Molecular Identification of a Papilloma Virus from Cutaneous Lesions of Captive and Free-ranging Florida Manatees

Authors: Woodruff, Rebecca A., Bonde, Robert K., Bonilla, J. Alfredo, and Romero, Carlos H.

Source: Journal of Wildlife Diseases, 41(2) : 437-441

Published By: Wildlife Disease Association

URL: https://doi.org/10.7589/0090-3558-41.2.437

Journal of Wildlife Diseases, 41(2), 2005, pp. 437–441

Molecular Identification of a Papilloma Virus from Cutaneous Lesions of Captive and Free-ranging Florida Manatees

Rebecca A. Woodruff,[1] **Robert K. Bonde,**[2] **J. Alfredo Bonilla,**[1] **and Carlos H. Romero**[1,3] [1] Department of Pathobiology, College of Veterinary Medicine, University of Florida, Gainesville, Florida 32610, USA; [2] US Geological Survey, Florida Integrated Science Center, Sirenia Project, Gainesville, Florida 32601, USA; [3] Corresponding author (email: romeroc@mail.vetmed.ufl.edu)

ABSTRACT: Cutaneous papillomatous lesions were biopsied from three captive Florida manatees (*Trichechus manatus latirostris*) at Homosassa Springs State Wildlife Park (HSSWP), Homosassa, Florida, USA, and from six free-ranging Florida manatees from Crystal and Homosassa rivers, Florida. Total DNA extracted from these lesions was assayed for the presence of papilloma virus genomes using the polymerase chain reaction (PCR) with primers that target the L1 capsid protein gene. The amplification generated DNA fragments 458 base pairs in length that encompassed a highly conserved domain within the L1 capsid protein and translated into identical polypeptides of 152 amino acids, suggesting the involvement of a single papilloma virus genotype. Multiple amino acid sequence and phylogenetic analyses of the L1 fragment indicated that the Florida manatee papilloma virus is a unique and quite distinct papillomavirus from other known papilloma viruses. The emergence of this new pathogen raises concerns about its potential impact on the already endangered Florida manatee.

Key words: Florida manatee, L1 gene, molecular characterization, papilloma virus, skin lesions.

Papilloma viruses are small, double-stranded DNA viruses that infect mucosal and cutaneous epithelial tissues and are associated with the development of benign warts and malignant neoplasia in humans (Walboomers et al., 1999) and domestic and wild animals (Sundberg, 1987). Papilloma viruses are also known to infect and cause genital and cutaneous lesions in several species of marine mammals (Kennedy-Stoskopf, 2001). The Florida manatee (*Trichechus manatus latirostris*) is a threatened marine mammal that occupies southeastern US waters and is listed as endangered at both the state and federal levels (US Fish and Wildlife Service, 2001). Recently, infection of the Florida manatee with papilloma virus was identified in a few individuals of a captive population housed at Homosassa Springs State Wildlife Park (HSSWP), Homosassa, Florida, USA (Bossart et al., 2002). In the present report, we extend these results by providing molecular evidence that the newly described manatee papilloma virus (TmlPV) is not only found in manatees in captivity, but also in free-ranging manatees in Florida waters.

Nine adult female Florida manatees comprised the captive population residing at Homosassa Springs State Wildlife Park (HSSWP) in Homosassa, Florida (Bossart et al., 2002), and skin lesions were biopsied from three of these animals (samples V369, V375, P31) in 1998 and preserved in 10% neutral buffered formalin at room temperature until processing in January, 2003. The site is a natural freshwater spring system at the headwaters of the Homosassa River in Citrus County, Florida. The manatees were confined to an enclosure of approximately two acres by an underwater fence placed at the junction of the spring and the river. Free-ranging manatees could occasionally be found at the outer perimeter of the underwater fence and are considered winter residents that use these springs for thermoregulation. Fresh samples were harvested from similar lesions (Fig. 1) on the skin of six free-ranging Florida manatees (samples V378, V389, V390, V396, V397, V408) in January and February of 2003. These six biopsies, each consisting of a small piece of the superficial cutaneous lesion, were obtained by a swimmer (R.K.B.) using a scalpel blade, immediately chilled in ice, and transported to the laboratory. Total DNA was extracted from approximately 25 mg of

FIGURE 1. Adult male free ranging Florida manatee (*Trichechus manatus latirostris*) with cutaneous papillomatous lesions on the rostrum, head, and anterior part of the body. Total DNA extracted from these lesions (V378) and amplified by PCR yielded a DNA fragment (458 bp) corresponding to the L1 capsid protein gene of a papilloma virus.

