# Notes

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## MOLECULAR IDENTIFICATION OF A NOVEL GAMMA HERPESVIRUS IN THE ENDANGERED HAWAIIAN MONK SEAL (*M0NACHUS SCHAUINSLANDI*)

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The Hawaiian monk seal (*Monachus schauinslandi*) is an endangered species of pinniped, which lives and breeds primarily on the remote Northwestern Hawaiian

Islands in six main subpopulations (Ragen and Lavigne 1999). Even though it has been protected under the U.S. Endangered Species Act since 1976, its numbers continue to decline; currently there are approximately 1,400 individuals in the population (Carreta *et al*. 2004). Recent studies have implicated trauma from entanglement in marine debris, shark attacks, and "mobbing" of females and pups by males, as factors contributing to deaths in Hawaiian monk seals (Banish and Gilmartin 1992). Although infectious diseases including parasitism and bacterial infections have been documented in postmortem findings (Banish and Gilmartin 1992), little is known about the viruses to which monk seals have been exposed or the role that viral diseases may play in their decline. In 1997 a mass mortality occurred among Mediterranean monk seals (*Monachus monachus*) in which almost half the population died. Although both a morbillivirus and saxitoxin were detected in dead seals, it is still unclear which was the primary cause of death in this event, as there are insufficient data on the epidemiology of either the virus or toxin exposure in monk seals (Osterhaus *et al*. 1997, Harwood 1998, Hernandez *et al*. 1998). While there are no reports of viral-associated disease in Hawaiian monk seals, clinical signs compatible with a possible viral etiology have been noted in juvenile monk seals that developed corneal ulcers while undergoing rehabilitation (Aguirre *et al*. 1999).

A recent study has indicated that a unique Hawaiian monk seal herpesvirus may be present in this population (Aguirre 2002). Cross-reactive antiherpes antibodies that were unable to neutralize phocine herpesvirus-1 (PhHV-1) were detected in sera collected from both wild and captive monk seals using an enzyme-linked immunosorbent assay (ELISA) developed to measure exposure to PhHV-1 in harbor seals (King *et al*. 2001, Aguirre 2002). As herpesviruses can cause reproductive failure, morbidity, and mortality, further information on such a virus is needed to determine its impact on Hawaiian monk seals. Furthermore, herpesviruses are important pathogens in other seal species. Phocine herpesvirus-1 was commonly isolated from harbor seals (*Phoca vitulina*) dying from pneumonia along the North Sea (Borst *et al*. 1986) and with adrenocortical necrosis in the Pacific (Gulland *et al*. 1997, King *et al*. 1998). The purpose of this study was to molecularly detect and classify this putative herpesvirus.

A technique for polymerase chain reaction (PCR)-based identification of PhHV-1 in nasal swabs collected from harbor seals (Goldstein *et al*. 2004) was adapted to analyze nasal swab samples collected from captive and free-ranging Hawaiian monk seals. Nasal swab samples were collected during 2000 and 2001 by the National Marine Fisheries Service from 122 apparently clinically healthy Hawaiian monk seals, nine of which were wild born but housed in captivity since 1995 due to an ocular disease that precluded their release from rehabilitation, and 113 that were free-ranging seals of both sexes and all ages from six of the Northwestern Hawaiian Islands (French Frigate Shoals [FFS], Laysan Island, Lisianski Island, Kure Atoll, Midway Atoll, and Pearl and Hermes Reef [PH]). Samples were collected by swabbing the nasal cavity of each animal with a clean cotton-tipped swab. The swabs were then placed into a sterile cryovial and frozen at −70°C until analyzed. DNA was extracted from the samples using previously described methods (Goldstein *et al*. 2004). Parallel reactions were run to confirm the presence of amplifiable DNA using conserved primers to amplify a 300-bp fragment of the seal ferritin gene. We used previously described degenerate

primers to amplify a 725-bp sequence of a conserved region of the herpesviral DNA polymerase gene, follwed by a secondary PCR reaction utilizing nested primers to amplify a 225-bp product of the gene (VanDevanter *et al*. 1996).

