URSID γ-HERPESVIRUS TYPE 1–RELATED VIRUS IN CAPTIVE BORNEAN SUN BEARS (*HELARCTOS MALAYANUS EURYSPILUS*) IN SABAH, MALAYSIA

Zahidah I. Zeid, DVM, Tom Hughes, Mei-Ho Lee, Jimmy Lee, MSc, Reza Tarmizi, DVM, Fernandes Opook, Alexter Japrin, Mohamed S.M. Azian, Muhammad Y. Wazlan, Nor A. Aziz, Ronald H.M. Tinggu, Emilly Sion, Siew T. Wong, MSc, Jum R.A. Sukor, Jonathan H. Epstein, DVM, MPH, PhD, and Peter Daszak, PhD

Abstract: The Bornean sun bear (*Helarctos malayanus euryspilus*) is the smallest subspecies of sun bear. Their numbers are declining, and more research is needed to better understand their health and biology. Forty-four bears housed at the Bornean Sun Bear Conservation Centre (BSBCC) in Sabah, Malaysia, were screened for known and novel viruses in November 2018. Ursid γ-herpesvirus type 1 (UrHV-1) is a herpesvirus that has been detected from swab samples of clinically healthy sun bears and biopsy samples of oral squamous cell carcinoma in sun bears. We detected an UrHV-1–related virus from throat and rectal swabs by molecular viral screening in samples from 15.9% of the sun bears at BSBCC. None of the bears with the UrHV-1–related virus in this study had oral lesions. There is no known report of UrHV-1 detection in the wild sun bear population, and its association with oral squamous cell carcinoma is not fully understood. Finding an UrHV-1–related virus in a rehabilitation center is a concern because conditions in captivity may contribute to spreading this virus, and there is the potential of introducing it into wild populations when a bear is released. This study demonstrates an urgent need to carry out similar surveillance for sun bears in captivity as well as those in the wild, to better understand the impact of captivity on the prevalence and spread of UrHV-1–related viruses. Positive bears also should be monitored for oral lesions to better understand whether there is a causal relationship.

INTRODUCTION

The Malayan sun bear (*Helarctos malayanus*) is the smallest bear in the world and resides in the forests of Southeast Asia; the Bornean sun bear (*Helarctos malayanus euryspilus*) is a subspecies, with a smaller skull and body size, and is found only on the island of Borneo.[15] Sun bears forage the forest for an array of invertebrates and fruits by using their exceptionally long tongues and keen sense of smell.[30] While foraging, their sharp claws create new tree cavities that other animals then use as nests. This activity, and their role in controlling pests and seed dispersal, has earned them the title "engineers of the forest." Sun bears are currently classified as Vulnerable on the International Union for Conservation of Nature Red List,[24] because they face threats from rapid

deforestation and the illegal wildlife trade. Historically, sun bears have received little conservation attention in the countries within their range,[30] but efforts are now being made to better understand and raise the profile of this important species. The Bornean sun bear is listed as a totally protected species in the Sabah Wildlife Conservation Enactment 1997.

The Bornean Sun Bear Conservation Centre (BSBCC) in Sandakan, Sabah, Malaysia, works with the Sabah Wildlife Department to house and rehabilitate rescued, confiscated, or surrendered sun bears to facilitate their return to the wild. The center consists of two bear houses and a visitor center for public viewing of the bears in their forest enclosures. Bears are kept in groups or individually and are monitored closely by the in-house veterinarian. The indoor enclosures provide a safe environment for bears and personnel while allowing ample natural light. Barriers between cages are made of metal bars, and cement flooring allows for proper cleaning and drainage. Bears can however interact between cages, and the possibility of pathogen transmission within an enclosure exists. Safe enrichments are provided for bears because the BSBCC strives to keep sun bears at the highest welfare standards.

