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SEROEPIDEMIOLOGY OF TmPV1 INFECTION IN CAPTIVE AND WILD FLORIDA MANATEES (*TRICHECHUS MANATUS LATIROSTRIS*)

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ABSTRACT: In 1997, cutaneous papillomatosis caused by Florida manatee (*Trichechus manatus latirostris* [Tm]) papillomavirus 1 (TmPV1) was detected in seven captive manatees at the Homosassa Springs Wildlife State Park, Florida, USA, and, subsequently, in two wild manatees from the adjacent Homosassa River. Since then, papillomatosis has been reported in captive manatees housed in other locations, but not in wild animals. To determine TmPV1 antibody prevalence in captive and wild manatees sampled at various locations throughout Florida coastal regions, virus-like particles, composed of the L1 capsid protein of TmPV1, were generated with a baculovirus expression system and used to measure anti-TmPV1 antibodies in an enzyme-linked immunosorbent assay. Serologic analysis of 156 manatees revealed a TmPV1 antibody prevalence of 26.3%, with no significant difference between captive ($n=39$) and wild ($n=117$) manatees (28.2% and 25.6%, respectively). No antibody-positive wild animal showed PV-induced cutaneous lesions, whereas papillomatosis was observed in 72.7% of antibody-positive captive manatees. Our data indicate that Florida manatees living in the wild are naturally infected by TmPV1 but rarely show TmPV1-induced papillomatosis. Hence, it appears that the wild population would not be harmed in a case of contact with captive animals without visible lesions and productive infections, which could be thus released into the wild.

Key words: Antibody prevalence, Florida manatees, TmPV1, virus-like particles (VLPs).

INTRODUCTION

Papillomaviruses (PVs) are species-specific viruses that infect cutaneous and mucosal epithelia, typically giving rise to benign lesions, such as cutaneous and genital papillomas (Sundberg et al., 2001; Handisurya et al., 2009). However, some PVs, such as high-risk human papillomaviruses (HPVs), may be associated with cancer development (zur Hausen, 2009). Papillomaviruses have been found in a wide range of mammals (Giri et al., 1985; Garland, 2002; Ghim et al., 2004; Zaugg et al., 2005; Rector et al., 2006; Nasir and Campo, 2008). Recently, these viruses have been detected in three marine mammal species: *Phocoena spinipinnis papillomavirus* type 1 (PsPV1) from Burmeister's porpoises (Van Bressem et al., 2007); *Tursiops truncatus papillomavirus* type 1, 2, and 3 (TtPV1, -2, and -3) from bottlenose dolphins (Rehtanz et al., 2006; Rector et al., 2008), and *Trichechus manatus latirostris (Tm) papillomavirus* type 1 (TmPV1) from Florida manatees (Bossart et al., 2002; Rector et al., 2004). Several studies suggested the possible existence of additional marine mammal PVs involved in the etiology of cutaneous and mucosal lesions in cetaceans, although studies to isolate and characterize these PVs have not been conducted (Flom et al., 1980; Lambertsen et al., 1987; Van Bressem et al., 1999, Bossart et al., 2005; Newman and Smith, 2006).

In sea mammals, PV infection predominantly causes benign hyperproliferative lesions. However, recent studies suggest that PV-related squamous carcinomas may

also develop, possibly because of malignant transformation of originally benign lesions (Bossart et al., 2005; Newman and Smith, 2006). Molecular comparison of existing marine mammal PVs to well-characterized carcinogenic HPVs indicates that at least one sea mammal PV (TtPV2) may possess oncogenic potential (Rehtanz et al., 2006).

In 1997, papillomatosis was detected for the first time in seven captive manatees in Florida, USA. The lesions primarily appeared as multiple, pedunculated, cutaneous papillomas over the dorsal surface and sometimes persisted for several years (Bossart et al., 2002). Clinical, histopathologic, electron microscopic, and immunohistochemical data indicated that the lesions were caused by PVs (Bossart et al., 2002). Subsequently, the DNA of a novel PV, TmPV1, was isolated from these lesions (Rector et al., 2004). The TmPV1 is the only virus fully characterized from a species of the order Sirenia. No other PV has been reported infecting the cutaneous or mucosal surfaces of Florida manatees.

