Available online at www.sciencedirect.com

SCIENCE DIRECT®

ELSEVIER

Tissue Distribution of Phocine Herpesvirus-1 (PhHV-1) in Infected Harbour Seals (*Phoca vitulina*) from the Central Californian Coast and a Comparison of Diagnostic Methods

T. Goldstein, J. A. K. Mazet*, L. J. Lowenstine[†], F. M. D. Gulland, T. K. Rowles[‡], D. P. King[§], B. M. Aldridge[†] and J. L. Stott[†]

The Marine Mammal Center, 1065 Fort Cronkhite, Marin Headlands, Sausalito, California 94965, [†]Department of Pathology, Microbiology and Immunology and [*]Wildlife Health Center, School of Veterinary Medicine, One Shields Ave, University of California, Davis, California, 95616 [‡]Office of Protected Resources, National Marine Fisheries Service, 1315 East-West Highway no.13736, Silver Spring, Maryland 20910, USA and [§]Institute for Animal Health, Ash Road, Pirbright, Surrey GU24 0NF, UK

Summary

The polymerase chain reaction (PCR) was used to determine the tissue distribution of phocine herpesvirus-1 (PhHV-1) DNA in 20 stranded Pacific harbour seals (17 pups and three seals older than one year) that died during rehabilitation. The aim was to begin to define stages of infection and to investigate the relation between the presence of PhHV-1 in tissues, histological lesions and serology. PhHV-1 DNA was detected in a wide range of tissues from 10/17 pups and 3/3 subadults or adults. Different clinical patterns emerged from the examination of ante- and post-mortem samples. These patterns probably represented pups with active PhHV-1 infection, pups recovering from infection, and older harbour seals with chronic, reactivated infection. As PhHV-1 DNA was detected in tissues in the absence of typical histological lesions in seven seals and in the absence of PhHV-1 specific antibodies in four seals, it is clear that both histological examination and serology underestimate the presence of infection. These results showed that infection can occur in the absence of obvious disease and that seroconversion may be associated with clinical recovery.

© 2005 Elsevier Ltd. All rights reserved.

Introduction

Phocine herpesvirus-1 (PhHV-1) was first isolated in 1985 from Atlantic harbour seal pups (*Phoca vitulina vitulina*) dying with pneumonia at a rehabilitation centre in The Netherlands (Osterhaus *et al.*, 1985). Clinical signs included nasal discharge, inflammation of the oral mucosa and general depression. Histopathological examination revealed interstitial pneumonia with mononuclear infiltrates and hepatic necrosis (Borst *et al.*, 1986). In 1996, a virus with similar antigenic properties and nucleotide sequence was isolated from Pacific harbour seal pups (*Phoca vitulina richardsii*) dying at a rehabilitation centre on the central Californian coast (Gulland *et al.*, 1997). Clinical signs in these pups included depression, failure to regulate body temperature, decreased body weight and seizures, but did not include the upper respiratory signs seen in The Netherlands. Histological lesions in pups in California most commonly consisted of multifocal adrenocortical necrosis, typically within the zona fasciculata, sometimes accompanied by random foci of hepatic necrosis and often (46% of

cases) associated with intranuclear inclusion bodies (Gulland *et al.*, 1997). Concurrent bacterial infections associated with interstitial (septicaemic) pneumonia, omphalophlebitis and meningitis were also common in affected pups, but inclusion bodies were not seen in the lungs, umbilicus or meninges. Other than the reported gross and histological lesions (Borst *et al.*, 1986; Gulland *et al.*, 1997), little is known regarding the distribution of PhHV-1 in tissues of infected harbour seals.

Antigenic and nucleotide characterization revealed that both the Atlantic and Pacific isolates of PhHV-1 belonged to the alpha herpesvirinae and were similar to each other (Frey *et al.*, 1989; King *et al.*, 1998). These seal isolates showed particularly close relationship to canine herpesvirus-1 (CHV-1), followed by feline herpesvirus-1 (FHV-1) (Lebich *et al.*, 1994; King *et al.*, 1998). Both CHV-1 and FHV-1 cause mild upper respiratory tract disease in young animals, infections tending to be subclinical in older animals (Gaskell and Willoughby, 1999). However, CHV-1 infections in neonatal dogs may cause widespread necrosis and death (Carmichael and Greene, 1990).

In North America, the prevalence of PhHV-1 antibodies in serum, albeit low in newborn harbour seals, was found by Goldstein *et al.* (2003) to become widespread with age. Conversely, young harbour seals were much more likely than subadult or adult animals to have detectable PhHV-1 viral DNA in mononuclear leucocytes or oropharyngeal swab samples (Goldstein *et al.*, 2004); however, not all pups with detectable viral DNA developed clinical disease. King *et al.* (2001) reported that in a PhHV-1 outbreak in Pacific harbour seals the majority of pups that died with herpesviral lesions had no measurable serum antibodies immediately before death. Thus, the relationship between antibody production, tissue distribution of the virus and clinical disease would seem to warrant further investigation.

