



Age-prevalence of Otarine Herpesvirus-1, a tumor-associated virus, and possibility of its sexual transmission in California sea lions

Elizabeth L. Buckles^{a,*}, Linda J. Lowenstine^a, Robert L. DeLong^b, Sharon R. Melin^b, Regina K. Vittore^a, Hong-Nei Wong^a, Gately L. Ross^a, Judith A. St Leger^c, Denise J. Greig, Rebecca S. Duerr^a, Frances M.D. Gulland^d, Jeffery L. Stott^a

^a Department of Pathology, Microbiology and Immunology, School of Veterinary Medicine, One Shields Avenue, University of California, Davis, CA 95616, USA

^b National Marine Mammal Laboratory, Alaska Fisheries Science Center, National Marine Fisheries Service, Seattle, WA 98115, USA

^c SeaWorld San Diego, 500 Sea World Drive, San Diego, CA 92101, USA

^d The Marine Mammal Center, Marin Headlands, 1065 Fort Cronkhite, Sausalito, CA 94965, USA

Received 27 January 2006; received in revised form 19 September 2006; accepted 4 October 2006

Abstract

Otarine Herpesvirus-1 (OthV-1) is a gammaherpesvirus routinely detected in urogenital tumor tissues of adult sea lions dying during rehabilitation. To investigate the epidemiology of this virus and guide the development of a mathematical model of its role in the multifactorial etiology of cancer in California sea lions, polymerase chain reaction (PCR) amplification of an OthV-1 specific fragment of the DNA polymerase gene was used to look for evidence of OthV-1 infection in urogenital and pharyngeal swabs and peripheral blood mononuclear cells (PBMC) of sea lions of different ages. Samples were also examined from pregnant females and their late term *in utero* or aborted fetuses to investigate potential for vertical transmission. Prevalence of infection in 72 adult females was 22%, whereas it was 46% in 52 adult males, and was significantly lower in 120 juvenile animals (6%). OthV-1 DNA was most often detected in the lower reproductive tract of the adult animals, especially the males, and rarely in the pharynx or urogenital tract of juvenile animals. These data suggest sexual transmission may be an important route of transmission. Additional studies are required to confirm this mode of transmission. Additionally, the virus was detected in a single prematurely born pup, suggesting the possibility of perinatal transmission. No indication of a PBMC associated viremia was evident in adults using standard PCR or in juveniles using standard and real time PCR.

© 2006 Elsevier B.V. All rights reserved.

Keywords: California sea lion; *Zalophus californianus*; Gammaherpesvirus; Epidemiology; Otarine Herpesvirus-1 (OthV-1)

* Corresponding author. Present address: Department of Biomedical Sciences, Cornell University, Ithaca, NY 14853, USA. Tel.: +1 607 253 3826.

E-mail address: elb36@cornell.edu (E.L. Buckles).

1. Introduction

Otarine Herpesvirus-1 (OtHV-1), a member of the subfamily *Gammaherpesvirinae*, genus *Rhadinovirus*, was first identified in association with urogenital carcinomas in adult California sea lions (*Zalophus californianus*) (Lipscomb et al., 2000; King et al., 2002). These cancers are diagnosed post mortem in about a fifth of both male and female stranded sexually mature sea lions examined (Gulland et al., 1996). Tumor cells in some of the cases contained intranuclear inclusion bodies, and herpesviral virions were detected by electronmicroscopy (Gulland et al., 1996; Lipscomb et al., 2000). Furthermore, all sea lion urogenital tumors examined to date have contained amplifiable fragments of OtHV-1 specific DNA (Lipscomb et al., 2000; King et al., 2002; Buckles et al., in press). These data indicate a strong association between viral infection and tumor presence; however, the epidemiology of the virus in the sea lion population has not been investigated. Gamma herpesvirus pathogenesis and transmission can be complex. Gammaherpesviruses are often shed from mucous membranes and undergo latency in circulating leukocytes (Corey, 2002). Thus, to establish important sites of viral infection and latency for OtHV-1, DNA from pharyngeal and urogenital swabs, and peripheral blood mononuclear cells was examined. Furthermore, because some herpesviruses are vertically transmitted, tissues from pregnant females and their fetuses or premature pups were evaluated for the presence of OtHV-1 DNA.