Infectious diseases are a key factor in population decline in wildlife species worldwide.[6] For example, the emergence of devil facial tumor

From Conservation Medicine, 47000 Sungai Buloh, Selangor, Malaysia (Zeid, Hughes, Lee, Lee, Opook, Japrin, Azian, Wazlan, Aziz, Tinggu, Sion); EcoHealth Alliance, New York, NY 10018, USA (Hughes, Lee, Lee, Opook, Epstein, Daszak); Borneo Rhino Alliance Berhad, c/o Faculty of Science and Natural Resources, Universiti Malaysia Sabah, 88400 Kota Kinabalu, Sabah, Malaysia (Tarmizi); Bornean Sun Bear Conservation Centre, 90000 Sandakan, Sabah, Malaysia (Wong); and Sabah Wildlife Department, 88100 Kota Kinabalu, Sabah, Malaysia (Sukor). Correspondence should be directed to Mr. Hughes (tom@conservationmedicine.org).

Table 1. Summary of previous reports on detection of herpesvirus infections in bears.

Subfamily	Virus	Species	Country
α-Herpesvirus	Equine herpesvirus 9[22]	Polar bear (*Ursus maritimus*)	United States[a]
α-Herpesvirus	Bovine herpesvirus 1[33]	Malayan sun bear (*Helarctos malayanus*)	Malaysia[a]
α-Herpesvirus	Equine herpesvirus 1[10,29]	American black bear (*Ursus americanus*) Polar bear (*U. maritimus*)	Germany[a]
γ-Herpesvirus	Ursid herpesvirus 1[12,18]	Malayan sun bear (*H. malayanus*)	United States,[a] Cambodia[a]
γ-Herpesvirus	Novel γ-herpesvirus[3]	American black bear (*U. americanus*)	United States[b]
α-Herpesvirus	Suid herpesvirus 1[1,23,32]	Polar bear (*U. maritimus*) Brown bear (*U. arctos*) American Black bear (*U. americanus*)	Spain[a] Italy[a] United States[a]

[a] Samples collected were from captive bears.
[b] Samples collected were from free-ranging bears.

disease in Tasmanian devils (*Sarcophilus harrisii*) in Australia has contributed to an approximately 80% decline in their population from 1996 to 2018.[13,31] In 1994, a canine distemper virus epidemic in Tanzania and Kenya caused a 30% decrease in the Serengeti and Mara African lions (*Panthera leo*).[21] Therefore, there is an urgent need to identify any disease agents within vulnerable sun bear populations. Conducting disease surveillance in wild sun bears is logistically challenging given their wide habitat range, shyness, and scarcity. The availability of a large population of captive sun bears at BSBCC helps provide insight as to what pathogens may be present in free-ranging populations and enables us to identify potentially harmful pathogens that could be reintroduced through rehabilitation.

The *Herpesviridae* family is a large and diverse group of DNA viruses, comprising three subfamilies designated α-, β-, and γ-herpesviruses.[26] Most herpesviruses are species specific; however, some have been proven to infect other species either experimentally or accidentally. The α-herpesviruses can infect a wide range of animal species and different cells (neurons, epithelial cells), the β-herpesviruses can infect different cells within the same animal species, and the γ-herpesviruses are the most species specific and cell specific.[26] There are limited studies or reports of herpesvirus infection in bears (Table 1).

Ursid γ-herpesvirus type 1 (UrHV-1) was first described in 2013, where molecular analysis of tissue samples from bears presenting with oral squamous cell carcinoma detected UrHV-1 in four of five bears from four zoological institutions in the United States.[12] In Cambodia, 18 of 31 sun bears had UrHV-1 detected via molecular analysis from swab and tissue samples.[18] Other reported viral infections in sun bears are canine adenovirus 1 in two captive sun bears in California[9] and foot and mouth disease virus in a captive sun bear in Vietnam.[17]

This sampling session was performed with BSBCC as part of PREDICT, a project of the U.S. Agency for International Development (USAID) Emerging Pandemic Threats program, to provide additional information beyond routine health screening to further improve welfare standards and help identify animals that might be suitable for release back into the wild.