In addition, published information on the relationship between PhHV-1-associated lesions and the distribution of virus in tissues is lacking. In young dogs the histological demonstration of typical CHV-1 lesions is usually diagnostic; less specific changes, however, such as those sometimes seen in newborn or older animals, may necessitate the use of more sensitive methods of detecting the virus (Schulze and Baumgärtner, 1998). The polymerase chain reaction (PCR) has been used to detect herpesviral DNA in tissues in other species (Reubel *et al.*, 1993; Burr *et al.*, 1996; Taouji *et al.*, 2002). A main objective of the present study was to use the PCR to evaluate the distribution of PhHV-1 DNA in tissues from neonatal and adult harbour seals as a contribution to defining the different stages of infection. Additionally, the study examined the relationship between PhHV-1 viral DNA in tissues, serum antibodies, and histological lesions.

Materials and Methods

Animals and Samples

Samples were collected between March 1999 and September 2001 from 20 harbour seals found stranded on the central Californian coast (37°42′ to 35° 59′ N, 123° 05′ to 121° 30′ W). The stranded animals were taken to a rehabilitation centre (The Marine Mammal Center, Sausalito, CA, USA), where they died. Upon admission all animals were weighed, measured, examined and classified (premature pup, full-term pup, weaned pup, subadult or adult). Pups were aged by the presence of a lanugo coat, presence of an attached umbilical cord, and by comparing their dates of admission with the known timing of pupping for the region. In California, pups are typically born between March and May, although premature births occur as early as February; the majority of newborn animals are weaned by the end of June (Bigg, 1969a; Boulva and McLaren, 1979; Tempte *et al.*, 1991). Subadults and adults were aged on the basis of standard length and weight, as defined by Bigg (1969b).

Seventeen of the seals were pups, details of which were as follows: five were premature (lanugo coat present), surviving in rehabilitation for 0 (dead upon arrival) to 12 days; five were full-term pups, aged up to 1.5 months, surviving 0–9 days; seven were weaned pups, aged 2–5 months, surviving 0–138 days. The remaining three seals (aged >1 year), which were in rehabilitation for 0–14 days before death, consisted of: one subadult male aged between 2 and 5 years; one adult male aged >5 years; one peri-parturient adult female aged >4 years. All pups presenting to the rehabilitation centre between March 1999 and September 2001 were included in the study if (1) clinical samples were collected before death, (2) necropsy was performed within 24 h of death, and (3) a full set of tissues was collected for PCR and histology. Because few subadult or adult harbour seals were found stranded or dead along the Californian coast during the study period, only three were available for examination.

Clinical samples were collected from each seal upon admission (within 48 h) to the rehabilitation

centre and then weekly until death. Samples included serum, whole blood to obtain mononuclear leucocytes, and nasal swabs. Blood was drawn from the extradural intravertebral vein or the interdigital vein in the rear flipper as described by Bossart *et al.* (2001) and placed into Vacutainer® tubes containing either ethylenediamine-tetra-acetic acid (EDTA) or serum separation gel (Becton Dickenson, Rutherford, New Jersey, USA). Nasal swab samples were collected with sterile cotton-tipped applicators, placed in sterile vials and frozen for DNA extraction. Duplicate sets of 21 tissues (tongue, tonsil, oral mucosa, salivary gland, retropharyngeal lymph node, trachea, lung, bronchial lymph node, liver, umbilical vein at portal entry, adrenal, spleen, mesenteric lymph node, ileo-caeco-colic junction, trigeminal ganglia, vagosympathetic ganglia, spinal ganglia [thoraco-lumbar area], brain stem, pituitary, genital mucosa and bladder) were collected at necropsy. One set of samples was fixed in 10% buffered formalin for histopathological examination, and the other set was frozen at −80 °C for DNA analysis.

Histological Examination

Formalin-fixed tissues were processed by routine methods for paraffin wax embedding, sectioned at 5 μm and stained with haematoxylin and eosin by the Histotechnique Laboratory, University of California, Davis (UCD) Veterinary Medical Teaching Hospital. Tissue sections were examined histologically by a single pathologist (LJL) to determine the cause of death, as well as to identify tissues with herpesviral inclusion bodies and other herpesvirus-associated changes. Lesions suggestive of PhHV-1 infection included: necrosis of adrenal gland and liver, with intranuclear inclusion bodies; adrenalitis or mineralization; regeneration or fibrosis of necrotic foci in adrenals; necrosis with inclusions in other tissues such as intestine, lung or brain.

Serological Analysis

Serum was harvested from centrifuged whole blood, frozen at −20 °C or lower, and examined by a previously established indirect ELISA to measure PhHV-1 specific antibodies (King *et al.*, 2001). All samples were tested in duplicate at a 1in 100 dilution, and compared with a standard curve generated from a positive reference sample with a PhHV-1 antibody concentration designated as 100 units/ml. Results were reported as a percentage of this standard, and samples with antibody concentrations greater than 1 unit/ml were considered to be positive.