The TmPV1 has a circular, double-stranded DNA genome of 7,722 base pairs (Rector et al., 2004). It contains seven open reading frames (ORFs) encoding for two structural proteins and five nonstructural proteins responsible for transcriptional regulation and viral DNA replication. According to a recent analysis, TmPV1 clusters by itself, close to the root of the PV phylogenetic tree, within the genus *Rhopapillomavirus*, and has a low similarity to PVs isolated from other sea mammals (Rector et al., 2008).

In this study, TmPV1 virus-like particles (VLPs) were produced in a baculovirus expression system and used as immunologic reagents in a serologic assay to compare the prevalence of antibody to TmPV1 between wild and captive manatees. Our findings could affect the management of captive manatees regarding papillomavirus infections.

MATERIALS AND METHODS

DNA extraction

We extracted TmPV1 DNA from biopsy material obtained from skin lesions of a captive manatee named Willoughby, as previously described (Rector et al., 2004). The DNA was suspended in TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) and used as a template to amplify the TmPV1 L1 ORF.

TmPV1 L1 cloning, expression, and detection

The full-length L1 ORF was amplified by PCR using the primers P1 (5′-ATGATGCTCGAGATGAATACGTGGTTACCG-3′) and P2 (5′-ATGATGCTGCAGAACAGCTATGACCATGAT-3′) to create *Xho*I and *Pst*I restriction sites (underlined) at the 5′ and 3′ ends, respectively. The reaction mixture consisted of 1 μl of DNA extract, 2.5 U of Platinum *Taq* DNA polymerase (Invitrogen, Carlsbad, California, USA), 2.5 mM $MgCl_2$, 0.4 μM of each primer, and 0.2 mM of each dNTP in 1× Platinum *Taq* DNA polymerase buffer (Invitrogen) to yield a final volume of 100 μl. The amplification was carried out using the following conditions: 1 min at 94 C, 30 cycles of 1 min at 94 C, 2 min at 55 C, and 3 min at 72 C. The PCR product was directionally cloned into the baculovirus transfer vector pBluebac4.5 (Invitrogen) downstream of the polyhedrin promoter. The integrity of the insert was confirmed by sequencing. The recombinant baculovirus (rL1bac) was produced by cotransfection of the recombinant transfer vector and the baculovirus-linearized DNA into *Spodoptera frugiperda* (Sf9) cells according to the manufacturer's recommendation (Bac-N-Blue™ Kit; Invitrogen) and as previously described (Ghim et al., 2004). The L1 expression in the Sf9 cells was confirmed by immunofluorescence. Briefly, Sf9 cells cultured on coverslips were infected with rL1bac. At 72 hr postinfection, cells were fixed with cold acetone for 5 min, washed with phosphate-buffered saline (PBS), and incubated for 1 hr at room temperature (RT) with a monoclonal antibody (mAb, 1H8) developed against disrupted *Bovine papillomavirus 1* (BPV-1) virions (Jenson et al., 1991) that cross-reacts with TmPV1. Fluorescein isothiocyanate-labeled anti-mouse immunoglobulin G (IgG; heavy and light chains [H+L]) serum (Roche Diagnostics, Indianapolis, Indiana, USA) was used as a secondary antibody. After mounting the coverslips on slides, the Sf9 cells were examined under a fluorescence microscope.

Western blot

Lysates of infected and uninfected cells were electrophoretically separated on a 10% sodium dodecyl sulfate polyacrylamide gel and transferred to a nitrocellulose membrane. After saturation with 20 mM Tris, 150 mM NaCl, and 0.4% Tween 20 for 1 hr at RT, the membrane was incubated with mAb 1H8 (Jenson et al., 1991), followed by incubation with alkaline phosphatase-tagged goat anti-mouse IgG (H+L) as a secondary antibody (Ghim et al., 1991). Naphthol AS-B1 phosphate and Fast Violet B AP-substrate (Sigma, St. Louis, Missouri, USA), dissolved in 100 mM Tris buffer containing 1μM $MgCl_2$, were used as a substrate.