MATERIALS AND METHODS

Animal information

In November 2018, samples for molecular viral screening were collected from 44 bears (17 males and 27 females) ranging between 11 mon and 17 yr old. All bears fasted for more than 8 h and were immobilized with tiletamine-zolazepam (Virbac, Carros, 06510, France) at an initial dose of 5 mg/kg, administered using a blow pipe or dart pistol (Telinject Dart Gun, Am Gewerbering, Dudenhofen, 67373, Germany). Dose calculations for induction were based on the most recent recorded body weight. Once the animal was anesthetized, current weight was recorded using a digital weighing scale (Accurate Measurement Sdn Bhd, Luyang, Sabah, 88784, Malaysia). An IV catheter was inserted to ease administration of a supplementary dose of tiletamine-zolazepam to achieve required anesthetic depth to complete the procedure, and vitals were monitored and recorded. A full physical examination, biometric measurements, photography of chest markings, and oral and dental inspection for the bears were performed, and a range of samples were collected from each animal. The sample collection protocols were in accordance with the Institutional Animal Care and Use Committee protocol 19300 and approved by Sabah Biodiversity Centre

(SaBC license JKM/MBS.1000-2/2JLD.7). In this study, we only screened the rectal and throat swabs, which were collected with sterile nonabsorbent mini-tip polyester-tipped applicators with flexible aluminum handles (Puritan, Guilford, CT 04443, USA). Collected swabs were placed in individual cryotubes containing 600 µl of TRIzol reagent (Invitrogen, Carlsbad, CA 92008, USA). Within 15 min of collection, all samples were stored in a liquid nitrogen dry shipper (Chart Biomed, Ball Ground, GA 30107, USA) at −196°C. These samples were later transported to the Wildlife Health, Genetic and Forensic Lab (WHGFL) in Kota Kinabalu, Sabah, a certified biosafety level 2 laboratory, for storage in an ultra-low freezer at −80°C.

PCR amplification and sequencing

The throat and rectal swab samples collected from each bear were used for the molecular viral screening carried out at the WHGFL. Total nucleic acid was extracted using the NUCLISENS MINIMAG system (bioMérieux, Marcy l'Etoile, Auvergne 69280, France) according to the manufacturer's protocol, with validated modifications. Complementary DNA (cDNA) of each sample was generated according to the manufacturer's protocol by using random hexamers from the SuperScript III First-Strand Synthesis System for reverse transcriptase polymerase chain reaction (PCR) (Invitrogen, Carlsbad, CA 92008, USA). The cDNA was used in PCR protocols to screen for different viral groups: adenovirus, alphavirus, arenavirus, astrovirus, bocavirus, bunyavirus, coronavirus, enterovirus, filovirus, flavivirus, hantavirus, henipavirus, herpesvirus, influenza virus, lyssavirus, nipah virus, orbivirus, orthopoxvirus, paramyxovirus, parapoxvirus, phlebovirus, polyomavirus, retrovirus-lentivirus, rhabdovirus, seadornavirus, and simian foamy virus (Supplemental Table 1). The PCRs were conducted using a SimpliAmp thermal cycler (Applied Biosystems, Foster City, CA 94404, USA). Reactions were carried out in a final volume of 20 µl, following the manufacturer's protocol (Qiagen, Hilden, North Rhine-Westphalia 40724, Germany) using 1 µl of the cDNA product as template and either Fast Cycling PCR kit or HotStarTaq *Plus* Master Mix with final concentration of 0.1 µM each primer and following manufacturer's protocols (Qiagen). Universal controls 1 and 2 and specific controls for filovirus and influenza virus were used (Supplemental Table 1). These controls were developed by core laboratories within the PREDICT project.