PCR Analysis

Tissue samples collected at necropsy were cut into small pieces with scissors disinfected with 10% sodium hypochlorite after handling each sample, to prevent DNA cross-contamination between tissues and between animals. Mononuclear leucocytes were isolated from whole blood samples as described by Goldstein *et al.* (2004). After overnight digestion with proteinase K, genomic DNA was extracted from tissues, mononuclear leucocytes and swab samples with a silica-gel-membrane-based kit (DNeasy Tissue Kit®; Qiagen Inc., Valencia, CA, USA). The DNA concentration for each tissue sample was determined by optical density at 260 nm, and approximately 500 ng of DNA were used as template in the PCR assay. PCR primers and amplification conditions to detect a 450 bp region of the phocine herpesviral glyco-protein B (gB) gene and a 544 bp fragment of the harbour seal ferritin gene (to confirm the suitability of the sample for the PCR) were those used previously by Goldstein *et al.* (2004). DNA extraction controls and positive (PhHV-1 DNA extracted from infected cell cultures) and negative (no template DNA) controls were included for all reactions.

The sensitivity of the PCR reaction was determined by titration of a linearized plasmid clone containing the gB insert and by spiking mouse DNA with the plasmid clone, showing that it was possible to detect a minimum of 166 copies of the viral DNA in samples. The specificity of the PCR reaction was confirmed by Southern blot and sequence analyses. For Southern blot analysis, the cloned 450 bp PhHV-1 gB fragment and a 507 bp equine herpesvirus-4 (EHV-4) gB fragment (kindly provided by Drs U. Balasuriya and N. J. MacLachlan), also an alphaherpesvirus (Borchers *et al.*, 1999), were labelled with digoxygenin and used as probes (DIG Probe Synthesis kit; Roche Diagnostics Corporation, Roche Applied Sciences, Indianapolis, IN, USA). Amplicons from the PhHV-1 and EHV-4 PCR were run out on an agarose gel, electroblotted on to nylon membranes (Hybond™-N; Amersham, Piscataway, NJ, USA) and hybridized with the PhHV-1 and EHV-4 DIG labelled probes. Hybridization was carried out overnight in DIG Easy HYB solution (Roche) at 42 °C, followed by one wash in 2x saline sodium citrate (SSC) containing sodium dodecyl sulphate (SDS) 0.1% and two washes in 0.5x SSC/0.1% SDS at room temperature. The resulting hybrids were detected with anti-DIG-alkaline phosphatase (AP) antibody (1 in 5000 dilution) followed by use of

the standard DIG detection reagents (DIG Nucleic Acid Detection Kit; Roche) showing that both the PhHV-1 and EHV-4 probes hybridized only with PhHV-1 and EHV-4 amplicons, respectively. Seven representative positive samples (bronchial lymph node, adrenal gland, liver, trigeminal ganglion, vagosympathetic ganglion, spinal ganglion, brain stem) from seven different young or adult harbour seals were directly sequenced by the chain termination method (Sanger *et al.*, 1977).

Results

Results from seven of the harbour seal pups (seals 1–7) were almost identical. Of these animals, three were premature, three full term and one weaned. While at the rehabilitation centre, all were negative for PhHV-1 antibodies in the serum ELISA test, and virus-negative as shown by PCR of mononuclear leucocytes and nasal swabs for PhHV-1 DNA. No PhHV-1 DNA was detected in any of the tissues from these seals (Table 1), and no inclusion bodies or herpesvirus-associated lesions were found histologically. The primary pathological findings ranged from meningitis to emaciation (Table 2).

Ten pups (seals 8–17) showed evidence of PhHV-1 infection. Seals 8 (full term) and 9 (premature) were negative for PhHV-1 antibodies in serum, but at the time of death had detectable PhHV-1 viral DNA in mononuclear leucocytes or nasal swab samples, or both. In addition, the viral DNA was detected (Table 1) in the umbilical vein of both seals, in the mesenteric lymph node of seal 8, and in the retropharyngeal and bronchial lymph nodes of seal 9. Phocine herpes-related disease did not appear to be the cause of death in these two pups, as no supporting histopathological evidence was seen; the primary histological findings were suppurative meningitis and pulmonary haemorrhage (Table 2).