TmPV1-VLPs production and purification

The Sf9 insect cells, cultured in supplemented Grace's medium (Gibco/Invitrogen, Carlsbad, California, USA) containing 10% fetal bovine serum, were incubated with the rL1bac for 2 hr. At 72 hr postinfection, the cells were harvested, suspended in Dulbecco's PBS (D-PBS, Invitrogen), Dounce-homogenized, and sonicated on ice for one min. The suspension was mixed with cesium chloride (CsCl) to achieve a final density of 1.30 g/ml and ultracentrifuged at 235,000 × G for 16 hr at 4 C (SW55Ti rotor, Beckman, Brea, California, USA). Fractions containing the VLPs were collected and dialyzed against D-PBS. Purified VLPs were stored at −80 C for further use. The quantity and quality of the VLPs were checked by titrating the protein concentration (Bio-Rad, Hercules, California, USA) and by electron microscopy of negatively stained VLPs (2% phosphotungstic acid, pH 6.8), respectively. For electron microscopy staining, 300-mesh formvar-carbon–coated copper grids were used.

Production of rabbit anti-manatee IgG serum

The production of rabbit anti-manatee IgG was performed as previously described for the production of anti–bottlenose dolphin IgG (Rehtanz et al., 2008). Briefly, IgG from 1.75 ml of combined serum from two TmPV1-negative, captive manatees were purified using protein A–Sepharose chromatography (Econo Column®; Bio-Rad) according to the manufacturer's recommendations. ImmunoPure® IgG elution buffer (Pierce, Rockford, Illinois, USA) was used to elute the IgG. Two New Zealand white rabbits were immunized three times at 2-wk intervals with 200 μg of manatee IgG together with 300 μl of adjuvant (TiterMax™ Gold; CytRx Corporation, Los Angeles, California, USA). Sera were collected 1 wk after the last immunization and titrated by enzyme-linked immunosorbent assay (ELISA).

TABLE 1. Status, age class, and sex distribution of the 156 Florida manatees (*Trichechus manatus latirostris*) included in the study.

Variable	Captive (n=39)	Wild (n=117)	Total (n=156)
Age class[a]			
Calf	2	21	23
Subadult	2	29	31
Adult	35	67	102
Sex			
Male	18	63	81
Female	21	54	75

[a] Classification based on total body length.

Serum samples collection and storage

A total of 176 sterile blood samples was obtained from 156 manatees (Table 1) from the brachial arteriovenous plexus located between the radius and ulna of the pectoral flipper (Walsh and Bossart, 1999). Blood samples were collected from wild manatees during multiple biomedical assessment captures conducted throughout Florida, USA, coastal regions (Table 2). For comparison, additional samples were collected from wild Antillean manatees in Belize. Samples from captive manatees were collected at nine facilities in Florida, USA (Table 2).

All samples were collected under the authority of US Fish and Wildlife Service research permits (MA791721, MA770191, and MA773494); samples from Belize were imported under authority of the Convention on International Trade in Endangered Species. Collection and processing of samples were conducted under Institutional Animal Care and Use Committee approval granted to the US Geological Survey (USGS/SESC-2010-06). Immediately after sample collection, serum was separated from the whole blood and stored at −70 C until analysis.

Enzyme-linked immunosorbent assay

The incubation steps were carried out in 100 μl per well at RT unless otherwise stated. Three to five washings were carried out between the incubation steps using 200 μl/well of PBS. The primary and secondary antibodies were diluted in PBS containing 1% bovine serum albumin (BSA). The

TABLE 2. Provenance of captive and wild, Florida manatees (*Trichechus manatus latirostris*), number of manatees sampled for each location, and number of sera tested.

Location	No. manatees[a] (No. sera)	Captive	Wild
Atlantic Coast	13 (13)		x
Belize	18 (20)		x
Cincinnati Zoo	2 (2)	x	
Columbus Zoo	4+2[b] (6)	x	
Crystal River	32 (34)		x
Everglades National Park	30 (32)		x
Homosassa Springs Wildlife State Park	6+2[b]+1[c] (17)	x	
Lowry Park Zoo	2 (2)	x	
Disney's Living Seas	2 (2)	x	
Mote Marine Laboratory	2 (2)	x	
Miami Seaquarium	9 (11)	x	
Sea World Orlando	3 (3)	x	
Sea World San Diego	6+1[c] (8)	x	
Tampa Bay	24 (24)		x
Total	156 (176)	39	117

[a] "No. manatees" is sample size for each group.

