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ANTIBODIES TO PHOCINE HERPESVIRUS-1 ARE COMMON IN NORTH AMERICAN HARBOR SEALS (*PHOCA VITULINA*)

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ABSTRACT: Phocine herpesvirus-1 (PhHV-1) has been associated with morbidity and high mortality in neonatal harbor seals (*Phoca vitulina*) along the Pacific coast of California (USA) and in northern Europe. Seals dying with PhHV-1 associated disease in California primarily have histopathologic evidence of adrenal necrosis or adrenalitis with herpesviral inclusion bodies. Little is known about prevalence of exposure to PhHV-1, modes of disease transmission, and viral pathogenesis in free-ranging harbor seal populations. To evaluate the prevalence in North America, 866 serum samples collected between 1994 and 2002 from harbor seals captured or stranded on the Pacific and Atlantic coasts of North America were assayed by enzyme linked immunosorbent assay (ELISA) for evidence of PhHV-1 exposure. Samples from three harbor seal age classes (pre-weaned, weaned, and subadults/adults) were obtained from each of four regions to compare exposure among sex, age class, and region. We found increasing prevalence with age as 37.5% of pre-weaned pups, 87.6% of weaned pups, and 99.0% of subadults and adults were seropositive. When accounting for age, no associations between seropositivity and sex or location of harbor seals were detected. These data indicate that PhHV-1 is endemic in the harbor seal populations of North America.

Key words: ELISA, harbor seal, herpesvirus, marine wildlife, PhHV-1, *Phoca vitulina,* serosurvey.

INTRODUCTION

Phocine herpesvirus-1 (PhHV-1) is an alpha herpesvirus (Frey et al., 1989; King et al., 1998), a subfamily of viruses that also includes canine and feline herpesviruses. Alphaherpesviruses are rapidly replicating, aggressive viruses that cause mass destruction of cells in humans and in domestic animals (Roizman, 1982). Phocine herpesvirus-1 was first isolated from neonatal harbor seals (*Phoca vitulina*) dying with clinical disease at rehabilitation centers in the Netherlands in 1984 (Osterhaus et al., 1985) and along the Pacific coast of North America in 1996 (Gulland et al., 1997; King et al., 1998). These outbreaks were associated with high mortality in young pups. Phocine herpesvirus-1 also contributed to deaths of free-ranging seals during the1988 mass mortality in northwest Europe (Frey et al., 1989). Annual outbreaks of disease can occur in newborn harbor seals undergoing rehabilitation, and clinical signs include respiratory infections, decreased thermoregulatory ability, seizures, and death (Harder et al., 1997; King et al., 2001). Primary lesions associated with disease in neonatal seals in California (USA) were adrenal necrosis or adrenalitis

with herpesviral inclusion bodies (Gulland et al., 1997). Additionally, intranuclear inclusions have been reported in adrenal glands of moribund harbor seals on rookeries in Oregon (USA) and California and in harbor seals in Prince William Sound, Alaska (USA) following the Exxon Valdez oil spill in 1989 (Spraker et al., 1994; Gulland et al., 1997).

Only one study has examined regional exposure of free-ranging harbor seals to PhHV-1. Zarnke et al. (1997) included serum samples collected from harbor seals in a multi-species marine mammal serosurvey measuring exposure to PhHV-1 and PhHV-2 in Alaska and Russia using a virus neutralization assay. Overall, 77% of those harbor seals were positive for antibody. Since disease and death are frequently associated with PhHV-1 infection in rehabilitating harbor seal pups and sporadically in free-ranging harbor seals along the Pacific coast of the US, a comprehensive epidemiologic survey was needed to evaluate considerations for disease management, as harbor seals are frequently rehabilitated and released throughout North America. Moreover, Zarnke et al. (1997) and Stuen et al. (1994) suggested that marine mammals may have an increased probability of being seropositive with age, although this has not been investigated in harbor seals. Consequently, our objectives were to compare the prevalence of exposure of harbor seals to PhHV-1 from four regions along the Atlantic and Pacific coasts of North America from three age classes of seals of both sexes, using a previously described and validated enzyme linked immunosorbent assay (ELISA) that measures PhHV-1 specific antibodies in serum samples (King et al., 2001).

