (lane M; Life Technologies) was employed as a size marker. (B) Digestion pattern of rolling circle amplification product by restriction endonucleases. Amplified DNAs were digested with each of 5 different enzymes: *Bam*H1 (lane 1), *Bgl*II (lane 2), *Eco*R1(lane 3), *Hin*dIII (lane 4), and *Pst*1 (lane 5). *Pst*1 cut the genomic DNA multiple times (8 cuts), *Bam*H1 did not cut it, and *Bgl*II and *Eco*RI had at least 2 cut sites. *Hin*dIII cut once, generating 1 fragment of between 7 and 8 kb. The 1 kb plus ladder (lane M, Life Technologies) was employed as a size marker

content. The BLAST search of GenBank confirmed that this is a novel PV, which is designated as *Trichechus manatus papillomavirus type 5* (TmPV5) according to the nomenclature system established by the International Committee on Taxonomy of Viruses (ICTV) for all new PVs (Table 1). Complete sequence information was deposited in GenBank (accession no. OK073977). This circular DNA virus has 6 ORFs, encoding E1, E2, E6, E7, L2, and L1 (Fig. 3) putatively with 2–3 more ORFs, and a 607 bp sized noncoding region is located between L1 and E6 ORFs.

3.4. Comparative and phylogenetic analyses of TmPV5

The complete genome of TmPV5 had 50.7, 48.9, 69.4, and 62.1 % bootstrap homologies with those of TmPV1, TmPV2, TmPV3, and TmPV4, respectively (Table 1). Phylogenetic analyses with the genomic DNA sequences revealed that TmPV5 is closest to TmPV3 and furthest from TmPV2 among the known

Table 1. General characteristics and percentage similarity of all West Indian manatee *Trichechus manatus* papillomaviruses (TmPVs). GenBank accession numbers of the types are given in parentheses

Subspecies	TmPV type	Length (nt)	Similarity of PV genome / L1 DNA (%) TmPV1 (NC006563)	TmPV2 (NC016898)	TmPV3 (NC038526)	TmPV4 (NC028267)	TmPV5 (OK073977)	Lesion type	Reference
T. m. manatus	TmPV5	7586	50.7	48.9	69.4	62.5	–	Mucosal	This study
T. m. latirostris	TmPV4	8241	50.2	48.8	61.5	–	70.7	Mucosal	Zahin et al. (2015)
T. m. latirostris	TmPV3	7622	49.4	48.5	–	69.3	71.6	Mucosal	Zahin et al. (2015)
T. m. latirostris	TmPV2	7855	58.3	–	58.4	56.1	57.7	Cutaneous	Unpublished
T. m. latirostris	TmPV1	7722	–	67.1	60.5	58.6	59.5	Cutaneous	Rector et al. (2004)