Four pups (seals 10–13) showed evidence of widespread herpesviral infection and herpesvirus-related disease (Table 1). These seals had detectable PhHV-1 DNA in mononuclear leucocytes or nasal swab samples, or both, at death. Pups 10 (premature) and 11 (weaned) were negative for PhHV-1 specific antibodies while under care at the rehabilitation centre, whereas pups 12 (full term) and 13 (weaned) were seropositive when tested on the day of admission. PhHV-1 DNA was detected in almost every tissue tested in all four of these pups, including their adrenal glands and livers. Histopathological examination of seal 10 led to a diagnosis of systemic phocine herpesviral infection, with extensive adrenocortical necrosis, multifocal hepatic necrosis, crypt necrosis of the small intestine, neuronal and cerebral necrosis, and tonsillar necrosis, commonly with intranuclear inclusions. Extensive areas of multifocal adrenocortical necrosis with mineralization and inclusion bodies were seen in seal 11, and hepatic necrosis with equivocal intranuclear inclusions was also noted. The primary finding and probable cause of death in this animal was septicaemia. No herpesviral inclusion bodies were found in tissues from seals 12 and 13; however, lesions consistent with herpesviral infection included foci of adrenal hyperplasia in seal 12 and multifocal subacute adrenalitis in seal 13. The primary lesions found in these animals were protozoal encephalitis and mandibular fracture (Table 2).

The remaining four pups (seals 14–17), which were all weaned, died in July or August. All four were seropositive at the time of death. Seals 14, 15 and 17 were positive on the day of admission, and seal 16 seroconverted on day 122 after admission. In these four pups, PhHV-1 DNA was demonstrated in mononuclear leucocyte or nasal samples during rehabilitation but had disappeared 9–34 days before death. After death, these four pups (seals 14–17) showed fewer PCR-positive sites (4–10) than did seals 10–13 (13–20 sites); moreover, the adrenal glands and livers of seals 14–17 were PCR-negative (Table 1), and no herpesviral inclusion bodies or associated lesions were found histologically in any tissue. The primary histological finding in seals 14, 15 and 17 was verminous pneumonia (Table 2). Pup 16 was humanely destroyed due to central nervous system (CNS) signs, the cause of which could not be determined histologically.

The one subadult and two adult animals (seals 18–20) were shown by ELISA to contain serum antibodies against PhHV-1. A nasal swab sample was not collected from the peri-parturient adult female (seal 18) on admission, but a sample taken at the time of death was PCR-positive; mononuclear leucocytes were PCR-negative, both on admission and one week later (Table 1). PhHV-1 DNA was detected in 10 tissue sites from this animal, including oral tissues and associated lymph nodes, as well as neural tissues (Table 1); moreover, necrotizing stomatitis with herpesviral inclusion bodies was noted on histopathological examination.

PhHV-1 DNA was not detected in mononuclear leucocytes from the subadult male (seal 19) and no nasal swab sample was collected; however, viral DNA was detected in four tissue sites (Table 1). Areas of adrenal nodular hyperplasia and fibrosis were noted histologically in the subadult male, suggestive of prior adrenal necrosis. PhHV-1 DNA

Table 1
Detection of PhHV-1 DNA by the PCR in 20 harbour seals

| | Results in harbour seal | | | | | | | | | | | | | | | | | | | | Total positive results |
| | pups | | | | | | | | | | | | | | | | | adults | | | |
Sample	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	
Salivary gland	−	−	−	−	−	−	−	−	−	ns	ns	+	+	−	+	+	−	+	−	+	6/18 (33.3%)
Tongue	−	−	−	−	−	−	−	−	−	+	+	ns	+	+	−	−	−	+	+	+	7/19 (36.8%)
Tonsil	−	−	−	−	−	−	−	−	−	+	+	+	+	−	+	−	−	+	−	+	7/20 (35.0%)
Oral mucosa	ns	ns	ns	−	−	−	−	−	−	ns	ns	ns	+	−	+	−	−	ns	−	+	3/13 (23.1%)
Retropharyngeal lymph node	−	−	−	−	−	−	−	−	+	+	+	+	+	−	+	−	+	−	−	+	8/20 (40.0%)
Trachea	−	−	−	−	−	−	−	−	−	+	+	+	+	−	+	+	−	−	−	+	7/20 (35.0%)
Lung	−	−	−	−	−	−	−	−	−	+	+	+	+	−	−	−	+	+	−	ns	6/19 (31.6%)
Bronchiolar lymph node	−	−	−	−	−	−	−	−	+	+	+	+	+	−	+	+	+	−	−	+	9/20 (45.0%)
Liver	−	−	−	−	−	−	−	−	−	+	+	+	+	−	−	−	−	−	−	+	5/20 (25.0%)
Adrenal gland	−	−	−	−	−	−	−	−	−	+	+	+	+	−	−	−	−	−	+	+	6/20 (30.0%)
Spleen	−	ns	−	−	−	−	−	−	−	+	+	+	+	−	−	+	−	ns	ns	+	6/17 (35.3%)
Mesenteric lymph node	−	−	−	−	−	−	−	+	−	+	+	+	−	−	+	−	−	−	−	+	6/20 (30.0%)
Ileo-caecal-colic junction	−	−	ns	−	−	−	−	−	−	+	+	−	+	+	+	+	−	+	+	+	9/19 (47.4%)
Trigeminal ganglia	−	−	−	−	−	−	−	−	−	+	+	+	+	+	−	+	−	+	ns	+	8/19 (42.1%)
Vagosympathetic ganglia	−	−	−	−	−	−	−	−	−	+	ns	−	+	+	+	−	ns	+	ns	+	6/17 (35.3%)
Spinal ganglia	−	−	ns	−	−	−	−	−	−	+	ns	−	+	−	+	+	+	+	+	+	8/18 (44.4%)
Brain stem	−	−	−	−	−	−	−	−	−	+	+	+	+	−	−	−	−	+	ns	+	6/19 (31.6%)
Pituitary	−	−	−	−	−	−	−	−	−	+	+	+	+	−	−	−	−	+	ns	+	6/19 (31.6%)
Umbilical vein	−	−	ns	−	−	−	−	+	+	+	ns	−	+	−	−	−	−	−	−	ns	4/17 (23.5%)
Bladder	ns	ns	ns	−	−	−	−	−	−	ns	ns	ns	+	−	−	−	−	ns	ns	+	2/12 (16.7%)
Genital mucosa	ns	ns	ns	−	ns	−	−	−	−	ns	ns	ns	+	ns	−	−	−	−	−	+	2/12 (16.7%)
Mononuclear leucocytes	−	−	−	−	−	−	−	+	+	+	ns	+	−	−	−	−	−	−	−	ns	4/18 (22.2%)
Nasal swab	−	−	ns	−	−	−	−	+	ns	−	+	ns	+	+	−	−	−	+	ns	ns	5/15 (33.3%)