The PCR products were loaded and run on 1% agarose gel electrophoresis (100 V, 30–45 min, and 0.5× Tris-acetate-EDTA buffer) (Vivantis Technologies Sdn. Bhd., Shah Alam, Selangor 40170, Malaysia). The gels were viewed on a transilluminator (Maestrogen, Hsinchu City, 30091, Taiwan), and expected size bands were excised and stored in separate microcentrifuge tubes. The PCR products that had the correct size bands were used as template for contamination control PCRs to check for contamination from the universal positive controls (Supplemental Table 1). Those products from PCRs using specific positive controls were not checked for control contamination. The PCR products from the contamination check were run using the same gel electrophoresis conditions. Those without the expected size bands indicated that there was no contamination from the controls. The products from the initial PCR of these samples were purified using Ultrafree-DA centrifugal filter units (Millipore, Carrigtohill, County Cork T45 RP21, Ireland). Purified PCR products were cloned using the dual-color selection Strataclone PCR cloning kit (Stratagene, La Jolla, CA 92037, USA) according to the manufacturer's protocol. Up to eight colonies containing the PCR product were picked and inoculated on Luria-Bertani agar slants, individually. Grown colonies were sent to a commercial company for direct colony sequencing.

Sanger sequencing was performed using the Prism 3730 Genetic Analyzer (Applied Biosystems, Foster City, CA 94404, USA). The nucleotide sequences were analyzed with Geneious 10.1.3 software (Auckland Central Business District, Auckland 1010, New Zealand) and compared with sequences in GenBank for homology analysis using the Basic Local Alignment Search Tool (BLAST; National Center for Biotechnology Information, Bethesda, MD 20894, USA).

Phylogenetic analysis

Using MEGA version 5 software,[27] a multiple sequence alignment of identified nucleic acid sequences and seven published herpesvirus DNA polymerase sequences was made with Clustal W. Phylogenetic analysis was performed using MEGA version 5 software[27] and the maximum likelihood method based on Kimura two-parameter model, and tree topology was verified with 1,000 bootstrap replicates.[7,11]

The following sequences retrieved from GenBank were used in the analysis: human herpesvirus type 4 (LN827582.1), human herpesvirus type 8 (HHV-8; LC200589.1), bovine γ-herpesvirus

Table 2. Summary of dental abnormalities recorded during dental inspection of sun bears at BSBCC.

Dental abnormality[2]	% of sun bears affected
Dental calculus (grade C3)	28.6
Gingivitis (grade G3)	9.5
Tooth mobility (grade M3)	9.5
Abrasion	33.3
Attrition	28.6
Missing teeth	45.6
Uncomplicated crown fracture	14.3
Complicated crown fracture	19.0
Caries	2.4
Enamel infraction	11.9

type 6 (KM438002.1), harp seal herpesvirus (HsHV) isolate (KF466473.1), phocid herpesvirus type 2 (PHV-2; GQ429152.1), gorilla rhadinovirus type 1 (RHV-1; AF250886.1), and UrHV-1 (JX220982.1).

RESULTS

Observations from health checks

Induction of anesthesia was smooth for all bears, and recovery went well. The mean temperature for all bears was recorded at 37.95°C, ranging between 36.70 and 39.20°C. The mean respiratory rate was recorded at 23 breaths/min, ranging from 10 to 36 breaths/min. The mean heart rate was recorded at 100 beats/min, ranging between 60 and 140 beats/min. Oral inspection of the bears revealed various dental abnormalities[2] (Table 2).

An oral lesion was observed in one bear and can be described as three irregular-shaped, dark pink erythema with smooth surface on the left lower labial mucosa (Fig. 1). An oronasal fistula was seen in one bear with a history of extraction of the right upper canine. Glossotrichia was suspected in three bears with hairs observed in the medial sulcus of the tongue.

PCR findings

Sequencing of PCR products amplified by the herpesvirus consensus primer of VanDevanter et al.[28] confirmed the detection of herpesviruses in seven bears aged between 11 mon and 12 yr (PSW02597, PSW02610, PSW02611, PSW02625, PSW02628, PSW02630, and PSW02631). Viral DNA was detected in throat swabs from two bears, in rectal swabs from four bears, and from the throat and rectal swab of one bear. DNA sequence alignments of amplicons from the seven animals by using Geneious software were 94.6% identical (Fig. 2), and BLAST analysis of these sequences revealed nucleotide similarities of >95% to UrHV-1.[12] All the UrHV-1–positive bears were clinically healthy, with no oral lesion. There were no significant findings from the complete blood count and serum biochemistry results for these seven bears. Viral screening for all the other virus families was negative.