[b] Same manatees (Holly and Willoughby).

[c] Same manatee (Oakley).

TmPV1-VLPs (100 ng/well in PBS) were immobilized on a microtiter plate (Dynatech Laboratories, Chantilly, Virginia, USA) overnight at 4 C. Blocking was performed with 200 µl/well of PBS–5% BSA for 2 hr at RT. Test sera at 1:100 dilution were added to the wells in duplicate and incubated for 1 hr. Rabbit anti-manatee IgG serum at 1:1,000 dilution was added and incubated for 1 hr, followed by incubation for 1 hr with a horseradish peroxidase–conjugated goat anti-rabbit IgG (Chemicon International Inc., Billerica, Massachusetts, USA) diluted 1:20,000. SureBlue Reserve TMB 1-Component Microwell peroxidase substrate (Kirkegaard and Perry Laboratories, Inc., Gaithersburg, Maryland, USA) was used as a chromogen. The reaction was stopped after 5–10 min by adding 0.6 N sulfuric acid. The optical density (OD) was read at 450 nm.

Before this serologic study, the conditions for the ELISA were optimized using sera from 64 captive and wild manatees, tested multiple times for their reactivity to TmPV1-VLPs. In these studies, two samples gave very reproducible results and were thus chosen as internal standards of the assay. Specifically, the serum from a lesion-free manatee that reproducibly gave a minimal OD value was used as a negative control, whereas the serum from an animal with viral papillomatosis having high anti-TmPV1 antibody levels and giving a maximal OD value was used as a positive control. The positive cutoff value was calculated using the OD of the negative control serum plus 2 SD and corresponded to 0.3 OD.

Statistical analysis

Serologic data were grouped by animal sex, age class, status (wild vs. captive), extent of exposure to manatees with papillomatosis, and presence of wart-like lesions. The association among those variables and antibody prevalence was analyzed with two-way contingency tables using the likelihood ratio χ^2 test for significance tests. Analyses were performed with Minitab 15 statistical software. P values <0.05 were considered statistically significant.

RESULTS

The TmPV1 L1 ORF was successfully amplified from TmPV1 genomic DNA and cloned into a baculovirus vector. The L1 protein was then overexpressed in the Sf9 insect cells. When infected cell lysates were analyzed by Western blot, a band of 55 kDa was detected (data not shown). This band, not detected in the lysates of uninfected cells, corresponds to a full-length L1 protein. Immunostaining of recombinant baculovirus-infected cells indicated a high level of expression of L1 protein with a homogenous nuclear distribution (data not shown). The recombinant TmPV1 L1 protein showed the ability to self-assemble into empty VLPs, as reported for L1 of all other PVs expressed using a baculovirus system (Kirnbauer et al., 1993; Rose et al., 1993; Rehtanz et al., 2008). Purified VLPs were approximately 55 nm in diameter and had the morphologic appearance of icosahedral particles (Fig. 1).

The TmPV1-VLPs were used as antigens in an indirect ELISA to measure anti-TmPV1 antibodies in the manatee sera. Because anti-manatee IgG serum is not commercially available, high-titer rab-

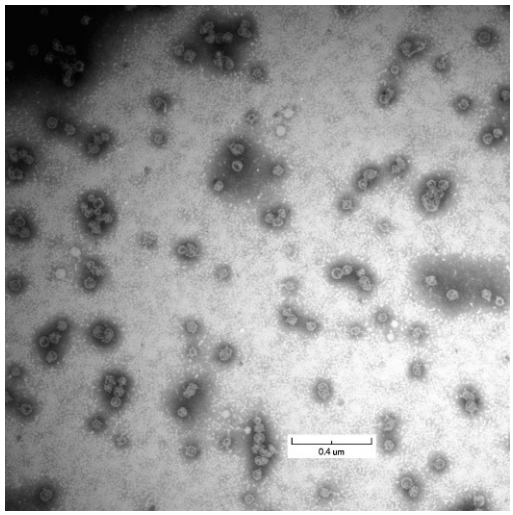

FIGURE 1. Electron micrograph of negatively stained *Trichechus manatus latirostris* papillomavirus 1 virus-like particles, purified by ultracentrifugation on cesium chloride (CsCl) continuous gradient (electron microscope at 80 kV and 18,000× magnification).