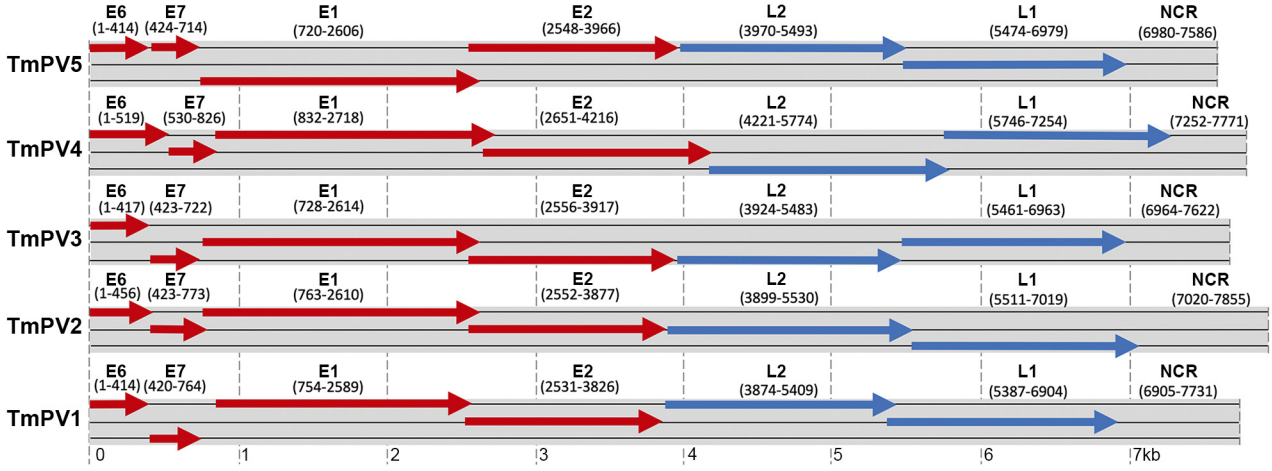

Fig. 3. Genomic organization of 5 *Trichechus manatus* papillomaviruses (TmPVs). Complete genomic DNA sequences were utilized for these analyses. All TmPVs encode open reading frames (ORFs) E6, E7, E1, E2, L2, and L1 that are represented by red and blue arrows. Red and blue arrows indicate the early- and late-replicating regions of the genome, respectively. The start and stop positions of ORFs are indicated inside the parentheses. The sizes of the corresponding ORFs were similar among types, except for TmPV4 E6, which was approximately 100 bp longer than the E6s of the other TmPVs. E4 and other putative ORFs are not shown here. TmPV5 genomic DNA is shortest of all 5 TmPVs. NCR: non-coding region

TmPVs (Fig. 4). When DNA sequences of all ORFs were analyzed, higher homogeneities were shown among 5 manatee PVs than other cetacean PVs (data not shown). The comparisons of the ORF DNA sequences indicated the low similarities among TmPVs, with values between 41.3 and 74.2%. The lowest similarity was found in E7 ORFs of TmPV1 and TmPV3. Homology higher than 70% was only found in E1 (74.2%), E7 (72.6%), and L1 (71.6%) DNA sequences between TmPV3 and TmPV5, and L1 DNA (70.7%) between TmPV4 and TmPV5 (L1 data are shown in Table 1). Overall results of peptide sequence comparisons were similar to those with DNA sequences. The highest homogeneity (75.2%) was found between L1 peptides of TmPV3 and TmPV5. Phylogenetic analyses using L1 peptide sequences of 13 cetacean and 5 sirenian PVs classified TmPV5 into the genus *Rhopapillomavirus* (Fig. 5), the classification of which was also concluded to be the same when done using the whole genomic sequences (Fig. 4).

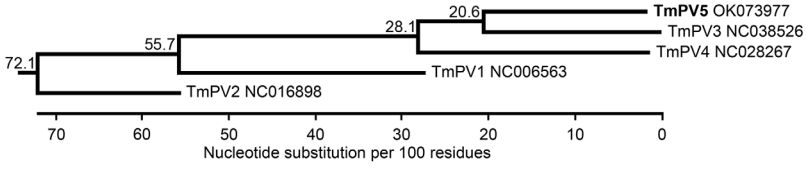

Fig. 4. Neighbor-joining phylogenetic analysis including GenBank accession numbers. The phylogenetic analysis using whole genomic sequences indicated that TmPV5 (highlighted in **bold**) is close to TmPV3 but further from TmPV2 and TmPV1. All TmPVs were classified into the genus *Rhopapillomavirus*. The scale on the bottom indicates the number of nucleotide substitutions per 100 nucleotides. Bootstrap numbers (%) are indicated on branches