Phylogenetic analysis

Analysis showed that the amplicons from the bear swab samples were more closely related to UrHV-1 than to members of the undefined genus such as PHV-2 and HsHV (Fig. 3). These data suggest that UrHV-1 and the bear herpesviruses found at the BSBCC are phylogenetically close.

DISCUSSION

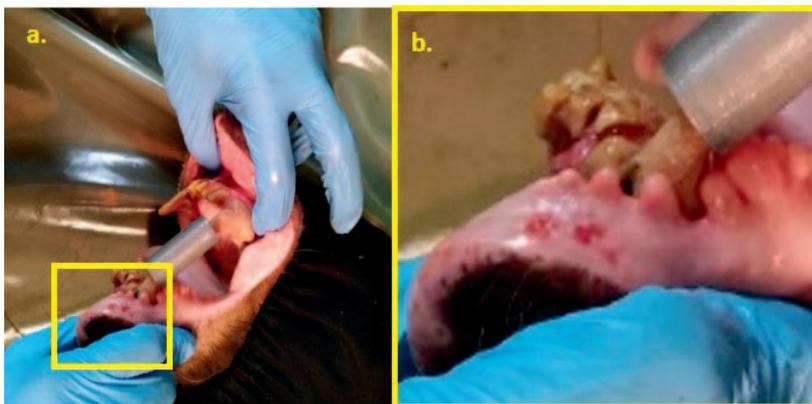This study provides data on the presence of a virus closely related to UrHV-1 in the captive

Figure 1. Bear PSW02601 with mild oral lesion on the left lower labial mucosa (**a**) and a close-up of the oral lesion (**b**). There is no evidence that this bear's oral lesion is cause by the UrHV-1–related virus.
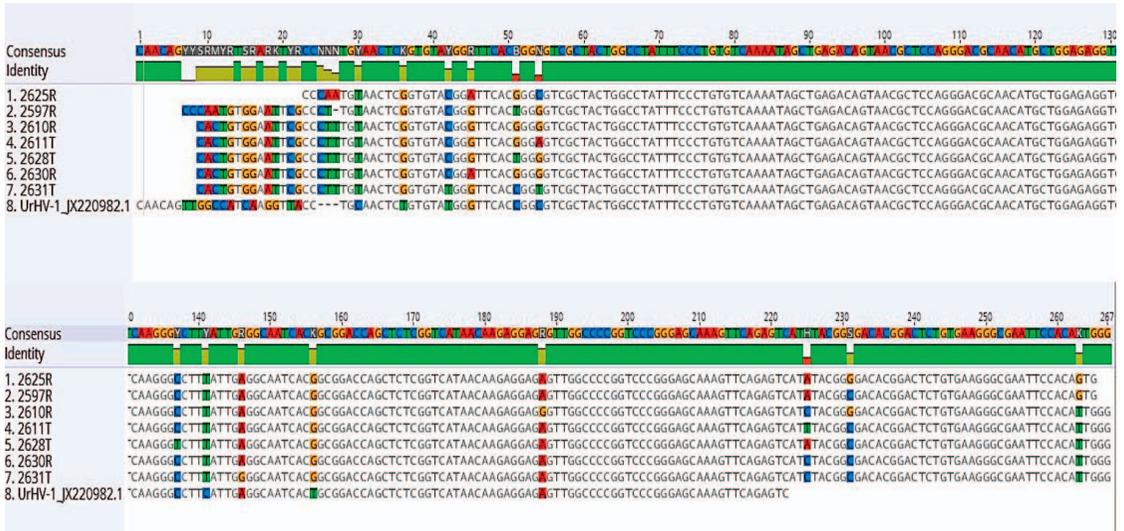
Figure 2. DNA sequence alignments of amplicons from herpesvirus consensus PCR analysis with UrHV-1 DNA sequence available in GenBank. 2625R, 2597R, 2610R, 2611T, 2628T, 2630R, 2631T: PCR products amplified from total DNA of swab samples from bears. UrHV-1: DNA sequence detected in the sun bear (accession JX220982). Green bar represents identical sequences.