Other factors, such as contaminant exposure, inbreeding and MHC genotype are also associated with the development of cancer in California sea lions (Acevedo et al., 2003; Bowen et al., 2004; Ylitalo et al., 2005). To explore the interactions amongst these factors, a mathematical model is currently in development. The aim of this paper is to present data on the epidemiology of OtHV-1 and discuss the factors that may influence viral prevalence in the sea lion population.

2. Materials and methods

Prevalence of OtHV-1 in adult and immature animals was explored by capturing, sampling, and

releasing animals on study sites in California and Washington. Age class was determined by standard body length (length from nose tip to tail tip), stage of sagittal crest development, and, in the case of smaller females, observation of breeding behavior. A survey of records at The Marine Mammal Center (TMMC, Sausalito, California) established that standard lengths > 143 cm for females and > 192 cm in males correlated with sexual maturity (Greig et al., in press). Adult female sea lions ($N = 47$), pups and immature individuals, ranging in age from 1 month to more than a year old ($N = 108$) were sampled just prior to and after the pupping season (April, May, October) while on the rookeries of San Miguel Island, California. Males ($N = 56$) from the California breeding populations were sampled in Puget Sound, Washington in April and October (prior to and after the breeding season). These migrant males are from the five island populations in California, including San Miguel Island. Four males were judged as immature based upon total length measurements. A single adult female was capture and sampled in Puget Sound. An additional 26 adult females, 12 juveniles and a single adult male stranding along the coast of California due to acute illness (i.e. intoxication or trauma) were also examined. Samples were taken from these animals on admission for rehabilitation to minimize the effects of captivity on viral shedding.

DNA samples were taken from all animals using a floating trap and squeeze cage for the males, and manual restraint for juveniles and females, followed by isoflourane anesthesia (Heath et al., 1997). Mucosal samples of the oropharynx, and vagina or prepuce, were taken with sterile cotton tipped-swabs and blood was collected via the caudal gluteal vein. Vaginal samples were obtained with the use of a plastic speculum to minimize fecal contamination. Peripheral blood mononuclear cells (PBMC) were separated from whole blood as previously described. (Bowen et al., 2002). All samples were initially held at -20°C and then stored at -80°C until analysis. After sample collection, the animals were allowed to recover and were returned to the beaches or open water, or to rehabilitation facilities in the case of stranded animals.

To determine whether there was evidence of vertical transmission of OtHV-1, 26 adult female animals and their late term fetuses were examined.

The females had been presented to rehabilitation centers in central and southern California after becoming intoxicated by domoic acid, an algal toxin that may result in stillbirth and abortion (Gulland et al., 2002). Nine of the adult females died as a result of the toxin, and thus tissue from multiple organs were available for herpesvirus evaluation from these animals. These tissues included salivary gland ($N = 9$), retropharyngeal lymph node ($N = 9$), tonsil ($N = 9$), skeletal muscle ($N = 1$) lung ($N = 9$), liver ($N = 9$), spleen ($N = 9$), kidney ($N = 9$), lumbar node ($N = 9$), ovary ($N = 9$), uterus ($N = 9$), cervix ($N = 9$), vagina ($N = 9$) and trigeminal ganglion ($N = 3$). Eighteen adult females survived the intoxication, and only DNA samples from vaginal swabs were taken. All 26 fetuses were dead; 9 were collected during necropsy of the mother and 17 were stillborn or died shortly after abortion. Tissues collected from the fetuses, conjunctiva ($N = 22$), retropharyngeal lymph node ($N = 22$), tonsil ($N = 23$), thymus ($N = 22$), spleen ($N = 25$), vagina/prepuce ($N = 23$), placenta ($N = 15$) and umbilicus ($N = 23$) represent probably routes of viral entry and are known to be sites of herpesvirus infection in neonates of other species. Additionally, salivary gland ($N = 4$), skeletal muscle ($N = 1$), liver ($N = 4$), kidney ($N = 11$), lumbar node ($N = 4$), urinary bladder ($N = 4$), gonad ($N = 2$), cervix ($N = 2$) and trigeminal ganglion ($N = 2$) were collected from some fetuses. DNA samples from urogenital swabs (vagina and prepuce) of an additional 38 dead premature pups, found on the rookeries during the breeding season, were also available for PCR analysis. Herpesvirus status as determined from vaginal samples, was known for five of the mothers of these pups.

