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Short Note

Genital Papillomatosis Associated with Two Novel Mucosotropic Papillomaviruses from a Florida Manatee (*Trichechus manatus latirostris*)

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Based on clinicopathologic findings, West Indian manatees (*Trichechus manatus;* Sirenia, Trichechidae) appear to be remarkably resistant to natural infectious disease and the sublethal effects of traumatic boat injury (Buergelt et al., 1984; Bossart, 2001; Bossart et al., 2003, 2004, 2012). These disease-resistant traits of manatees may partially result from a remarkably efficient and responsive immune system (Bossart, 1999). In 2002, the authors (SG, ABJ, and GDB) and colleagues reported the first viral disease in Florida manatees (*T. m. latirostris*) caused by a papillomavirus (PV) that was later sequenced and named *Trichechus manatus* papillomavirus type 1 (TmPV1) (Rector et al., 2004b). TmPV1 caused cutaneous papillomas with the help of immunosuppression in the host (Bossart et al., 2002). Recent data from Dona et al. (2011) indicate that Florida manatees living in the wild can be naturally infected by TmPV1 but rarely show TmPV1-induced cutaneous papillomatosis. This note presents the first report of multiple genital papillomavirus infections associated with two novel mucosotropic PVs in a Florida manatee.

A juvenile male Florida manatee was rescued on 12 February 2008 in Wakulla Springs, Florida, with severe cold stress and blunt boat impact pulmonary trauma. Due to the thoracic injury, the manatee developed negative buoyancy but appeared otherwise clinically normal. The manatee was housed with another negatively buoyant male at two different rehabilitation facilities in Florida from 2008 to 2010. The manatee companion died in 2010 from abscess complications from its blunt impact trauma. One of these facilities housed other manatees that previously had cutaneous papillomas associated with TmPV1, although they were never in contact due to a double-layer fence. The manatee of this case report was seronegative for TmPV1 by an enzyme-linked immunosorbent assay (ELISA) in November 2010 and remained clinically unchanged and otherwise healthy (Dona et al., 2011). It was transported to Puerto Rico on 9 December 2010 and quarantined at the Puerto Rico Zoo in Mayagüez. On 1 April 2011, the manatee was moved to the Puerto Rico Manatee Conservation Center (PRMCC), Inter American University, Bayamón, to act as a surrogate animal with the manatee rehabilitation program at this facility.

Upon routine physical examination on 16 September 2011 and 13 January 2012, the manatee was noted to have multiple white round superficial sessile lesions in the preputial ostium measuring approximately 2 to 5 mm in diameter (Figure 1). The surface of these lesions was velvety and non-ulcerative. The manatee remained otherwise clinically unchanged. On subsequent physical examinations from September 2012 to May 2013, no genital lesions were observed.

Two lesions designated as Tm1 and Tm3 from September 2011 and January 2012, respectively, were biopsied and bisected with half kept at -70° C for molecular tests to identify and characterize manatee PVs possibly present in the lesions (Rector et al., 2004a, 2004b) and the other half processed for light microscopy and immunohistochemistry (IHC) as previously described (Bossart

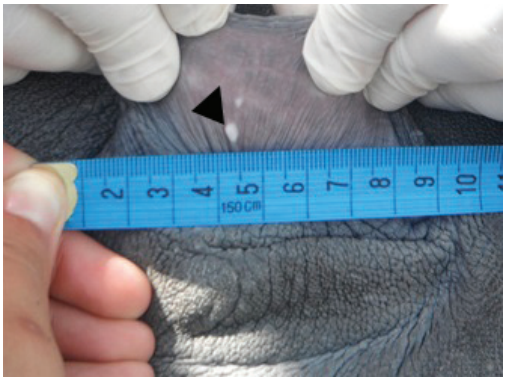

Figure 1. A sessile papilloma of the genital mucosa from the prepuce of a male Florida manatee; the lesion is focal, irregular, slightly raised, and white with a velvety and non-ulcerative mucosa (arrowhead).