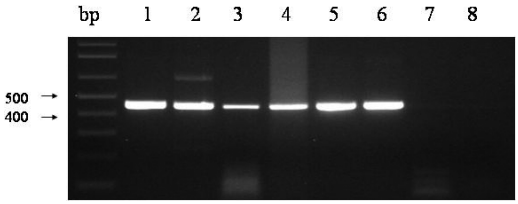

FIGURE 2. Agarose gel electrophoresis of amplified DNA fragments corresponding to the L1 capsid protein gene of the Florida manatee papilloma virus (TmlPV) from cutaneous lesions of captive and free ranging manatees. bp=100 base-pair ladder; Lane 1=V375 DNA; 2=V396 DNA; 3=P31 DNA; 4=V389 DNA; 5=V390 DNA; 6=V397 DNA; 7=DNA from uninfected manatee skin; 8=no DNA in the PCR reaction tube.

each skin lesion using the DNeasy tissue kit (Qiagen Inc., Valencia, California, USA), following the manufacturer's protocol. The polymerase chain reaction (PCR) targeting the L1 capsid protein gene was used to amplify a 458-base pair DNA fragment using consensus primers MY11/MY09 (Manos et al., 1989), modified by us to contain deoxyinosine at positions of nucleotide degeneracy. Reactions were performed in 100 μl volumes and contained: 200 nM of each primer, 2 mM $MgCl_2$, 100 μM of each deoxynucleoside triphosphate, 20 mM Tris-HCl pH 8.4, 50 mM KCl, 2 units of Taq DNA polymerase (Invitrogen Life Technologies, Carlsbad, California, USA), and 0.5–1 μg of DNA template. The reaction was performed in a PTC-100 thermal cycler (MJ Research, Inc., Waltham, Massachusetts, USA) as follows: one initial denaturation step at 94 C for 1 min; 40 cycles of denaturation at 94 C for 1 min, annealing at 50 C for 1 min, and extension at 72 C for 2 min; and one final extension step at 72 C for 10 min. A new set of primers was designed based on newly generated L1 sequences. These primers were: forward primer ΔL1TmlPV 5′–CAG GGG CAT AAG AAT GGT ATT G–3′ and reverse primer ΔL1TmlPV 5′–GAG GGG AGA CTG ATC GAG TTC TG–3′. Cycling conditions were similar to those for PCR with primers MY11/MY09, but with temperatures of 60 C for annealing and 68 C for extension with Accuprime enzyme (Invitrogen). Amplified DNA fragments were resolved by electrophoresis of 40 μl of PCR products in 1.2% agarose containing ethidium bromide (0.5 μg/ml) and visualized under UV light. PCR products of the expected (~458 bp) size (Fig. 2) were purified and cloned into the TOPO pCR 2.1 T/A cloning kit (Invitrogen) and used to transform One-Shot chemically competent *Escherichia coli* cells (Invitrogen). Plasmid DNA was extracted from overnight cultures using the 10-minute mini-prep protocol (Zhou et al., 1990). Recombinant plasmids containing inserts of the expected size were identified after restriction with HindIII, EcoRI, and the combination of ApaI and BamHI enzymes. The recombinants were further propagated in One Shot *E. coli* competent cells and purified plasmid DNA prepared using a Midi-Prep kit (Qiagen). Approximately 100 fmol of each DNA clone were sequenced with forward and reverse M13 primers in the Beckman-Coulter CEQ 2000XL (Fullerton, California, USA). Chromatograms were evaluated with the Chromas software (Technelysium Pty Ltd., Queensland, Australia) and the assembled sequences were analyzed using the seqed, gap, and translate functions of the Wisconsin Package Version 10.0 (Genetics Com-