+, Positive; −, negative; ns, no sample. PhHV-1 antibodies demonstrated by ELISA in serum of seals 12–20, seals 1–11 being negative.

Table 2
Primary pathological and herpesvirus-associated findings in 20 harbour seals

Harbour seals	Age	Lesions
Pups		
1	Premature	Septic meningitis (bacterial) resulting from omphaloarteritis
2	Full term	Protozoal meningoencephalitis (*Sarcocystis neurona*)
3	Weaned	Interstitial verminous pneumonia and bronchitis (*Otostrongylus circumlitus*)
4	Full term	Inanition, diffuse systemic congestion suggestive of shock
5	Full term	Bilateral mandibular fracture resulting in emaciation
6	Premature	Suppurative interstitial pneumonia, hepatic lipidosis, thymic atrophy
7	Premature	Severe thymic and hepatocellular atrophy consistent with emaciation
8	Full term	Severe suppurative meningitis and a brain abscess
9	Premature	Severe pulmonary haemorrhage, thymic atrophy, splenic lymphoid depletion
10	Premature	Phocine herpesvirus-associated necrosis with inclusions in multiple organs
11	Weaned	Septicaemia, multifocal hepatic and adrenal necrosis with inclusions
12	Full term	Protozoal meningoencephalitis (*S. neurona*), adrenocortical hyperplasia
13	Weaned	Mandibular fracture resulting in emaciation, multifocal subacute adrenalitis
14	Weaned	Interstitial verminous pneumonia (*O. circumlitus*), peritonitis
15	Weaned	Verminous pneumonia (*Parafilaroides decorus*), diaphragmatic hernia
16	Weaned	Euthanasia—CNS signs, cause not determined histologically
17	Weaned	Verminous pneumonia (*O. circumlitus, P. decorus*)
Adults		
18	> 4 yr	Encephalitis as a result of septicaemia, stomatitis with herpesviral inclusions
19	2-5 yr	Protozoal meningitis (*S. neurona*), adrenal fibrosis and hyperplasia
20	>5 yr	Suppurative bronchopneumonia resulting in sepsis and necrotizing hepatitis

was detected in all tissues collected from the adult male (seal 20, Table 1), but no leucocyte or swab samples were collected from this animal as it was dead upon arrival; no herpesviral inclusion bodies or associated lesions were found in any tissue sample from this animal. These three older seals were severely ill due to protozoal encephalitis, sepsis or bronchointerstitial pneumonia (Table 2).

Overall, PhHV-1 DNA was detected by PCR in tissues from 10/17 seal pups (two premature, two full term, six weaned) and all three older animals. Forty percent (4/10) of the pre-weaned pups (premature and full term combined) and 86% (6/7) of the weaned pups had detectable PhHV-1 DNA in tissue samples. The PCR results from all 20 seals showed that PhHV-1 DNA was found at least once in each tissue tested. The tissues most commonly positive, however, were ileo-caeco-colic junction (9/19; 47%), bronchial lymph node (9/20; 45%), spinal ganglia (8/18; 44%) and trigeminal ganglia (8/19; 42%) (Table 1). All samples of the seven tissues from seven different animals submitted for sequencing were identical with each other, as well as with the previously described gB fragment (King *et al.*, 1998).

Regardless of age, all ELISA-positive seals gave PCR-positive results from tissues (Table 3). However, four of the 11 seronegative seals had detectable viral DNA in tissues (Table 3). With the PCR assay in tissues as the "gold standard" for detecting infection, the ELISA had a sensitivity of

69% and specificity of 100%, while histological examination had a sensitivity of 46% and specificity of 100%. Herpesviral inclusion bodies were found in only three of 13 animals that had detectable PhHV-1 DNA in tissues, and no evidence of herpesvirus-associated lesions was found in seven of 13 animals with PCR-positive tissue (Table 3).