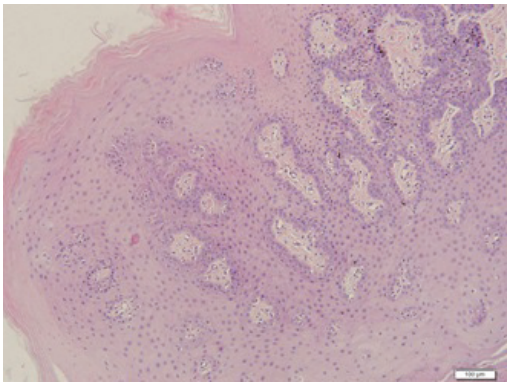

Figure 2. Photomicrograph of an H&E-stained histologic section from the genital lesion shown in Figure 1: a heavily keratinized sessile plaque composed of a focally extensive proliferation well-differentiated squamous epithelium forming irregular papillae which project into the submucosa

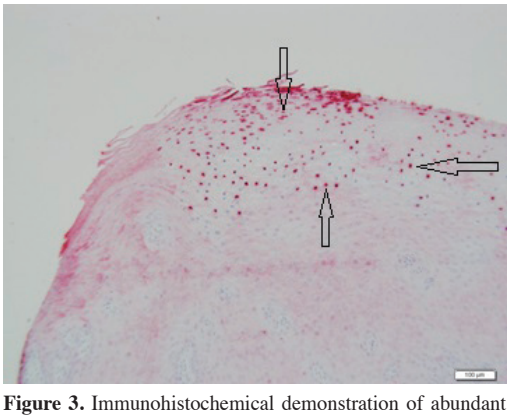

Figure 3. Immunohistochemical demonstration of abundant papillomavirus antigen within the nuclei of superficial proliferating squamous cells of the histological sample shown in Figure 2 (arrows); immunohistochemistry (IHC) was carried out using rabbit polyclonal antibody generated against denatured bovine papillomavirus type 1 (BPV1) virions.

et al., 2002). For light microscopy, biopsies were fixed in 10% neutral buffered formalin, routinely processed, embedded in paraffin, sectioned at 5 μm, and stained with hematoxylin and eosin. For IHC, additional 4 μm paraffin sections were deparaffinized, reacted with a commercially available rabbit polyclonal antibody generated against denatured bovine papillomavirus type 1 (BPV1) (DAKO Corp., Carpenteria, CA, USA), and treated with a standard avidin-biotin-peroxidase staining technique. The major capsid protein of BPV1 has at least 40 distinct linear epitopes that are conserved to various degrees among the mammalian and avian PVs (Jenson et al., 1991). This immunohistochemical technique has been routinely used to screen animal tissues for evidence of productive PV infections (Jenson & Lancaster, 1991; Jenson et al., 1997) and has been validated for the demonstration of TmPV1 in manatee papillomas (Bossart et al., 2002). Microscopically, the prepucial lesions were characterized by sessile plaques composed of a focally extensive proliferation well-differentiated and occasionally dysplastic squamous epithelium forming irregular papillae which projected into the submucosa (Figure 2). The surfaces of the lesions were heavily and diffusely keratinized. Frequent squamous cells had vacuolated cytoplasm and pleomorphic round vesicular nuclei that were centrally or eccentrically located which were consistent with koilocytes as previously observed in TmPV1 cutaneous lesions (Bossart et al., 2002). With IHC, strong positive staining for papilloma viral antigen was noted within the nuclei of superficial proliferating squamous cells (Figure 3).

Each frozen sample was examined by two different molecular methods to determine PV infection (Rector et al., 2004b). The samples were first examined by PCR methodology. For this, a portion of each bisected sample was treated with proteinase K (100 μg/ml) in proteinase K digestion buffer (50 mM Tris pH 8.5, 1mM EDTA, 0.5% Tween 20) at 55° C for overnight, followed by inactivation of proteinase K by heating for 10 min at 95° C. Extracted DNA samples from each biopsy were then tested for PV DNA by PCR using degenerated primers that anneal to either the E1 or L1 ORFs. Lesions Tm1 and Tm3 were positive for the predicted size of PV DNA (approximately 370 bp) identified by the set of primers (E1F2/E1R3: 5'-ATG GTN CAG TGG GCN TAT GA-3', 5'-GGN CCN CCN AAT ASW GGN AA-3') that target E1 (Figure 4), although they were negative using L1-specific primer sets (data not shown). The sequencing data