puter Group [GCG], University of Wisconsin, Madison, Wisconsin, USA). Sequencing of the nine cloned DNA fragments yielded nucleotide sequences that were 100% identical (GenBank accession numbers: AY455940, AY455941, AY496568, AY496569, AY496570, AY496571, AY496 572, AY496574, AY496575) and translated correctly from the first nucleotide of forward primers MY11 and FPΔL1TmlPV into protein fragments consisting of 152 amino acids. Sequences were entered into the Basic Local Alignment Search Tool (BLAST) software of the National Center for Biotechnology Information Website (NCBI, Bethesda, Maryland, USA, www.ncbi.nlm.nih.gov) to identify papilloma virus homologues with the highest similarity and identity to TmlPV L1. This exercise demonstrated that the amplified TmlPV fragments spanned a highly conserved domain within the L1 capsid protein of papilloma viruses. Individual comparisons of the TmlPV L1 152-amino acid sequence with homologues from the most similar papilloma viruses using the GAP function of the GCG Genetic Package demonstrated similarities that ranged between 58% and 75% and identities that ranged between 47% and 55% (not shown). To investigate the phylogenetic relationship of TmlPV to that of other human and animal papilloma viruses, 40 amino acid sequences were aligned and compared. The sequences were aligned using ClustalW and phylogenetic analysis was performed with PAUP (Phylogenetic Analysis Using Parsimony) v4.0.0d55 (Swofford and Berlocher, 2002) using the heuristic search for maximum parsimony trees. Bootstrap analysis was carried out with 100 replicates to assess branch topology. The starting tree was obtained via stepwise addition, and by performing tree–bisection–reconnection branch swapping. Phylogenetic and molecular evolutionary analyses were also conducted using MEGA version 2.1 (Kumar et al., 2001) and PHYLIP (Felsenstein, 1985). However, these programs gave nearly identical tree topologies as PAUP, and are not shown.

Our results provide molecular evidence for the existence of a manatee papilloma virus (Bossart et al., 2002) and confirm and extend the above findings by showing that papillomatous skin lesions from three captive and six free-ranging Florida manatees contained viral genomic sequences corresponding to the L1 capsid protein gene of a papilloma virus. Multiple sequence and evolutionary analysis of the L1 protein fragment showed that the TmlPV is a unique virus that clades by itself in a robust phylogenetic tree (Fig. 3), and is evolutionarily most related to another marine papilloma virus, the porpoise papilloma virus (PsPV-1) and papilloma viruses of humans (HPV-4, HPV-65, HPV-15, HPV-9, HPV-5, HPV-20, HPV-21) most commonly associated with epidermodysplasia verruciformis, common verrucous warts, and keratotic flat lesions of the skin (zur Hausen, 2000). These preliminary data suggest that the described TmlPV genotype found in cutaneous papillomatous lesions in captive and free-ranging manatees in the Crystal and Homosassa rivers and at Homosassa Springs State Wildlife Park, Florida, might not have the full genetic potential to induce the invasive malignancies observed in humans infected with papilloma viruses (HPV-11, HPV-16, HPV-18, HPV-30) evolutionarily distant from TmlPV (Fig. 3). It is not known at this time whether the recent appearance of skin lesions induced by TmlPV in captive and free-ranging Florida manatees might be a consequence of immunosuppression due to water pollution with chemicals or toxins or to long-term exposure to cool water (Bossart et al., 2002). In humans, immunosuppression has long been suspected as the underlying cause of disease triggered by papilloma viruses (zur Hausen, 2000). Papilloma viruses have also been demonstrated to occur in normal skin of domestic and wild animals in the absence of overt lesions (Antonsson and Hansson, 2002), although these findings need to be con-