DNA was extracted from mucosal swabs and lymphocytes using the Qiagen, DNeasy Tissue (Qiagen Inc., Valencia, California) kit with the following modifications: sample lysis overnight at 55 °C and final elution using two washes with 50 ml EB solution. Due to the small amount of DNA in these samples, DNA concentrations were not normalized, rather samples were used at full elution concentration for PCR analysis. DNA was extracted from fresh frozen tissues either as described by Lockridge et al. (1999) or by use of a commercially available phase lock gel DNA purification system (Eppendorf, Westbury, New York) per manufacturer's instructions. The

tissue digestion steps of both protocols were modified to include an alpha-amylase (Sigma, St. Louis, Missouri) treatment (10% by volume for 1–2 h, 37 °C) in order to remove proteoglycans present in marine mammal tissues. Tissue DNA was normalized to 50 ng/ml using a GeneQuant II spectrophotometer (Amersham Biosciences, Pharmacia Biotech, and Piscataway, New Jersey).

OtHV-1 primers specific for a 697 bp fragment of the DNA polymerase gene (for 5'-TTACTTCTTACGTGATGG-3', and rev 5'-CA ATGATACTGGACGAAGA) were designed based on the published sequence (GenBank accession # AF236050). Reactions were performed at a 25 μ l final volume using 2.5 mM Mg concentration (Epicentre Master Mix B, Madison, Wisconsin) and Qiagen HotStar Taq (Qiagen Inc., Valencia, California) for 35 cycles on a PTC 200 Peltier Thermal Cycler (MJ Research, Waltham, Massachusetts). Cycles were composed of 40 s at 94 °C, 40 s at 60 °C and 40 s at 72 °C. A final elongation step of 10 min at 72 °C was performed after the completion of the amplification cycles. PCR products were resolved via electrophoresis through a 1.5% ethidium bromide stained agarose gel. PCR sensitivity was established by cloning the entire published OtHV-1 DNA polymerase sequence into a plasmid using the Topo TA vector kits (Invitrogen Technologies, Carlsbad, California). PCR performed on serial dilutions of these plasmid constructs determined that the reaction could detect a minimum of 256 copies of the gene. As there was neither a way to quantify the amount of virus in the tissues, nor could OtHV-1 be grown and quantified in culture, the plasmid experiment was used as a model to approximate the sensitivity of PCR detection in the fresh tissue. Sequencing of PCR product from 10 positive sea lions was performed using an ABI 3730 capillary electrophoresis genetic analyzer (Applied Biosystems, Foster City, California) to ensure specificity of the amplification (sequencing performed by the University of California, Davis, Division of Biological Sciences Sequencing Facility).

To demonstrate amplifiable DNA in the extracts, and decrease the probability of false viral negatives, PCR was performed using either sea lion specific interleukin-2 primers (Genbank accession # AY354470) or mammalian ferritin primers developed in our lab (Genbank accession # AF246195). Since

both these genes should be found in all sea lions, a positive result indicated that the sample DNA was of satisfactory quality for PCR. Reactions were performed under similar conditions to those described for the DNA polymerase amplicon.