bit anti-manatee IgG serum was generated to serve as a secondary antibody in the ELISA. A total of 176 sera collected from 156 manatees ($n=117$ wild and $n=39$ captive) was analyzed. Status, age class, sex, and location of the manatees included in this study are outlined in Tables 1 and 2.

The overall antibody prevalence was 26.3% (41/156); anti-TmPV1 antibodies were detected in 28.2% (11/39) of captive animals and 25.6% (30/117) of wild manatees (Table 3). This difference was not statistically significant ($P=0.754$). Calves showed the lowest antibody prevalence in comparison with the other age classes (Table 3), and those differences were statistically significant ($P=0.007$). The antibody prevalence was lower among male than among female captive manatees (16.7% and 38.1%, respectively), although that difference was not statistically significant ($P=0.132$). The difference between the prevalences of anti-TmPV1 antibodies in wild males and females was also not statistically significant (28.6% and 22.2%, respectively; $P=0.432$). Overall, the percentages of seropositive male and female animals were 25.9% and 26.7%, respectively. That difference was not statistically significant ($P=0.916$), suggesting no significant association between TmPV1 antibody prevalence and sex.

Among the captive manatees, 66.7% (26/39) had a history of papillomatosis, displayed wart-like lesions, or had direct contact with animals with papillomatosis housed in the same pools ("exposed" animals in Table 4). The antibody prevalence was significantly higher among the "exposed" animals in comparison with the "not exposed" group (42.3% vs. 0%, $P=0.001$). The antibody prevalence was significantly higher among manatees with

TABLE 3. Prevalence of antibodies to *Trichechus manatus latirostris* papillomavirus 1 in captive and wild, Florida manatees by age class, sex, and overall.

Variable	No. antibody-positive manatees/No. tested (%)			*P* value
	Captive ($n=39$)	Wild ($n=117$)	Total ($n=156$)	
Age class[a]				
Calf	0/2 (0)	1/21 (5)	1/23 (4)	0.007[b]
Subadult	0/2 (0)	7/29 (24)	7/31 (23)	
Adult	11/35 (31)	22/67 (33)	33/102 (32.4)	
Sex				
Male	3/18 (17)	18/63 (29)	21/81 (26)	0.916
Female	8/21 (38)	12/54 (22)	20/75 (27)	
Overall	11/39 (28)	30/117 (25.6)	41/156 (26.3)	0.754[c]

[a] Classification based on total body length.

[b] Antibody prevalence of calves compared with antibody prevalence of subadults and adults.

[c] Antibody prevalence of captive manatees compared with antibody prevalence of wild manatees.

TABLE 4. Prevalence of antibody to *Trichechus manatus latirostris* papillomavirus 1 (TmPV1) in captive manatees by presence of wart-like lesions (cutaneous papillomatosis) and exposure to TmPV1.

	No. manatees[a] (%)					
	Exposed[b]			Lesions		
	Yes	No	Total	Yes	No	Total
Seropositive	11 (42.3)*	0 (0)	11 (28.2)	8 (66.7)*	3 (11.1)	11 (28.2)
Seronegative	15 (57.7)	13 (100)	28 (71.8)	4 (33.3)	24 (88.9)	28 (71.8)
Total	26 (100)	13 (100)	39 (100)	12 (100)	27 (100)	39 (100)

[a] "No. manatees" is sample size for each group.

[b] "Exposed" group includes manatees with a history of papillomatosis, with current papillomatosis, or with previous direct contact with manatees with papillomatosis.

* $P<0.001$.

lesions than among lesion-free animals (66.7% vs. 11.1%, $P=0.001$). In 33.3% (4/12) of the animals with papillomatosis, anti-TmPV1 antibodies were not detected. However, for one of those animals, the OD value was close to the positive cutoff. The manatees housed at Disney's Living Seas, Mote Marine Laboratory, and Lowry Park Zoo were all negative, whereas there were antibody-positive individuals in all the other locations.