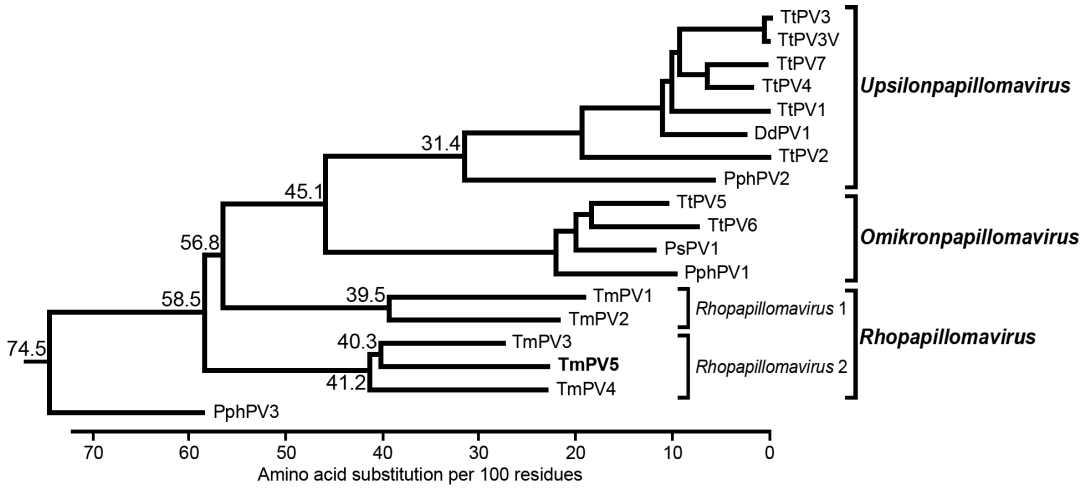

Fig. 5. Neighbor-joining phylogenetic analysis with L1 peptide sequences of manatee and cetacean papillomaviruses (PVs). A neighbor-joining phylogenetic tree was reconstructed based on the L1 peptide sequence alignment of 13 cetacean and 5 sirenian PVs (Tm: *Trichechus manatus*; Tt: *Tursiops truncatus*; Dd: *Delphinus delphis*; Pph: *Phocoena phocoena*; Ps: *Phocoena spinipinnis*). Bootstrap numbers >30% are indicated on branches. TmPVs cluster under the genus *Rhopapillomavirus* as shown in the analysis with whole genomic sequences. As shown in Fig. 4, TmPV5 (highlighted in **bold**) is close to TmPV3

4. DISCUSSION

Manatees are an ancient tropical aquatic species (~17–20 million yr old; Domning 2018, Suárez et al. 2021) and considered sentinels of coastal health (Bonde et al. 2004, Bossart 2011). They are Endangered, due to anthropic actions, with estimated population numbers for the West Indian species between 11 000 and 16 000 individuals (Morales-Vela et al. in press).

Manatee PVs, including TmPV1 to TmPV4, have been previously characterized in Florida manatees (Bossart et al. 2002a, Rector et al. 2004, Donà et al. 2011, Ghim et al. 2014, Zahin et al. 2015, 2018). According to the seroepidemiological study on Florida manatee TmPV1 infections (Donà et al. 2011), TmPV1 antibody prevalence was approximately 26% with no significant difference between captive and wild manatees. Therefore, it was suggested that TmPV was widespread among Florida manatees. In the latter study, it was observed that 72% of seropositive captive manatees manifested papillomatosis while no wild manatees had evidence of papillomatosis, suggesting that captive-induced immunosuppression might play a role in symptomatic TmPV1 infections (Donà et al. 2011, Owen et al. 2018). Antibody-positive evidence for TmPV1 was discovered from wild Antillean manatees in Belize by serological testing (Donà et al. 2011). Although they tested positive by serology, these animals had low antibody prevalence and low levels of anti-TmPV1 antibodies, and in addition, none of the antibody-positive wild animals had active papillomatosis. The existence of detectable antibodies to TmPV1 in wild Antillean manatees did not correlate with the absence of lesions in the few opportunistic observations during health assessments, suggesting that Antillean manatees may be infected by other PVs with low antigenic cross-reactivity with TmPV1 (Donà et al. 2011) or that the PV virus is in a latent state until activated by a stressor. The present case, in Salinas, Puerto Rico, had a history of rescue as a newborn calf 15 yr prior to presentation and had no contact with juvenile or adult wild manatees during captivity, and had surveillance health exams since release in 2010, 2011, and 2012. Based on the findings, this is the first papillomatosis identified and characterized genetically in a free-ranging Antillean manatee.

PV types are numerous among humans, other mammals, and birds. The spectrum of gross and histologic changes tends to reflect PV type (Egawa et al. 1993). Consistent with this view, gross and microscopic lesion features and distribution of viral antigen stained by immunohistochemistry in the present case were similar to those reported for genital papillomatosis in Florida manatees (Ghim et al. 2014, Zahin et al. 2015) and cetaceans (Lambertsen et al. 1987, Van Bressem et al. 1996, Bossart et al. 2002b, 2005), corresponding to PV lesions instigated by phylogenetically closely related PVs. PV infections typically induce benign epithelial proliferation, which may persist, spontaneously resolve, or in rare instances,