Peripheral blood mononuclear cells were examined using real time quantitative PCR in order to detect low copy numbers of latent virus. PBMC DNA was available from fewer than 50% of the animals captured during this study, but archived frozen PBMCs were obtained from Dr. Brad Fenwick and Maureen Ridder at the Kansas State University, College of Veterinary Medicine (Manhattan, Kansas). These animals were 4 months old at the time of initial sampling. Additional samples of 30 of these individuals at 6 months old and 12 of these animals at 11 months old were also analyzed to give a longitudinal illustration of OtHV-1 status.

Real time quantitative PCR (qPCR) utilized primers specific for a 65 bp segment of the OtHV-1 DNA polymerase gene designed based on the published sequence of the OtHV-1 polymerase gene (for 5'-GCGGGAACGCAACTATATCCT-3' rev 5'-TCCAAGTGGCTACCAGGGAG-3') and optimized to work at similar efficiency to the sea lion GAPDH (for 5'-TGAGAACGGGAAGCTTGTC-3' rev 5'-GACAATCTTGAGGGAGTTGTCA-3'), a single copy number genomic sequence.

Standard curves to quantify gene copy numbers were established by cloning target sequences of OtHV-1 DNA polymerase and GAPDH sequence into a plasmids (Topo TA vector kits, Invitrogen Technologies, Carlsbad, California) and performing real time reactions on serial plasmid dilutions containing known gene copy numbers. The resultant standard curves were used to correlate Ct value (the number of cycles after which the exponential phase of the replication cycle begins) and copy number of the target sequence (Niesters, 2001). Reactions were performed using the Qiagen Sybreen quantitative PCR system per manufacturer's instructions (Qiagen Inc., Valencia, California) on a Biorad I-cycler Real Time PCR machine (BIO-RAD Laboratories, Hercules, California). Reaction conditions were similar to those used for the standard PCR but included a data cycle at the end of the program. Three replicates of each reaction were performed and the mean and standard deviation of Ct value for each dilution were calculated.

Quantitative PCR reactions on test samples were performed in 25 μ l final volumes and three replicates per sample were used to calculate Ct value. Number of cells examined was established based on standard curve analysis of the GAPDH gene and number of viral copies per cell was calculated using the standard curve generated for OtHV-1 viral polymerase gene. Melt curve analysis was used to validate the specificity of the reactions. Plasmid constructs (see above) were used as positive controls on each reaction plate.

Mean, standard deviation, χ^2 values and p values were calculated using Sigma Plot version 8 (Aspire Software International, Leesburg Virginia) and EpiInfo 2002 version 3.2.1 (United States Centers for Disease Control, Epidemiology Program Office, Atlanta, Georgia). Results were considered significant if $p < 0.05$.

3. Results

Evaluation of the DNA samples from the adult animals revealed a distinct sex-related difference in the prevalence of OtHV-1 DNA. Detection frequency of OtHV-1 in genital swabs from free-ranging or acutely stranded female sea lions was 22.2% (16/72). OtHV-1 was present in the reproductive tract of 46% of adult males ($N = 26/54$). This prevalence was significantly higher than in the adult females ($\chi^2 = 6.72$, $p < 0.05$) (Table 1). Pharyngeal DNA was available from 95 free ranging adult animals (44 females, 51 males). OtHV-1 was detected in one male with concurrent prepuccial infection (Table 1). OtHV-1 was not detected in any samples of circulating PBMCs from free ranging adult sea lions ($N = 60$, 21 F, 39 M). Dilution analysis showed that this PCR technique could detect as few as 256 copies of OtHV-1 DNA per sample.

There was also a significant difference between the prevalence of OtHV-1 in immature animals and that in the adult animals ($\chi^2 = 40.6$). One hundred and twenty sexually immature animals were collected, sampled and released. This study population consisted of pups under one month old ($N = 8$), pups between 4 and 10 months old ($N = 70$) and 42 juveniles older than 1 year. Overall, OtHV-1 was detected in a total of seven animals (5.8%). Viral DNA was amplified from the urogenital tract of a single pup under one month old, in

Table 1

The prevalence of OtHV-1 in urogenital, pharyngeal and PBMC samples from free ranging sea lions captured on San Miguel Island (SMI), Puget Sound (PS) and acutely stranded sea lions sampled at The Marine Mammal Center (TMMC) positive for OtHV-1