Discussion

Different clinical patterns emerged as a result of examining ante-and post-mortem samples from the 20 harbour seals in this study. These included uninfected and infected animals, the latter consisting of active, recovering, chronic or recrudescent cases with evidence of in-utero or post-natal transmission. Pups 1–7 were considered to be uninfected, as they were seronegative for PhHV-1, PCR-negative for viral DNA in both clinical samples and post-mortem tissue samples, and showed no histological evidence of infection. These data

Table 3
Relationship between presence of PhHV-1 DNA (detected by PCR), PhHV-1 antibodies, and histological evidence of PhHV-1 infection (inclusion bodies, typical lesions) in 20 harbour seals

	Number of seals with	
Tissue PCR result in 20 seals	PhHV-1 antibodies	histological evidence of PhHV-1 infection
Positive (*n*=13)	9/13	6/13
Negative (*n*=7)	0/7	0/7

confirm previous suggestions that not all harbour seals are infected with PhHV-1 at birth (Goldstein *et al.*, 2003).

Six pups (seals 8–13) showed evidence of active PhHV-1 infection at death, with viral DNA in ante-mortem clinical samples as well as post-mortem tissue samples. Two of these pups (8 and 9) were newborn animals that died early in the year, shortly after admission, at a time when no other PhHV-1-infected seals were detected in the hospital. They had detectable PhHV-1 DNA only in umbilical veins and in a small number of lymphoid tissues. These pups may have acquired the infection *in utero*; alternatively, infection may have been acquired during parturition, as has been noted in human herpes simplex infection and canine herpesvirus infection (Hashimoto *et al.*, 1979; Jeffries, 1991). In-utero or peri-parturient infection does not necessarily result in severe neonatal disease.

Other actively infected pups (10–13) showed PCR evidence of much wider tissue distribution of the virus, often accompanied by histological lesions. In one seal (10) there was overwhelming infection, with histological changes in multiple organs. Widespread infection, with or without lesions, has been noted with alphaherpesviral infections in other species (Weigler *et al.*, 1995; Schulze and Baumgärtner, 1998; Taouji *et al.*, 2002). Since seal 10 was a premature pup that died within 7 days of admission, at a time when no other PhHV-1 infected seals were present, it is likely that this pup was infected *in utero* or perinatally.

Based on previous histological studies (Gulland *et al.*, 1997), it would appear that the adrenal gland and liver are the usual target organs for PhHV-1 disease, and PhHV-1 DNA was detected in these organs in all four of the actively infected pups (10–13). Seals 10 and 11, which were seronegative, had adrenal and liver necrosis with herpesviral inclusions, while seals 12 and 13 both had measurable PhHV-1 specific antibodies and histological evidence of healing adrenal gland damage. Seals 12 and 13 may therefore have been mounting a protective response against the virus.

Four pups (14–17) were considered to be recovering from clinical infection. These were all weaned animals with no histological evidence of PhHV-1-associated lesions. All had detectable antibodies against the virus, and PhHV-1 DNA was detected in mononuclear leucocytes and nasal swabs before death; all four had detectable PhHV-1 DNA in tissues but not in the adrenal gland or liver. The lack of PhHV-1 DNA and histological lesions in these two target organs may have been due to (1) a protective immune response that prevented entry of the virus, or (2) clearance of the virus from these tissues.

The subadult and two adult harbour seals were antibody-positive and PCR-positive. Since most harbour seals are exposed to PhHV-1 at a young age (Goldstein *et al.*, 2003) and most herpesvirus infections persist for the lifetime of the host (Roizman *et al.*, 1992), these three older seals probably represented chronic cases. Additionally, all three animals had serious clinical illness and one was peri-parturient. Illness and pregnancy precipitate recrudescence of latent herpesviral infections in other species (Whitley *et al.*, 1998), and it is therefore possible that reactivation of chronic infections was being detected in these animals.

Further studies directed at the detection of viral gene expression with reverse transcriptase PCR or in-situ hybridization, as has been done in other species (Borchers *et al.*, 1999; Kennedy *et al.*, 2000), and serial sampling of seals of different ages, would be the next logical step to determine whether or not latency occurs in harbour seals. The best source of tissue samples for such studies would be healthy adult free-ranging harbour seals that died trauma-tically. However, such carcasses are rarely found along the Californian coast and were not available for the present study.

Four of the six pups that appeared to have active PhHV-1 infections at death did not have measurable PhHV-1 specific antibodies, supporting previous findings (King *et al.*, 2001). This may indicate that PhHV-1 disease can progress rapidly to death in some seal pups if they are unable to mount an effective immune response. However, because seroconversion takes approximately 7–9 days (Horvat *et al.*, 1989; Goldstein *et al.*, 2004), seals dying from some other cause shortly after exposure to PhHV-1 would be antibody-negative. For this reason, the ELISA was only moderately sensitive (69%).