Bornean sun bear population in Sabah. An investigation using degenerate PCR primers of samples from rectal and throat swabs resulted in amplification of DNA polymerase gene sequences with homology to γ-herpesviruses. DNA sequence alignment (Fig. 2) and phylogenetic (Fig. 3) analyses show that PCR amplicons from the swab samples have the highest homology to UrHV-1. Because of the lack of references in public databases, we are unable to conclude whether the herpesviruses detected are in fact UrHV-1 or a new herpesvirus strain within the γ-herpesvirus family.

Previous studies revealed a high prevalence of UrHV-1 in bears with erythematous oral lesions. The virus was first reported in 2013 after detection in four of five sun bears in North America with erythematous oral lesions that were confirmed as squamous cell carcinoma.[12] Officer et al.[18] revealed the overall prevalence of oral lesions was 68.3% (69/101) for sun bears at a Cambodia rescue center; however, there were also four bears with no oral lesions that were positive for UrHV-1. None of the bears in the current study with the UrHV-1–related virus had oral lesions at the time of sampling. There was only one bear (PSW02601) that exhibited a mild oral lesion, but the virus was not detected in this animal from the samples collected. There is no evidence that this bear's oral lesion is due to the UrHV-1–related virus. There were no previous occurrences
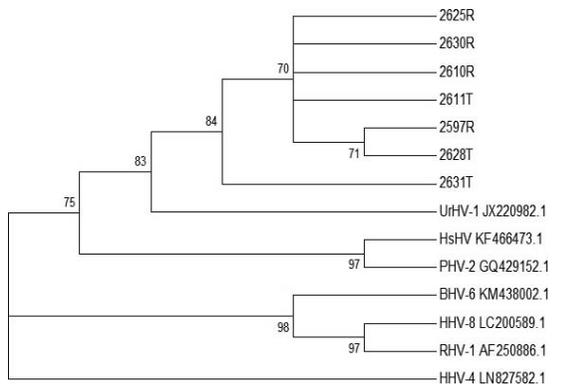

Figure 3. Phylogenetic tree of the DNA sequences of the DNA polymerase subunit. HHV-4 = human herpesvirus type 4; HHV-8 = human herpesvirus type 8; BHV-6 = bovine γ-herpesvirus type 6; HsHV = harp seal herpesvirus isolate; PHV-2 = phocid herpesvirus type 2; RHV-1 = gorilla rhadinovirus type 1; UrHV-1 = Ursid γ-herpesvirus type 1; 2625R = amplicon from bear PSW02625 rectal swab; 2597R = amplicon from bear PSW02597 rectal swab; 2628T = amplicon from bear PSW02628 throat swab; 2610R = amplicon from bear PSW02610 rectal swab; 2611T = amplicon from PSW02611 bear throat swab; 2630R = amplicon from bear PSW02630 rectal swab; 2631T = amplicon from bear PSW02631 throat swab. The accession numbers used for DNA polymerase coding sequence analysis are given after each virus name.

of oral lesions or any form of oral cancer at BSBCC (Wong, pers. comm.). The bear that presented with a mild oral lesion showed improvement and the lesion resolved without treatment after 4 mon (Yeoh, pers. comm.). It is common that animals infected with herpesvirus show no clinical signs or are in a latent form of infection whereby symptoms start to show much later.[26] Our study was carried out to identify novel and known viruses in sun bears and was not focused specifically on diagnosing lesions. Animals suffering from oral squamous cell carcinoma experience pain and discomfort, leading to poor quality of life without treatment.[14,19] In previous reports, sun bears with squamous cell carcinoma were treated with the combination of surgical resection, intralesional injection of chemotherapy drugs, and radiation therapy.[12,16] The association of UrHV-1 with oral carcinoma is still poorly understood, and a broader study of UrHV-1 and squamous cell carcinoma in sun bears could help identify a causal relationship.