Most of the antibody-positive captive animals had a high reactivity against TmPV1 (arbitrarily defined as OD>0.5; Fig. 2). Only the sera from the manatees

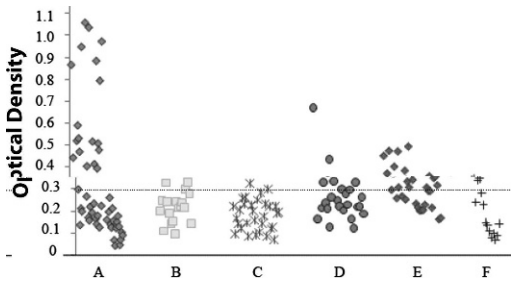

FIGURE 2. Scatter plot of optical density (OD) values for enzyme-linked immunosorbent assay for antibodies to *Trichechus manatus latirostris* papillomavirus 1 in serum (1:100 dilution) of Florida manatees. The OD values at 450 nm for all 176 serum samples included in this prevalence study are shown. Group A=captive manatees; groups B=Belize manatees; C–F=wild manatees. Group A includes captives from all locations and includes multiple samples from individuals.

housed at Miami Seaquarium had a low, although positive, reactivity. With the exception of two cases, all animals with high-reactive sera presented or had been previously diagnosed with papillomatosis (data not shown).

Papillomavirus-induced lesions were observed in only 1.7% (2/117) of the wild manatees. Those two animals, sampled in Crystal River, were both antibody-negative. Clinical signs of a TmPV1 infection were not evidenced in any of the 30 antibody-positive wild manatees. With the exception of one animal, which had a highly reactive serum (OD>0.5), the other antibody-positive wild manatees generally had low anti-TmPV1 reactivity (Fig. 2). The highest antibody prevalence was found in the Everglades National Park population (15/30=50%).

Serial serum samples were collected from eight manatees during a period of 1–8 yr. All serial samples gave the same serologic result, either positive or negative, with the exception of one captive manatee that seroconverted during monitoring (Table 5).

DISCUSSION

Since the identification of TmPV1 as the causal agent of papillomatosis among captive Florida manatees, the interinstitutional exchange program for these animals

TABLE 5. Anti–*Trichechus manatus latirostris* papillomavirus 1 serum reactivity in captive, Florida manatees repeatedly sampled over time.[a]

Manatee	Date	OD_{450nm}	ELISA
Amanda	January 2000	0.299	neg
	May 2008	0.126	neg
Ariel	August 2002	0.470	pos
	May 2008	0.414	pos
Betsy	August 2002	0.267	neg
	May 2008	0.224	neg
Holly	August 2002	0.948	pos
	October 2005	0.866	pos
	May 2008	0.794	pos
Lorelei	January 2000	0.443	pos
	June 2002	0.590	pos
	May 2008	0.394	pos
Oakley	June 2002	0.531	pos
	February 2008	0.972	pos
Sarah	July 2007	0.139	neg
	March 2008	0.125	neg
Willoughby	January 2000	0.137	neg
	February 2001	0.520	pos
	October 2005	1.058	pos

[a] OD = optical density; ELISA = enzyme-linked immunosorbent assay; neg = negative; pos = positive.

has been closely regulated to prevent the spread of this virus among wild and other captive manatees. This is the first report, to our knowledge, on the prevalence of anti-TmPV1 antibodies in wild and captive manatees.

We employed VLPs composed of the major capsid protein L1 of TmPV1 as antigens in our serologic assay. The TmPV-VLPs are the optimal reagent to measure anti-TmPV1 antibodies resulting from both previous and current infections, whereas DNA detection only reflects the current status (Marais et al., 2000; Stone et al., 2002). In the past, VLP-based ELISAs were successfully used to evaluate PV antibody prevalence in other marine mammal species (Rehtanz et al., 2010).