Histological examination was also of low sensitivity, giving a positive result in fewer than half of the seals that had PCR-positive tissues. When typical lesions of herpesviral infection are absent, a more sensitive diagnostic method is needed. The PCR is such a method, but it is important to note that a positive result does not always indicate active disease. Causes of death other than phocine herpesvirus infection were found in almost all seals, supporting documented findings that although PhHV-1 can cause fatal disease, it may often play no more than a contributory role in mortality (Gulland *et al.*, 1997; King *et al.*, 2001). It is unclear whether PhHV-1 infection occurs first,

making animals more susceptible to other diseases, or vice versa. Specificity was 100% for both ELISA and histopathological diagnosis.

In conclusion, PhHV-1 infections (active, recovering, persistent and recrudescent cases) were successfully diagnosed in harbour seals, the presence of viral DNA in tissues being more widespread than the presence of lesions. Preweaned pups (40%) and weaned pups (85.7%) showed evidence of PhHV-1 infection; these findings accord with the proportions of exposed preweaned and weaned pups reported by Goldstein *et al.* (2003). The results suggested that vertical transmission occurs in small numbers of newborn animals but that primary exposure occurs after birth in the majority of pups. Primary PhHV-1 infection probably occurs as a result of initial entry of the virus into the mucosal tissues or blood. A mononuclear leucocyte-associated viraemia, like that associated with CHV-1 infection in dogs (Carmichael and Greene, 1990), favours spread of the virus to lymph nodes and mucosal-associated lymphoid tissue (e.g., tonsil, Peyer's patches), followed by dissemination to parenchymal organs. Seroconversion occurs in animals that survive primary infection and appears to be associated with clinical recovery but not necessarily with clearance of the virus. Viral reactivation may occur in subadult or adult seals that are physiologically stressed, as in illness or pregnancy. Once infected, harbour seals probably retain the ability to infect naïve animals during periods of viral reactivation. Such reactivation may play an important role in the persistence of the virus in free-living harbour seal populations and in the epidemiology of PhHV-1 disease in young seals.

References

Bigg, M. A. (1969a). Clines in the pupping season of the harbour seal, *Phoca vitulina*. *Journal of the Fisheries Research Board of Canada*, **26,** 449–455.

Bigg, M. A. (1969b). The harbour seal in British Columbia. *Bulletin of the Fisheries Research Board of Canada*, **172,** 1–33.

Borchers, K., Wolfinger, U. and Ludwig, H. (1999). Latency-associated transcripts of equine herpesvirus type 4 in trigeminal ganglia of naturally infected horses. *Journal of General Virology*, **80,** 2165–2171.

Borst, G. H., Walvoort, H. C., Reijnders, P. J. H., van der Kamp, J. S. and Osterhaus, A. D. M. E. (1986). An outbreak of herpesvirus infection in harbor seals (*Phoca vitulina*). *Journal of Wildlife Diseases*, **22,** 1–6.

Bossart, G. D., Reiderson, T. H., Dierauf, L. A. and Duffield, D. A. (2001). Clinical pathology. In: *Handbook of Marine Mammal Medicine*, L. A. Dierauf and F. M. D. Gulland, Eds, CRC Press Inc., Boca Raton, Florida, pp. 383–448.

Boulva, J. and McLaren, I. A. (1979). Biology of the harbor seal, *Phoca vitulina*, in eastern Canada. *Bulletin of the Fisheries Research Board of Canada*, **200,** 1–24.

Burr, P. D., Campbell, M. E. M., Nicolson, L. and Onions, D. E. (1996). Detection of canine herpesvirus 1 in a wide range of tissues using polymerase chain reaction. *Veterinary Microbiology*, **53,** 227–237.

Carmichael, L. E. and Greene, C. E. (1990). Canine herpesvirus infection. In: *Infectious Diseases of the Dog and Cat*, C. E. Greene, Ed., W.B. Saunders, Philadelphia, pp. 252–258.

Frey, H.-R., Liess, B., Haas, L., Lehmann, H. and Marschall, H.-J. (1989). Herpesvirus in harbour seals (*Phoca vitulina*): isolation, partial characterization and distribution. *Journal of Veterinary Medicine B*, **36,** 699–708.

Gaskell, R. and Willoughby, K. (1999). Herpesviruses of carnivores. *Veterinary Microbiology*, **69,** 73–88.

Goldstein, T., Gulland, F. M. D., Aldridge, B. M., Harvey, J. T., Rowles, T., Lambourn, D. M., Jeffries, S. J., Measures, L., Yochem, P. K., Stewart, B. S., Small, R. J., King, D. P., Stott, J. L. and Mazet, J. A. K. (2003). Antibodies to phocine herpesvirus-1 in North American harbor seals (*Phoca vitulina*). *Journal of Wildlife Diseases*, **39,** 487–494.