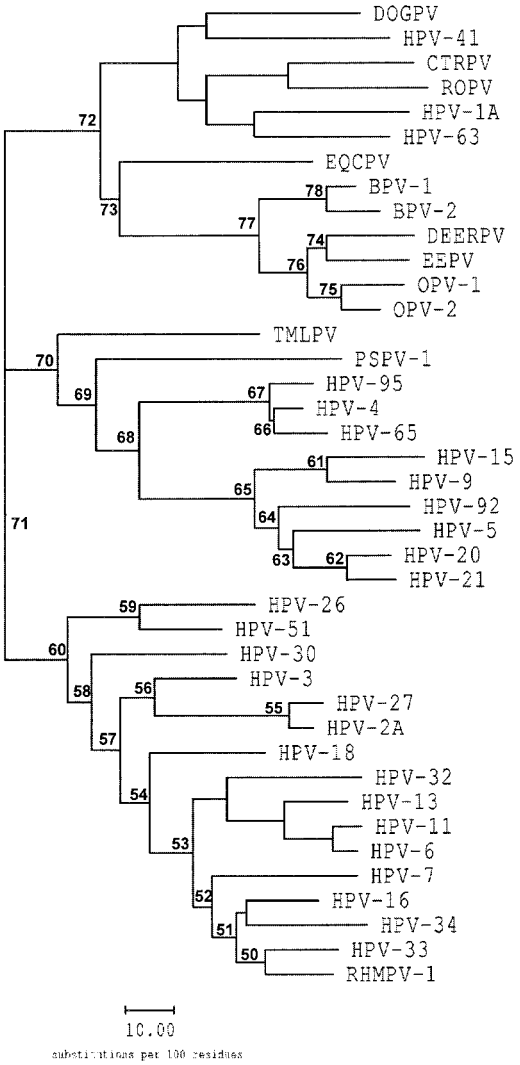

FIGURE 3. Phylogenetic tree of papilloma viruses including the 39 most closely related to the Florida manatee papilloma virus (TmlPV) as determined by deduced amino acid sequences of the L1 capsid protein fragments. Human papilloma virus type 1a (HPV-1a) (GenBank accession number V01116), HPV-2a (X55964), HPV-3 (X74462), HPV-4 (CAA50163), HPV-5 (M17463), HPV-6 (AAF00066), HPV-7 (X74463), HPV-9 (X74464), HPV-11 (M14119), HPV-13 (CAA44654), HPV-15 (X74468), HPV-16 (K02018), HPV-18 (X05015), HPV-20 (AAA79393), HPV-21 (AAA79400), HPV-26 (X74472), HPV-27 (X74473), HPV-30 (X74474), HPV-32 (X74475), HPV-33 (M12732), HPV-34 (X74476), HPV-41 (X56147), HPV-51 (M62877), HPV-63 (X70828), HPV-65 (CAA50177), HPV-92 (NC_004500), HPV-95 (CAF05708), bovine PV type 1 (BPV-1) (X02346), BPV-2 (M20219), canine oral PV (DogPV) (L22695), cottontail rabbit papilloma virus (CtRPV) (CAB96121), deer PV (DeerPV) (M11910), Florida manatee papilloma virus (TmlPV), *Equus caballus* PV (EqcPV) (NC_003748), European elk PV (EePV) (M15953), ovine PV type 1 (OPV-1) (U83594), OPV-2 (U83595), rabbit oral papilloma virus (ROPV) (AAF67129), rhesus monkey PV type 1 (RhmPV-1) (M60184), and Burmeister's porpoise PV-1 (PsPV-1) (AJ006302). The tree was constructed using PAUP (Swofford and Berlocher, 2002). Bootstrap values, greater than 50%, obtained by 100 replicates, are indicated at respective nodes.

firmed. It is plausible, then, that when the Florida manatee's immune system is compromised by ill-defined immunosuppressive factors, the fine balance between the virus and the host might be affected, thus enabling the papilloma virus to suddenly become invasive and produce cutaneous lesions. Most, if not all papillomatous lesions found in the Florida manatee were cutaneously localized and no mucosal genital, oral, or respiratory lesions have been observed, as is the case with human papilloma viruses and the Burmeister's porpoise papilloma virus (Van Bressem et al., 1996). Sequencing of the complete genome of several TmlPVs from different free-ranging manatees and from different geographical locations is necessary in order to investigate the possible existence of more than one genotype and better predict its oncogenic potential.