undergo malignant transformation (Munday 2014). In humans, squamous cell carcinoma (SCC) can occur in orogenital mucosa infected with high-risk PVs, particularly alpha-PVs (HPV16 and -18) or beta-PVs in immunosuppressed individuals (Hines et al. 1995a,b, Munday 2014, Scarth et al. 2021). Similarly, PV-associated genital papilloma in horses may later develop into SCC (Lange et al. 2013), PV-associated lingual papilloma may progress to SCC in common bottlenose dolphins *Tursiops truncatus* (Bossart et al. 2005), and PV-associated cutaneous hyperplastic plaques may give rise to SCC or basal cell carcinoma in felids (Sundberg et al. 2000, Rector et al. 2007). Many of the instances of PV-associated malignancy involve the genital tract or lack papillary morphology, instead initially consisting of flat (planar or sessile) warts presenting as plaques (Sundberg et al. 2000, Bossart et al. 2005, Munday 2014), similar to the present and previously described PV lesions in manatees (Bossart et al. 2002a, Ghim et al. 2014). However, there are no reports of cancerous lesions related to TmPVs and malignant neoplasms involving external genitalia in sirenians despite the existence of a long-term manatee surveillance program in Florida. Genital papillomatosis resolved within 2 mo of initial diagnosis in the present case, confirming spontaneous regression.

Transmission of manatee PVs is believed to occur through direct contact (Bossart et al. 2002a), and venereal transmission is presumed for genital papillomatosis given the lesion location. Tumors of the reproductive tract are a concern to wildlife population health since they may impede copulation, gravidity, or parturition. Instances of genital papillomatosis have been considered severe enough to potentially compromise reproductive success in cetaceans (Van Bressem et al. 1996). Maternal HPV6 or HPV11 infection can result in recurrent respiratory papillomatosis in children, particularly when genital warts are present at parturition (Silverberg et al. 2003, Derkay & Wiatrak 2008). These consequences of genital papillomatosis have yet to be described with TmPVs, if at all present. While it is believed that symptomatic papillomatosis in manatees is an issue observed only in captivity from immunosuppressed individuals (Owen et al. 2018), the present case involving a free-ranging manatee with active papillomatosis would suggest that occurrence in free-living manatees can occur.

The lesions from this manatee contained 1 distinctive PV type, which was designated as TmPV5. The genomic organization of all TmPVs showed typical forms for most other PVs, while cetacean PVs, for example, have a unique genome without E7 but with a long E6. TmPV5 is the shortest among TmPVs, but the significance of these species' genetic conservatism is unknown at this time. Because TmPV3 and -4 were isolated from mucosal lesions while TmPV1 and -2 were from cutaneous tissues, a mucosotropic TmPV5 was anticipated to be genetically closer to TmPV3 and -4 rather than to TmPV1 and -2. Thus, the tissue-tropism of the PVs seemed to be the critical factor for PV grouping. The phylogenetic analysis allocated mucosotropic PVs (TmPV3–5) to the *Rhopapillomavirus* 2 subgroup and cutaneotropic ones (TmPV1 and -2) to the *Rhopapillomavirus* 1 subgroup. The L1 peptide-based PV classification confirmed this different grouping of mucosotropic and cutaneotropic TmPVs. Research studies suggest that PVs co-evolved and co-speciated together with their hosts (Chan et al. 1997, Rector et al. 2007). In our phylogenetic analysis of TmPVs, we found no host-specific evidence indicating PV co-evolution at the subspecies level of the host, but rather at the species level. Surveillance and testing of the Florida subspecies during veterinary exams of manatees under human care, wild population health assessments, or during necropsies as part of the regional carcass recovery program could determine if TmPV5 is circulating within the population.

5. CONCLUSION

Activation of latent infection and re-inoculation from active infection are hypothesized to generate cutaneous PVs in Florida manatees, with simultaneous immunosuppression as a mediator in disease etiology. As shown in Florida manatees and this Antillean manatee case, boat-strike trauma is implicated in the induction of the immunocompromised condition that may have triggered the appearance of the novel TmPV5 lesions previously acquired in the wild through a venereal process. As sentinel species, manatees are an excellent biomarker in evaluating coastal health ecosystems and current or future negative health impacts at an individual and population level. Environmental degradation, including increased activity of watercraft and its impact on the environment and directly on wildlife, can cause the emergence or re-emergence of diseases with the potential to have epizootic consequences. Whether this is a singular case from an immunocompromised individual or newly emerging to the population, future monitoring of the wild populations of Antillean manatees in Puerto Rico is needed to understand the spread, virulence, and epidemiology of this manatee papillomatosis. Addi-

tionally, further monitoring of sister manatee species, such as the Amazonian manatee *Trichechus inunguis*, the African manatee *T. senegalensis*, and the Florida manatee subspecies *T. manatus latirostris* is warranted to better understand the role and significance that PVs may have in the sirenian clade.

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