Goldstein, T., Mazet, J. A. K., Gulland, F. M. D., Rowles, T., Harvey, J. T., Allen, S. G., King, D. P., Aldridge, B. M. and Stott, J. L. (2004). The transmission of phocine herpesvirus-1 in rehabilitating and free-ranging Pacific harbor seals (*Phoca vitulina*) in California. *Veterinary Microbiology*, **103,** 131–141.

Gulland, F. M. D., Lowenstine, L. J., Lapointe, J. M., Spraker, T. and King, D. P. (1997). Herpesvirus infection in stranded Pacific harbor seals of coastal California. *Journal of Wildlife Diseases*, **33,** 450–458.

Hashimoto, A., Hirai, K., Okada, K. and Fujimoto, Y. (1979). Pathology of the placenta and newborn pups with suspected intrauterine infection with canine herpesvirus. *American Journal of Veterinary Research*, **40,** 1236–1240.

Horvat, B., Willhaus, T. H., Frey, H.-R. and Liess, B. (1989). Herpesvirus in harbour seals (*Phoca vitulina*); transmission in homologous host. *Journal of Veterinary Medicine B*, **36,** 715–718.

Jeffries, D. (1991). Intra-uterine and neonatal herpes simplex virus infection. *Scandinavian Journal of Infection*, **78**(Suppl.)**,** 21–26.

Kennedy, P. G. E., Grinfeld, E. and Bell, J. E. (2000). Varicella-zoster virus gene expression in latently infected and explanted human ganglia. *Journal of Virology*, **74,** 11893–11898.

King, D. P., Lie, A. R., Goldstein, T., Aldridge, B. M., Gulland, F. M. D., Haulena, M., Adkison, M. A., Lowenstine, L. J. and Stott, J. L. (2001). Humoral immune responses to phocine herpesvirus-1 in Pacific harbor seals (*Phoca vitulina richardsii*) during an outbreak of clinical disease. *Veterinary Microbiology*, **80,** 1–8.

King, D. P., Parselles, R., Gulland, F. M. D., Lapointe, J. M., Lowenstine, L. J., Ferrick, D. A. and Stott, J. L. (1998). Antigenic and nucleotide characterization of a herpesvirus isolated from Pacific harbor seals (*Phoca vitulina richardsii*). *Archives of Virology*, **143,** 2021–2027.

Lebich, M., Harder, T. C., Frey, H.-R., Visser, I. K. G., Osterhaus, A. D. M. E. and Liess, B. (1994). Comparative immunological characterization of type-specific and conserved B-cell epitopes of pinniped, felid and canid herpesviruses. *Archives of Virology*, **136,** 335–347.

Osterhaus, A. D. M. E., Yang, H., Splijkers, H. E. M., Groen, J., Teppema, J. S. and Van Steenis, G. (1985). The isolation and partial characterization of a highly pathogenic herpesvirus from the harbor seal (*Phoca vitulina*). *Archives of Virology*, **86,** 239–251.

Reubel, G. H., Ramos, R. A., Hickmann, M. A., Rimstad, E., Hoffmann, D. E. and Pedersen, N. C. (1993). Detection of active and latent feline herpesvirus 1 infections using polymerase chain reaction. *Archives of Virology*, **132,** 409–420.

Roizman, B., Desrosiers, R. C., Fleckenstein, B., Lopez, C., Minson, A. C. and Studdert, M. J. (1992). The family Herpesviridae: an update. *Archives of Virology*, **123,** 425–443.

Sanger, F., Nicklen, S. and Coulson, A. R. (1977). DNA sequencing with chain terminating inhibitors. *Proceedings of the National Academy of Sciences of the USA*, **74,** 5463–5467.

Schulze, C. and Baumgärtner, W. (1998). Nested polymerase chain reaction and in situ hybridization for diagnosis of canine herpesvirus infection in puppies. *Veterinary Pathology*, **35,** 209–217.

Taouji, S., Collobert, C., Gicquel, B., Sailleau, C., Brisseau, N., Moussu, C., Breuil, M.-F., Pronost, S., Borchers, K. and Zientara, S. (2002). Detection and isolation of equine herpesvirus 1 and 4 from horses in Normandy: an autopsy study of tissue distribution in relation to vaccination status. *Journal of Veterinary Medicine B*, **49,** 394–399.

Tempte, J. L., Bigg, M. A. and Wiig, O. (1991). Clines revisited: the timing of pupping in the harbour seal (*Phoca vitulina*). *Journal of Zoology, London*, **224,** 617–632.

Weigler, B. J., Scinicariello, F. and Hilliard, J. K. (1995). Risk of venereal B virus (Cercopithecine herpesvirus 1) transmission in rhesus monkeys using molecular epidemiology. *Journal of Infectious Diseases*, **171,** 1139–1143.

Whitley, R. J., Kimberlin, D. W. and Roizman, B. (1998). Herpes simplex viruses. *Clinical Infectious Diseases*, **26,** 541–555.