Upon acquiring herpesviral sequences from monk seal nasal swab samples, monk seal herpesviral-specific PCR primers were designed to confirm the sequence identity (sense: 5′-GGGAATGTTTCCTTGCGTTAA-3′, antisense: 5′-CCTTAAAACTGGCATCATTCTC-3′). These primers were used in combination with the degenerate primer pair to obtain additional sequence of the monk seal herpesviral DNA polymerase gene. The obtained sequences were then compared among samples from individual monk seals and to four known marine mammal herpesvirus sequences including two gamma herpesvirus isolates, phocine herpesvirus-2 (PhHV-2) from an Atlantic harbor seal, and otarine herpesvirus-1 (OtHV-1) from a California sea lion (*Zalophus californianus*); and two alpha herpesviruses, both PhHV-1 from Atlantic and Pacific harbor seals. Positive PCR products were cloned into a plasmid vector and sequenced by the chain termination method (Sanger *et al*. 1977) using an automated sequencer (Prism, Perkin Elmer, Norwalk, CT). These sequences were then compared to other published herpesviral sequences in the GenBank Database (National Center for Biotechnology Information, National Library of Medicine, Bethesda, MD). The phylogenetic relationship between the newly detected virus and other pinniped herpesviruses was established using the NEIGHBOR program of PHYLIP (Phylogeny Inference Package, version 3.5c, University of Washington, Seattle, WA) and were tested by SEQBOOT and CONSENSE programs to determine the degree of support for the particular tree nodes.

DNA was obtained from nasal swab samples from 95 monk seals. These 95 samples were used for the subsequent PCR analysis. Initial DNA analysis using the degenerate primers identified the viral sequence (225 bp) as a herpesvirus belonging to the gamma herpesvirus subfamily. Using the monk seal-specific primer pair (150 bp), positive PCR products were obtained from samples from 20.0% (19/95) of the seals. These included six of the nine seals in captivity and 13 of the 86 free-ranging animals (Table 1). Positive test results were obtained from almost all sampling locations, both sexes, all age classes, and at all times of the year. A significantly higher proportion of the captive seals tested positive compared to the free-ranging seals for all sampling locations combined ($P < 0.001$, Fisher's exact test). There was no association between age and testing positive ($\chi^2 = 4.53, P = 0.2$); however, a higher proportion of females tested positive compared to males ($\chi^2 = 4.42$, $P = 0.04$). There was no correlation between testing positive for antibodies in the ELISA assay and testing positive with the PCR test (kappa statistic = 0.26). This lack of correlation is not surprising as the ELISA assay was developed to test for exposure to PhHV-1 in harbor seals and the herpesvirus detected in the Hawaiian monk seal population is distinct from PhHV-1.

A 497-bp sequence (Genbank accession number DQ093191) was obtained (using the sense degenerate primer described by VanDevanter *et al*. 1996 and the monk seal herpesviral-specific antisense primer) and used in the subsequent analysis to better identify and classify the molecular isolate. Comparison of the sequence to other known herpesviruses confirmed its classification as a gamma herpesvirus, with similarity to black rhinoceros herpesvirus (62.9% similarity), gorilla rhadinovirus

*Table 1.* Prevalence of herpesviral DNA detected by PCR in nasal swab samples collected from Hawaiian monk seals (*Monachus schauinslandi*) ($n = 95$) by location, age and sex. Percentages of occurrence and sample sizes are indicated.

| | Location | | | | | | | Age | | | | Sex | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Overall prevalence | Captive | French Frigate Shoals | Kure | Laysan | Lisianki | Midway | Pearle & Hermes | Pup (<1 yr) | Juvenile (1–2 yr) | Subdult (3–4 yr) | Adult (>5 yr) | M | F |
| 20.0% (19/95) | 66.7% (6/9) | 11.5% (3/26) | 0% (0/2) | 13.0% (3/23) | 17.7% (3/17) | 23.1% (3/13) | 20.0% (1/5) | 12.9% (4/31) | 15.6% (5/27) | 41.7% (5/12) | 25.0% (5/25) | 10.4% (5/48) | 29.8% (14/47) |