	OtHV-1 in adult sea lions					
	Urogenital swabs		Pharyngeal swabs		Lymphocytes (PBMC)	
	<i>N</i>	#OtHV-1+	<i>N</i>	#OtHV-1+	<i>N</i>	#OtHV-1+
Female (<i>N</i> = 72)						
TMMC	26	7 (27.0%)	0	0	0	0
SMI	45	9 (19.6%)	44	0	21	0
PS	1	0	1	0	0	0
Total	72	16 (22.2%)	45	0	21	0
Male (<i>N</i> = 61)						
TMMC	1	0	0	0	0	0
PS	59	26 (44.1%)	51	1 (2.0%)	39	0
Total	60	26 (43.3%)	51	1 (2.0%)	39	0

The parenthetical *N* value after the age in column 1 indicates the number of animals captured and sampled for the category. The *N* value for each sample type indicates the number of samples for which adequate DNA was available for analysis.

the urogenital tract of a single pup between 4 and 10 months old, the urogenital tract of four animals over 1 year and the pharynx of one animal greater than 1 year old (Table 2).

Quantitative PCR revealed no evidence of PBMC associated viremia in the immature sea lions tested. This assay proved to be much more sensitive than standard PCR. Standard curve generation showed that the PCR assay could detect a minimum of 86 copies of the target OtHV-1 polymerase sequence compared to 256 copies detectable with standard PCR. Blood cells from 42 juvenile animals were examined and, using the GAPDH standard curve, it was determined that an average of 4.2×10^8 cells were examined per animal. No OtHV-1 was detected in any animal. One replicate

from three animals revealed a weak signal at a high threshold crossing. However, when analyzed on a 1.5% agarose gel, the resultant product produced an indistinct band, which could not be cloned into *E. coli*. Additionally, analysis of these samples with qualitative PCR and primers targeting a longer, more specific portion of the polymerase gene revealed no evidence of OtHV-1 DNA. Thus, this signal was probably a false positive.

OtHV-1 DNA was found in the vagina of one of the 38 (2.6%) premature pups found on the rookery. This pup's mother had been captured and sampled for the prevalence portion of this study and OtHV-1 DNA was detected in her vagina. Examination of tissues or fetal membranes from the dead pups of mothers presented

Table 2

Prevalence of OtHV-1 DNA samples from the urogenital tract, pharyngeal area and PBMCs of non-sexually mature individuals

Age	Prevalence of OtHV-1 in immature California sea lions					
	Urogenital swabs		Pharyngeal swabs		Lymphocytes (PBMC)	
	<i>N</i>	#OtHV-1+	<i>N</i>	#OtHV-1+	<i>N</i>	#OtHV-1+
<1 month (<i>N</i> = 8)	8	1 (12.5%)	7	0	0	0
4–10 months (<i>N</i> = 70)	67	1 (1.5%)	62	0	20	0
>1 year (<i>N</i> = 42)	41	4 (9.8%)	25	1 (4%)	23	0
Total	116	6 (5.2%)	104	1 (0.1%)	43	0

Prevalence is significantly less than that seen in adult animals. The parenthetical *N* value after the age in column 1 indicates the number of animals captured and sampled for the category. The *N* value for each sample type indicates the number of samples for which adequate DNA was available for analysis.

at rehabilitation revealed no evidence of OtHV-1. OtHV-1 DNA was detected in three of the dams (11%) sampled for this part of the study, in a single sample from each (uterus, cervix and vaginal swab).