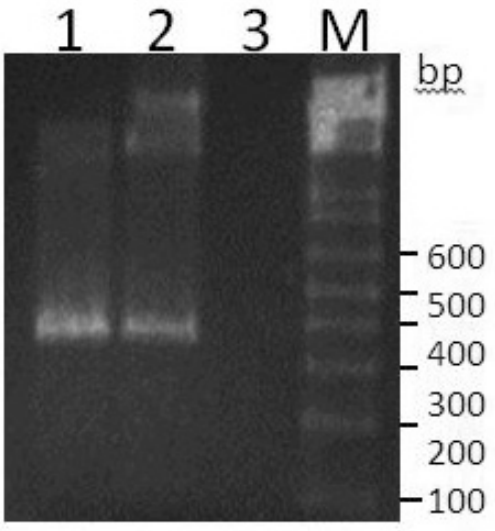

Figure 4. Detection of PV DNA by PCR amplification; DNAs extracted from tissue biopsies were used as templates of PCR. Lesions Tm1 (lane 1) and Tm3 (lane 2) produced amplicons of approximately 370 bp by a set of degenerated PV primers (E1F2/E1R3; Rector et al., 2004b), indicating the presence of PV DNAs. Water was used as a negative control (lane 3). 1 kb plus ladder (M; Life Technologies) was employed as a size marker.

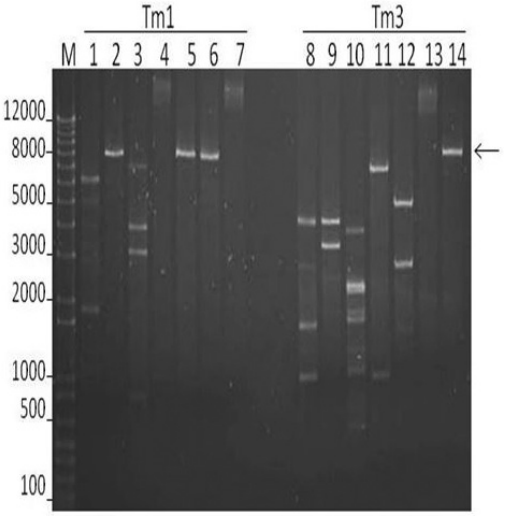

Figure 5. Two different digestion patterns of amplified PV genomic DNA; PV circular genomic DNAs of two PVs from Tm1 and 3 were amplified by the rolling circle amplification (RCA) technique (GE Life Sciences) and digested with each of seven different restriction endonucleases: KpnI (lanes 1 & 8), XbaI (lanes 2 & 9), EcoRI (lanes 3 & 10), BamHI (lanes 4 & 11), HindIII (lanes 5 & 12), SacI (lanes 6 & 13), and SalI (lanes 7 & 14). Amplified DNAs from sample Tm1 (lanes 1 to 7) and Tm3 (lanes 8 to 14) presented distinctively different digestion patterns, which were also different from the predicted sizes of TmPV1 (AY609301; Rector et al., 2004b). Unique enzyme sites, cutting only once amplified-small circular DNA from Tm1 tissue, were Xba1 (lane 2)/ Hind III (lane 5)/Sac 1 (lane 6), and for that from Tm3 tissue was Sal1 (lane 14). The full length digested linear DNAs were approximately 8 kb, which is the size of the genome of most of the other PVs. 1 kb plus ladder (M; Life Technologies) was employed as a size marker.

of these PV DNAs revealed that the identity in pairwise alignment between them was rather low at 66.6% and that their identities with the prototype TmPV1 were 42.0 and 43.7%, respectively. Since PVs are typically species-specific, and no similar DNA sequences were found in Genbank (www.ncbi.nlm.nih.gov), we concluded that the PVs were novel and different from each other. To confirm our conclusions, we then used the rolling circle amplification (RCA) technique to amplify PV genomic DNA in DNA extracts from each sample (GE Life Sciences, Pittsburgh, PA, USA) (Rector et al., 2004a). The RCA products were digested with various restriction enzymes (KpnI, XbaI, EcoRI, BamHI, HindIII, SacI, and SalI; Life Technologies, CA, and New England Biolabs Inc., Ipswich, MA, USA) and loaded on 1% agarose gel. Identifiable DNA products that showed different digestion patterns from the predicted digestion pattern of TmPV1 (Genbank locus number: NC_006563.1) of approximately 8 kb size were observed from samples Tm1 and 3, while no smaller rolling circle DNA products were observed (Figure 5). RCA data were consistent with the PCR sequencing results (data not shown), confirming the existence of a different PV in each biopsy of lesion Tm1 and 3. L1 sequences of these two new TmPVs were submitted to Genbank (Accession numbers: KF574428 and KF574429), and full genomic sequencing of these two novel TmPVs are in progress.