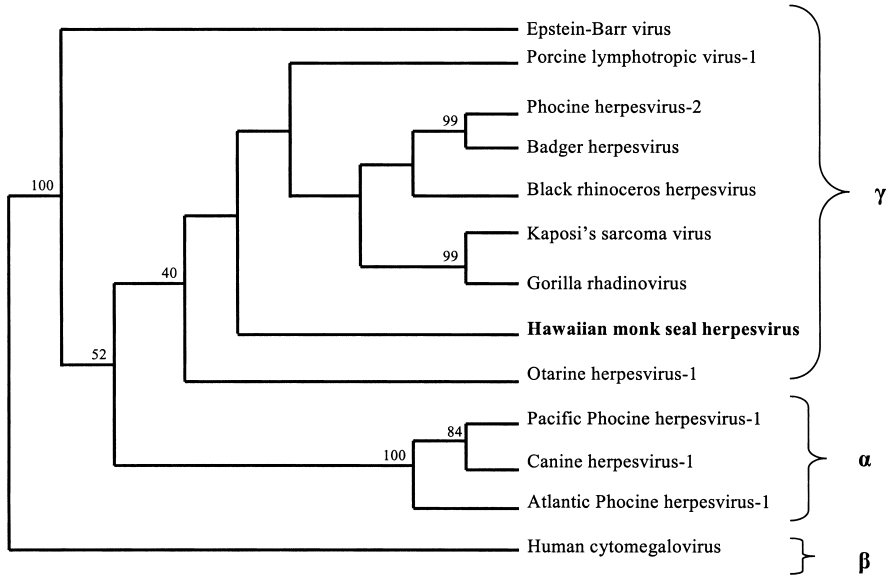


*Figure 1.* Consensus phylogram representing the relationship between the newly identified Hawaiian monk seal herpesvirus (Genbank accession number DQ093191) and other known herpesviruses. Sequences of viruses (accession numbers shown) are: Epstein–Barr virus (V01555), porcine lymphotropic virus-1 (AF191042), phocine herpesvirus-2 (T. Goldstein, unpublished data), badger herpesvirus (AF275657), black rhinoceros herpesvirus (AF287948), Kaposi's sarcoma herpesvirus (NC_003409), gorilla rhadinovirus (AF250886), otarine herpesvirus-1 (AF236050), Pacific phocine herpesvirus-1 (U92269), canine herpesvirus (X89502), Atlantic phocine herpesvirus-1 (T. Goldstein, unpublished data), human cytomegalovirus (AF133597). This analysis was performed using a 497-bp region of the DNA polymerase gene obtained by using the sense degenerate primer described by VanDevanter *et al*. 1996 and the monk seal herpesviral-specific antisense primer. Significant bootstrap values (>70%) or those that designate subfamily branches are shown. Viruses designated as α, β, γ belong to the alpha, beta, and gamma herpesvirus subfamilies, respectively.

(58.2% similarity), badger herpesvirus (57.9% similarity), and porcine lymphotropic virus (43.4% similarity). Comparisons to other known pinniped herpesviruses showed a 43.1% similarity to OtHV-1, 40.5% similarity to PhHV-2, and 39.4% and 38.5% similarity to the PhHV-1 Pacific and Atlantic isolates, respectively (Fig. 1).

In summary, a new molecular herpesviral isolate has been identified in nasal swab samples collected from captive and free-ranging Hawaiian monk seals, belonging to the gamma herpesviral subfamily. Herpesviruses in this subfamily are generally associated with respiratory disease and leukocytosis, and tend to be predominantly lymphotropic (Roizman 1996). Further studies are needed to determine the association of this newly identified herpesvirus with disease. It is interesting to note that 20.0% (19/95) of the samples tested positive for the herpesviral DNA, and that a significantly higher proportion of the seals admitted for rehabilitation and that remained in captivity tested positive than all free-ranging seals combined. As herpesviruses are known to reactivate during periods of stress (Roizman *et al*. 1981, 1992), the

positive tests may be indirect evidence of direct transmission between animals in close proximity or under stressful circumstances (*e.g.*, captivity, concurrent illness). These results demonstrate the presence of the virus in five of the six subpopulations (only two samples were tested from seals from Kure Atoll, thus the sample size was likely too low to determine whether the virus is present in this subpopulation). Therefore, there does not appear to be any risk of introduction of the virus within the population from future seal translocation efforts. However, herpesviruses have been known to cause death and disease particularly in young or stressed marine mammals. Therefore, a further understanding of the natural history of Hawaiian monk seal herpesvirus infection and the ability to detect it may be useful in managing the recovery of this endangered species.

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