4. Discussion

The results of this study provide important insight into the ecology of OtHV-1 infection of California sea lions and also suggest possible modes of viral transmission. The higher prevalence of OtHV-1 DNA in the adults, especially males, (22.2% for females and 46% for males) than in the sexually immature animals (5.8%) and the detection of the virus most often in urogenital secretions suggest viral transmission occurs via sexual activity. These findings are consistent with epidemiological information available on HHV-8, a closely related oncogenic gammaherpesvirus endemic in certain human populations. Serologic and PCR studies document that prevalence of endemic HHV-8 infection increases after the onset of puberty and sexual activity, and viral DNA can be detected in the prostatic and vaginal secretions (Hengge et al., 2002; de Sanjose et al., 2002; Eltom et al., 2002). Based on this information, it is believed that sexual activity serves as the main method by which HHV-8 is maintained in some populations. The differences in viral prevalence between adult male and female sea lions are also mirrored in the epidemiology of HHV-8. In human populations with endemic HHV-8 infection, the virus is most prevalent in males and correlates with the number of sexual partners per individual (de Sanjose et al., 2002; Eltom et al., 2002). The data presented here do suggest that sexual transmission is a mode of OtHV-1 transmission, however, additional studies looking for viral structural genes, and gene expression are needed in order to confirm this finding.

Despite detailed epidemiological studies on HHV-8, much of the viral life cycle remains obscure. For instance, detection of the virus in genital secretions points to sexual transmission, but this has only been proven for homosexual encounters. Viral DNA is also found in saliva of infected individuals and the relative roles of oral rather than genital contact in heterosexuals are unclear (Eltom et al., 2002). Further exploration into the epidemiology of OtHV-1,

combined with knowledge of the animal's breeding behavior may lend clues to the mechanisms by which sex specific differences develop. As in humans, the breeding behavior of male and female sea lions is different. The polygynous breeding strategy of the sea lions results in successful breeding male sea lions having at least twice the lifetime genital contacts of females (DeLong, Melin, unpublished), hence, potentially more opportunities to acquire OtHV-1. However, some adult males are excluded from breeding by this social system, limiting probability of venereal transmission of OtHV-1. Thus, although number of sexual contacts may play a role in determining the sex-specific viral prevalence, other factors are likely involved.

It is possible that the different prevalence in adult males and females observed in this study resulted from non-random sampling. There was not a significant difference in prevalence of OtHV-1 between females sampled on the San Miguel rookery (20%) and those stranded on the California coast (27%) (Fisher's exact test $p = .56$). All of the adult males sampled in Puget Sound were permanently marked and represent migrants from up to five breeding populations in Southern California. Subsequent observations have documented that 16 of the 52 marked adult males have been observed on San Miguel Island during the summer breeding season through 2005 (Melin, DeLong, unpublished data). Seven of those 16 males (44%) were positive for OtHV-1. This proportion is clearly not different from the 46% prevalence in the total adult male sample. Thus, it appears that the higher prevalence in adult males is real and not an artifact of sampling.

On a cellular level, the difference in infection prevalence may be a result of different microenvironments in male and female lower genital tracts. *In vitro* studies have shown human prepuccial cells to be more susceptible to HIV-1 infection than cervical cells, and hormonally mediated fluxes in vaginal immunity can also alter cellular permissiveness to viral infections (Brabin, 2002; Patterson et al., 2002). Additionally, differences in exposure to anthropogenic chemicals may predispose male sea lions to viral infection. Many of the contaminants found in sea lion tissues and in association with tumors are known to be immunosuppressive, as well as oncogenic, are found at higher levels in males compared to females, and could

increase the susceptibility of the males to OtHV-1 infection (Hong et al., 1998; Gauthier et al., 1999; Ylitalo et al., 2005).

Although sex specific behavioral and cellular differences may account for the differences in prevalence of the OtHV-1 and HHV-8 in adults, they cannot account for the low levels of infection seen in immature sea lions and children. OtHV-1 is found oral and genital secretions of occasional juveniles. HHV-8 infection is found in some children exhibiting mild illness and there is a strong correlation between the infection status of mothers and children and between siblings residing in the same household (Andreoni et al., 2002; Cook et al., 2002). It is thought that the virus must be transmitted via an oral/nasal route in these cases. This theory might also apply to the sea lions, since pups and juveniles are exposed to infected vaginal secretions at birth and saliva during close contact on the rookery. OtHV-1 was detected in oral secretions of rare individuals and in the urogenital secretions of one premature pup born to an OtHV-1 infected mother. It is not known if this individual was infected with the virus or simply covered with infected vaginal secretions from the dam. Nonetheless, it does indicate that perinatal viral exposure does occur and could lead to infection of at least a small number of non-sexually mature individuals.