The pathologic and immunohistologic findings were consistent with a diagnosis of multiple genital sessile papillomas associated with PV antigen (Wititsuwannakul et al., 2013), and the results of DNA analysis indicated that the lesions biopsied from September 2011 and January 2012 were caused by two different and novel PVs. This is the first report of genital papillomas associated with PVs in this endangered species. So far, three types of PVs infecting this species have been identified: a cutaneous type (TmPV1) previously described (Bossart et al., 2002; Rector et al., 2004b; Woodruff et al., 2005) and two mucosotropic PVs identified from this study.

Papillomaviruses are transmissible, nonenveloped epitheliotropic DNA viruses that typically

induce benign tumors in a wide range of mammalian and bird species (Sundberg et al., 2001; Handisurya et al., 2009; Bernard et al., 2010; Rector & Van Ranst, 2013). Papillomas associated with PVs have been reported in other marine mammals, including Atlantic bottlenose dolphins (*Tursiops truncatus*) (Bossart et al., 2005, 2008; Rector et al., 2008; Rehtanz et al., 2012), sperm whales (*Physeter macrocephalus*) (Lambertsen et al., 1987), and killer whales (*Orcinus orca*) (Bossart et al., 1996, 2002). With few exceptions, PVs are species-specific and anatomic site-restricted (de Villiers et al., 2004; Doorbar, 2007; White & Howley, 2013). All PVs have the same basic genetic organization of their DNA genome (Jenson & Lancaster, 1991; Chan et al., 1995; Doorbar, 2007). Mucosotropic genital papillomas in other domestic mammalian species may spontaneously regress (Nicholls & Stanley, 2000; MacLachlan & Kennedy, 2002). However, in humans, some PV types, particularly those found in mucosotropic lesions of the oropharynx and genital tract can be associated with malignant transformation (Howley, 2006; zur Hausen, 2009; Doorbar et al., 2012). Our previous studies also suggest that PV-related lingual squamous carcinomas in Atlantic bottlenose dolphins may also develop because of malignant transformation of originally benign sessile papillomas (Bossart et al., 2005).

In the case reported herein, spontaneous regression of all of the genital lesions suggested the role of an effective immunologic response which eliminated the tumors. Immunosuppression and latent viral infection were previously implicated in the pathogenesis of cutaneous and mucosotropic papillomatosis in other species, including manatees and humans (Harwood et al., 2000; Bossart et al., 2002; Palefsky, 2006, 2007; Bossart, 2011; Dona et al., 2011; Doorbar, 2013; Dugué et al., 2013; Maglennon et al., 2013). Recent seroepidemiologic data by Dona et al. (2011) indicated that Florida manatees living in the wild are naturally infected by TmPV1 but rarely show TmPV1-induced papillomatosis. The prevalence of TmPV1 antibody among manatees with absence of lesions suggests an immunologic response that effectively controls productive PV infection and/or rapidly resolves lesions. In the present case, the complications of chronic thoracic trauma and/or the transport from Florida to Puerto Rico may have resulted in a temporary stress-induced immunosuppressive state, resulting in activation of latent infection, productive viral infection, and lesion formation. Once the immunologic perturbation resolved, spontaneous regression of the tumors may have occurred.

Papillomaviruses are thought to have coevolved with their hosts (Chan et al., 1997; Rector et al., 2007; van Doorslaer, 2013). In manatees with cutaneous papillomatosis associated with TmPV1, it was suggested that TmPV1 was not transmitted to manatees from other species but, rather, coevolved with the species itself (Rector et al., 2004b). Further studies are required to provide additional insights into the prevalence and natural history of PV infections in manatees and to clarify the immunopathogenesis of infection.

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