In humans, a link has been hypothesized between herpesvirus infections and periodontal diseases, because infected individuals are immunosuppressed, which leads to growth of periodontopathic bacteria.[25] Previous studies have described the role of herpesviruses in periodontal disease and in immune suppression in a range of mammals;[5,20,22] however, more common contributors to periodontal disease such as diet and age factors should not be overlooked.[8] Sun bears are omnivores and feed on a wide range of food available in their habitat.[30] Dietary and behavioral factors related to captivity could explain the high occurrence of dental diseases seen in captive bears. A high sugar diet leads to the formation of plaque and calculus, whereas stereotypic behavior in bears such as bar biting leads to abrasion, attrition, and fractures in teeth.[8] The sun bears first detected with UrHV-1 by Lam et al.[12] had a history of periodontal diseases, and one bear was reported to frequently rub its lips with its claws. In this study, the seven bears in which UrHV-1–related virus was detected had mild-to-moderate periodontal disease. Bears in captivity with various degrees of periodontal disease experience discomfort and have been observed to rub their face against hard structures, leading to oral lesions that may progress to squamous cell carcinoma. This also may contribute to the transmission of orally shed herpesviruses, such as UrHV-1, via surface contamination. Therefore, it is particularly important to carry out routine dental inspection in animals and practice routine disinfection in animal enclosures to achieve the highest welfare standards, eliminate complications from periodontal diseases, and reduce the risk of infectious disease transmission among animals housed together.

The bears in this study that tested positive for the UrHV-1–related virus were aged between 11 mon and 12 yr. Previous studies have found UrHV-1 in sun bears ranging from 8 to 19 yr old[12] and from 5 to 20 yr old.[18] UrHV-1 is present in bears with a wide age range and no sex predilection.

The HHV-8 and macacine herpesvirus type 5 are related to the UrHV-1 within the rhadinovirus family, and HHV-8 was reported to be transmitted through oral and genital discharges.[4] Previous studies revealed that UrHV-1 can be detected in various sample types, including oral swabs and lip, tongue, tonsil, and vulval biopsies.[18] In this study, the UrHV-1–related virus was detected from throat and rectal swabs. Based on our findings and these previous studies, we can hypothesize that the UrHV-1–related virus can be shed via oral, genitourinary, and rectal discharges.

Ideally, the seven sun bears shedding the UrHV-1–related virus should be isolated to curb spread within the center. However, because of a lack of space such isolation is not an option. The small sample size and lack of similar surveillance in other captive sun bears make it difficult to conclude the impact of finding the UrHV-1–related virus at the BSBCC and how widespread the virus is in captive and wild sun bears. There is a possibility that this virus was contracted before coming to the BSBCC or that it is present in sun bears in a latent form that could be reactivated under conditions in captivity. Screening future bears before they are integrated into the BSBCC may provide crucial information and is highly recommended. Developing a serology test for UrHV-1 in sun bears will be valuable to determine whether clinically healthy sun bears or those with oral lesions have been exposed to this virus.[18] With the availability of serology testing, samples that we have collected can be tested retrospectively to gain a better understanding of the exposure of sun bears to UrHV-1.

The prevalence of UrHV-1 infection in wild sun bear populations is not known nor are the occurrence of oral lesions. Captivity-related stress may contribute to the development of oral lesions and the transmission of UrHV-1.[18] Finding an UrHV-1–related virus in a rehabilitation center raises concern over the risk of introducing the

virus to the wild population when a bear is released. Future research can incorporate whole genome sequencing and virus characterization to better understand this UrHV-1–related virus in Bornean sun bears. A better understanding of the association of this virus with periodontal diseases, oral lesions, and the development to oral squamous cell carcinoma will be valuable in the effort to understand the overall health impacts of infection with this virus. Monitoring positive bears for lesions is an essential part of understanding what the health implications are to the bears carrying this virus. This will allow for the development of clear guidelines on whether positive bears should be released into the wild. This study attests to the need for continued health surveillance of captive and wild sun bears to define risk factors and prevalence of UrHV-1 as well as conducting studies of pre- and postrehabilitation infection rates to assist rehabilitation centers in carrying out risk assessments for the future release of sun bears and creating better species conservation plans for governmental or nongovernmental organizations working on sun bear conservation.

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