One significant difference between OtHV-1 and HHV-8 is that thus far, there is no evidence of a PBMC associated viremia with OtHV-1. Since OtHV-1 could have established a latent, low copy number infection in PBMCs a highly sensitive quantitative PCR was used to examine these samples. Thus, the more sensitive qPCR was used to screen PBMCs of juvenile animals for low copy number infection (Corey et al., 2002). Even with the increased sensitivity of this test, no PBMC associated viremia was evident in any animal. The false positive reactions found in four individuals were likely the result of the small target sequence (Niesters, 2001). Further studies to detect for viremia are needed. TaqMan real time PCR, might prove to be a more sensitive and specific assay and could further clarify the role viremia may play in viral pathogenesis (Niesters, 2001).

Taken together, the results of this study begin to elucidate the ecology of OtHV-1 in sea lions. Moreover, the epidemiologic parallels between OtHV-1 and HHV-8 are intriguing. Given the paucity

of experimental data on naturally occurring gamma-herpesvirus infections further studies into OtHV-1 could contribute important insights into gammaherpesvirus biology. This is particularly relevant in so much that OtHV-1 is associated with neoplasia, and as top of the food chain predators, sea lions are exposed to many of the same environments as humans. Development of a serologic test for OtHV-1, exploration of the viral genome and further studies into sea lion genetics and toxin exposure will be invaluable in determining how gammaherpesviruses are circulated in a populations and how multiple factors may effect viral prevalence and potentially contribute to cancer development.

References

- Andreoni, M., Sarmati, L., Nicastrì, E., El Sawaf, G., El Zabalani, M., Uccella, I., Bugarini, R., Parisi, S.G., Rezza, G., 2002. Primary human herpesvirus 8 infection in immunocompetent children. *J. Am. Med. Assoc.* 287, 1295–1300.
- Bowen, L., Aldridge, B.M., Gulland, F., Woo, J., Van Bonn, W., DeLong, R., Stott, J.L., Johnson, M.L., 2002. Molecular characterization of expressed DQA and DQB genes in the California sea lion (*Zalophus californianus*). *Immunogenetics* 54, 332–347.
- Brabin, L., 2002. Interactions of the female hormonal environment, susceptibility to viral infections, and disease progression. *AIDS Patient Care STDS* 16, 211–221.
- Buckles, E.L., Lowenstine, L.J., Funke, C., Vittore, R.K., Wong, H. N., St Leger, J.A., Greig, D., Duerr, R., Gulland, F.M.D., Stott, J.L., in press. Otarine Herpesvirus-1, not Papillomavirus, is associated with endemic tumors in California Sea Lions (*Zalophus californianus*). *J. Comp. Pathol.*
- Cook, R.D., Hodgson, T.A., Waugh, A.C., Molyneux, E.M., Borgstein, E., Sherry, A., Teo, C.G., Porter, S.R., 2002. Mixed patterns of transmission of human herpesvirus-8 (Kaposi's sarcoma-associated herpesvirus) in Malawian families. *J. Gen. Virol.* 83, 1613–1619.
- Corey, L., Brodie, S., Huang, M.L., Koelle, D.M., Wald, A., 2002. HHV-8 infection: a model for reactivation and transmission. *Rev. Med. Virol.* 12, 47–63.
- de Sanjose, S., Marshall, V., Sola, J., Palacio, V., Almirall, R., Goedert, J.J., Bosch, F.X., Whitby, D., 2002. Prevalence of Kaposi's sarcoma-associated herpesvirus infection in sex workers and women from the general population in Spain. *Int. J. Cancer* 98, 155–158.
- Eltom, M.A., Mbulaiteye, S.M., Dada, A.J., Whitby, D., Biggar, R.J., 2002. Transmission of human herpesvirus 8 by sexual activity among adults in Lagos. *Nigeria Aids* 16, 2473–2478.
- Gauthier, J.M., Dubeau, H., Rassart, E., Jarman, W.M., Wells, R.S., 1999. Biomarkers of DNA damage in marine mammals. *Mut. Res.* 444, 427–439.