Our data provide serologic evidence for TmPV1 infection among wild manatees (25.6% antibody prevalence) with no significant difference compared with captive animals (28.2% prevalence; $P=0.754$). Antibody-positive wild manatees were found in all five sampling locations—Belize, Crystal River, Tampa Bay, Everglades National Park, and the Atlantic Coast—although low antibody prevalence and low levels of anti-TmPV1 antibodies were observed among the manatees of Belize. These manatees belong to the subspecies *T. m. manatus*, and they have never shown evidence of papillomatosis. They may not be susceptible to TmPV1 infection or may be infected by other PV(s) with low antigenic cross-reactivity with TmPV1. Alternatively, it is possible that manatees from this population do not have detectable anti-TmPV1 antibodies because they may not have been exposed to this virus.

Most of the antibody-positive wild manatees, regardless of their location, had low anti-TmPV1 antibody levels (Fig. 2). In contrast, almost all antibody-positive captive animals had a high reactivity to TmPV1, both those with and without lesions.

Our results indicate that the presence of detectable antibody to TmPV1 in wild manatees does not correlate with the presence of lesions because none of the antibody-positive wild animals had papillomatosis. The presence of detectable anti-TmPV1 antibodies in healthy animals may indicate either a previous, active infection or a relatively high frequency of current asymptomatic infections among the wild manatees. Conversely, 72.7% of the antibody-positive captive manatees displayed a cutaneous papillomatosis, indicating a significant association between detection of an anti-TmPV1 humoral immune response and presence of lesions in this group ($P=0.001$). The absence of detectable antibodies against TmPV1 does not provide assurance of absence of infection, as also shown for HPV (Park et al., 1998). In fact, anti-TmPV1 serum reactivity was not observed in four captive animals with evident PV-induced lesions. The only two wild manatees that displayed wart-like lesions containing TmPV1 DNA (Woodruff et al., 2005) were also antibody-negative. The appearance of papillomatosis in those manatees may depend on

the ineffective generation of anti-TmPV1 neutralizing antibodies on which protective immunity against PV infection mainly relies (Christensen et al., 1994; Christensen et al., 1996). An ineffective immune response to PVs may depend on the peculiar characteristics of the viral life cycle, which is exclusively intraepithelial. Papillomavirus infections are not known to be associated with viremia, and no inflammation usually accompanies the infection. Viral proteins are not expressed in professional antigen-presenting cells (Kanodia et al., 2007). Conversely, they are expressed at high levels only in terminally differentiated keratinocytes, shed from the epithelial surface. All these features have an important effect on PV recognition by the immune system because they limit access of the immune cells to the PV antigens. Moreover, it has been reported that HPVs evolved active strategies to evade host immunity (Kanodia et al., 2007). A similar scenario cannot be excluded in TmPV1 infections.

For captive manatees, it is also possible that stress-driven defects in the immune surveillance system hampered the development of an effective response to TmPV1 and favored the appearance of the lesions. It has been reported that adverse environmental conditions, such as inappropriate water temperature, may induce immunosuppression in Florida manatees (Halvorsen and Keith, 2008). Immunosuppression, either genetically or environmentally induced, is a well-recognized risk factor for the development of PV-associated diseases (Madkan et al., 2007; Palefsky, 2007). However, seroconversion may occur months after infections, as frequently observed in HPV-infected women (Dillner, 1999; Coursaget, 2003); therefore, we cannot exclude that blood samples from antibody-negative, lesion-positive animals were collected before seroconversion. Finally, negative antibody results might also indicate the failure to detect anti-TmPV1 antibodies because of the presence of extremely low antibody levels, possibly because of low levels of viral replication. Moreover, antibodies may be present that bind to TmPV1-VLPs with low affinity, so that they are only detected if present at high concentrations.