This research was supported by The Training Program in the Care of Marine Mammals, College of Veterinary Medicine and The Whitney Laboratory, University of Florida, with funding provided by Florida Fish and Wildlife Conservation Commission. The Institutional Animal Care and Use Committee of the University of Florida approved the use of animal tissues in this study. Samples were collected and analyzed under authority of the US Fish and Wildlife Service research permit MA-791-721/2 issued to the USGS Sirenia Project.

LITERATURE CITED

ANTONSSON, A., AND B. G. HANSSON. 2002. Healthy skin of many animal species harbors papilloma-

viruses which are closely related to their human counterparts. Journal of Virology 76: 12537–12542.

BOSSART, G. D., R. Y. EWING, M. LOWE, M. SWEAT, S. J. DECKER, C. J. WALSH, S. GHIM, AND A. B. JENSON. 2002. Viral papillomatosis in Florida manatees (*Trichechus manatus latirostris*). Experimental and Molecular Pathology 72: 37–48.

FELSENSTEIN, J. 1985. Confidence limits on phylogenies: An approach using the bootstrap. Evolution 39: 783–791.

KENNEDY-STOSKOPF, S. 2001. Viral diseases. *In* CRC Handbook of Marine mammal medicine, L. A. Dierauf and F. M. D. Gulland (eds.). CRC Press, Boca Raton, Florida, pp. 285–307.

KUMAR, S., K. TAMURA, I. B. JAKOBSEN, AND M. NEI. 2001. MEGA2: Molecular evolutionary genetics analysis software. Bioinformatics 17: 1244–1245.

MANOS, M. M., Y. TING, D. K. WRIGHT, A. J. LEWIS, T. R. BROKER, AND S. M. WOLINSKY. 1989. The use of polymerase chain reaction amplification for the detection of genital human papillomaviruses. Cancer Cells, Chapter 7. *In* Molecular Diagnostics of Human Cancer. M. Greaves (ed.). Cold Spring Harbor Laboratory, Woodbury, New York, pp. 209–214.

SUNDBERG, J. P. 1987. Papillomavirus infections in animals. *In* Papillomaviruses and human disease, K. Syrjaenen, L. Gissmann, and L. G. Koss (eds.). Springer Verlag, Berlin, Germany, pp. 40–103.

SWOFFORD, D. L., AND S. H. BERLOCHER. 2002. Inferring evolutionary trees from gene-frequency data under the principle of maximum parsimony. Systematic Zoology 36: 293–325.

US FISH AND WILDLIFE SERVICE. 2001. Florida Manatee Recovery Plan, (*Trichechus manatus latirostris*), 3rd Revision, U.S. Fish and Wildlife Service. Atlanta, Georgia, 144 pp. + appendices.

VAN BRESSEM, M. F., K. VAN WAEREBEEK, G. PIERARD, AND C. DESAINTES. 1996. Genital and lingual warts in small cetaceans from coastal Peru. Diseases of Aquatic Organisms 26: 1–10.

WALBOOMERS, J. M., M. V. JACOBS, M. M. MANOS, F. X. BOSCH, J. A. KUMMER, K. V. SHAW, P. J. SNIJDERS, J. PETO, C. J. MEIJER, AND N. MUNOZ. 1999. Human papilloma virus is a necessary cause of invasive cervical cancer worldwide. Journal of Pathology 189: 12–19.

ZHOU, C., Y. YANG, AND A. Y. JONG. 1990. Mini-prep in ten minutes. BioTechniques 8: 172–173.

ZUR HAUSEN, H. 2000. Papilloma viruses causing cancer: Evasion from host-cell control in early events in carcinogenesis. Journal of the National Cancer Institute 92: 690–698.

Received for publication 27 March 2004.