- Greig, D.J., Gulland, F.M.D., Kreuder, C., in press. A decade of live California sea lion (*Zalophus californianus*) strandings along the central California coast: causes and trends, 1991–2000. *Aquat. Mammals*.
- Gulland, F.M., Trupkiewicz, J.G., Spraker, T.R., Lowenstine, L.J., 1996. Metastatic carcinoma of probable transitional cell origin in 66 free-living California sea lions (*Zalophus californianus*), 1979 to 1994. *J. Wildlife Dis.* 32, 250–258.
- Gulland, F.M.D., Haulena, M., Fauquier, D., Langlois, G., Lander, M.E., Zabka, T., Duerr, R., 2002. Domoic acid toxicity in Californian sea lions (*Zalophus californianus*): clinical signs, treatment and survival. *Vet. Rec.* 150, 475–480.
- Heath, R.B., DeLong, R., Jameson, V., Bradley, D., Spraker, T., 1997. Isoflurane anesthesia in free ranging sea lion pups. *J. Wildlife Dis.* 33, 206–210.
- Hengge, U.R., Ruzicka, T., Tying, S.K., Stuschke, M., Roggendorf, M., Schwartz, R.A., Seeber, S., 2002. Update on Kaposi's sarcoma and other HHV8 associated diseases. Part 1: epidemiology, environmental predispositions, clinical manifestations, and therapy. *Lancet Infect. Dis.* 2, 281–292.
- Hong, C.S., Xiao, J., Bush, B., Shaw, S.D., 1998. Environmental occurrence and potential toxicity of planar, mono-, and di-ortho polychlorinated biphenyls in the biota. *Chemosphere* 36, 1637–1651.
- King, D.P., Hure, M.C., Goldstein, T., Aldridge, B.M., Gulland, F.M., Saliki, J.T., Buckles, E.L., Lowenstine, L.J., Stott, J.L., 2002. Otarine herpesvirus-1: a novel gammaherpesvirus associated with urogenital carcinoma in California sea lions (*Zalophus californianus*). *Vet. Microbiol.* 86, 131–137.
- Lipscomb, T.P., Scott, D.P., Garber, R.L., Krafft, A.E., Tsai, M.M., Lichy, J.H., Taubenberger, J.K., Schulman, F.Y., Gulland, F.M., 2000. Common metastatic carcinoma of California sea lions (*Zalophus californianus*): evidence of genital origin and association with novel gammaherpesvirus. *Vet. Pathol.* 37, 609–617.
- Lockridge, K.M., Sequer, G., Zhou, S.S., Yue, Y., Mandell, C.P., Barry, P.A., 1999. Pathogenesis of experimental rhesus cytomegalovirus infection. *J. Virol.* 73, 9576–9583.
- Niesters, H.G.M., 2001. Quantitation of viral load using real-time amplification techniques. *Methods* 25, 419–429.
- Patterson, B.K., Landay, A., Siegel, J.N., Flener, Z., Pessis, D., Chaviano, A., Bailey, R.C., 2002. Susceptibility to human immunodeficiency virus-1 infection of human foreskin and cervical tissue grown in explant culture. *Am. J. Pathol.* 161, 867–873.
- Ylitalo, G.M., Stein, J.E., Hom, T., Johnson, L.L., Tilbury, K.L., Hall, A.J., Rowles, T., Greig, D., Lowenstine, L.J., Gulland, F.M.D., 2005. The role of organochlorines in cancer-associated mortality in California sea lions (*Zalophus californianus*). *Mar. Pollut. Bull.* 50, 30–39.