The rarity of papillomatosis among wild animals (1.7%) suggests that the wild population is capable of efficiently controlling TmPV1 infection without developing clinical disease. In contrast, noticeable signs of TmPV1 infection were documented in 30.8% of the captive animals. Although we observed that most of the captive manatees with papillomatosis developed a humoral response to TmPV1, antibodies are not involved in the resolution of established infections, which is based on cellular immunity. The higher prevalence of lesions in captive manatees may thus depend on defects in cellular immune responses. The clinical history of some of the captive animals in which papillomatosis was first evidenced, with lesion persistence or regression and reappearance at new sites, was strongly suggestive of immunosuppression (Bossart et al., 2002). These manatees had significantly lower lymphocyte proliferation stimulation indices compared with healthy control captive manatees, suggesting an underlying immunosuppressive state that may be involved in the pathogenesis of papillomatosis (Bossart et al., 2002). Immunosuppression combined with a higher chance for horizontal transmission among peers housed in the same facilities may reasonably explain the higher prevalence of clinical lesions in captive manatees. Although TmPV1 infections might be equally common among captive and wild animals, our data suggest that wild manatees might be more resistant to TmPV1-associated disease than stressed, possibly immunocompromised, captive animals. Similarly, HPV-related diseases, including cervical cancer, are more prominent among transplant recipients and HIV-positive patients, suggesting that individuals with functional defects of the immune

system are more vulnerable to PV infections and associated diseases (Harwood et al., 2000; Palefsky, 2006, 2007).

The TmPV1-induced papillomatosis was initially identified in seven captive manatees that were clinically monitored to track the progression or resolution of their lesions. During follow-up, serial serum samples were obtained from these animals to monitor changes in their serologic status. Only one of these animals (Willoughby, from which TmPV1 was originally isolated) changed serologic status over time (Table 5). At the time the cutaneous lesions were detected, the animal was antibody-negative. One year later, papillomas were still present, but the serum had high levels of detectable anti-TmPV1 antibodies. As previously emphasized, (neutralizing) anticapsid PV antibodies are responsible for the prevention of the viral infection but are not involved in the regression of PV-induced lesions, which mainly depends on a cell-mediated immune response (Kadish et al., 2002; Palefsky and Holly, 2003). Most humans and animals that undergo spontaneous regression of a productive wart or papilloma have a simultaneous increase in antibody titer against PV virions. For instance, regressing warts have been associated with high serum levels of neutralizing anti-PV antibodies in beagles (*Canis lupus familiaris*) infected with *Canine oral papillomavirus* (CPV1, previously abbreviated as COPV; Ghim et al., 2000). Therefore, as papillomas regress because of a specific cell-mediated immunity, an abundant release of PV virions at the peak of the regression may induce a high-titer humoral response (Ghim et al., 2000).

Studies have shown that PVs may have coevolved and cospeciated with their hosts (Chan et al., 1997; Rector et al., 2007). Rector et al. (2004) suggested that TmPV1 was not transmitted to manatees from other species, but rather coevolved with the species itself. Thus, TmPV1 could have been present in manatees and passed from generation to generation for millions of years. The low rate of clinical disease among wild manatees suggests that as long as manatees live in optimal conditions, they are able to efficiently control the virus or rapidly resolve possible lesions. Although the oncogenic potential of TmPV1 has not been defined and all biopsied lesions caused by this virus have been classified as benign, malignant transformation is still an important complication of PV infection in immunosuppressed individuals (Bouwes Bavinck and Berkhout, 1997). Hence, maintenance of low-stress living conditions may help prevent the emergence of PV-induced lesions and favor their resolution. Indeed, captive manatees with papillomatosis resolved their lesions when relocated to facilities with better environmental conditions (increased water temperature, etc.).

Further epidemiologic surveys are needed to provide additional insights into the prevalence of TmPV1 in Florida manatees and to clarify the natural history of the infection. Serodiagnosis of TmPV1 may remain an important research tool to better understand the emergence, transmission, and immune response against TmPV1 and the significance of PV-induced diseases in Florida manatees. The possible existence of unknown PVs that may cross-react serologically with TmPV1 also should be considered.

In conclusion, captive and wild manatees had a similar prevalence of anti-TmPV1 antibodies. However, captive manatees seem more apt to exhibit papillomatosis because the frequency of PV-induced lesions was higher in captive than in wild manatees. These data seem to indicate the ability of wild animals to effectively control the viral infection and may thus influence management decisions concerning the release of captive manatees. Healthy, captive manatees that have been closely monitored for an appropriate period to ensure the absence of active TmPV1-induced lesions would not pose a risk of spreading TmPV1-related disease among wild animals.

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