MATERIAL AND METHODS

Between 1994 and 2002, we obtained 866 serum samples from free-ranging harbor seals captured for ongoing studies and from orphaned or sick animals upon admission to rehabilitation facilities. Four geographic regions around North America were represented: 1) the northeast along the Atlantic coastline from eastern Canada (48°22′N, 68°27′W) to New Jersey (USA; 39°25′N, 74°22′W; $n=140$), 2) the Alaska region (Bristol Bay; 57°28′N, 157°40′W to 58°10′N, 57°28′W; $n=30$), 3) the northwest along the Pacific coast from British Columbia (Canada; 48°55′N, 123°23′W) to the Oregon border (43°40′N, 124°11′W; $n=286$), and 4) the southwest along the Pacific coast of California (41°45′N, 124°11′W to 33°41′N, 118°17′W; $n=410$). We classified samples as coming from pre-weaned pups (<2 mo old, includes suckling free-ranging pups and orphaned pups that may not have nursed; $n=269$), weaned pups (2–11 mo; $n=177$), and animals >1 yr of age (yearlings, subadults, and adults; $n=420$). Twenty-seven samples were also obtained from adult harbor seals permanently housed at four facilities in the US (SeaWorld Adventure Parks, Ohio; Brookfield Zoo, Illinois; National Aquarium, Baltimore, Maryland; and Mystic Aquarium, Connecticut). Blood was drawn from either the extradural intravertebral vein or the plantar interdigital vein of the rear flippers from physically restrained seals using either a 20 gauge 38 mm or 63 mm or 18 gauge 89 mm needle (Bossart et al., 2001). Blood samples were placed into vacutainers containing serum separation gel (Vacutainer®, Becton Dickinson, Rutherford, New Jersey), allowed to clot, and then centrifuged within 4 hr of collection at 3,000×G for 10 min. The serum was subsequently transferred to sterile vials and frozen at −20 C until assayed for PhHV-1 specific antibodies by ELISA (King et al., 2001).

The PhHV-1 antigen used in the ELISA assay (Pacific isolate—HS950; King et al., 1998) was propagated as previously described (Gulland et al., 1997; King et al., 1998) and purified as described by King et al. (2001). Microtiter plates (Pro-bind®, Falcon, Becton Dickinson, Franklin Lakes, New Jersey) were coated overnight with 1.4 μg/ml of purified virus. The plates were then blocked with 1% bovine serum albumin (Sigma, St. Louis, Missouri, USA), and antibody binding was detected by sequential incubation with 0.5 μg/ml biotinylated anti-grey seal (*Halichoerus grypus*) immunoglobulin G-specific monoclonal antibody-H49a that cross reacts with harbor seal immunoglobulin G (King et al., 1993), 0.83 μg/ml horseradish peroxidase conjugated strepavidin (HRP-Strepavidin®, Zymed, San Francisco, California) and *O*-phenylinediamine dihydrochloride (O.P.D., Sigma, St. Louis, Missouri, USA). Optical densities of the color-change proportional to the concentration of PhHV-1 specific antibody present in the samples were read at 490 nm with a ultraviolet max kinetic microplate reader. Results were analyzed using

Softmax® software (Version 3.0 Molecular Device, Menlo Park, California). All samples were tested in duplicate at a 1:100 dilution and compared with a positive reference sample with a PhHV-1 specific antibody level designated at 100 units/ml. Results were reported as a percent of this standard and samples with antibody concentrations >5 units/ml were considered positive.

Seroprevalence of exposure to PhHV-1 was calculated for each age class, sex, and geographic region separately and then for age classes and sex within each geographic region and for rehabilitation status (free-ranging versus rehabilitating). Association of seropositivity with age, sex, location, and rehabilitation status was evaluated using either a Chi-square test for association (Fleiss, 1981) or a Fisher's exact test (Fisher, 1935). Odds ratios (OR) and 95% confidence intervals (CI) were used to estimate the strength of associations between seropositivity and sex or age (Epi Info 2000 software, Version 1.1.2, June 2000, Centers for Disease Control and Prevention, Atlanta, Georgia, USA and Medcalc® Statistical software, Version 6.0-1993, Broekstraat 52, 9030 Mariakerke, Belgium).

RESULTS

Prevalence of exposure of harbor seals to PhHV-1 across all four regions was 37.5% (101/269) of pre-weaned pups, 87.6% (155/177) of weaned pups, and 99.0% (416/420) of animals >1 yr (Table 1). Seropositivity increased significantly with age (χ^2=369.52; P<0.001). Adults were more than 100 times more likely to have antibodies than pre-weaned pups (OR=173, 95% CI=60.7–477.4). However, no significant association was found between sex and seropositivity (P=0.38).

Table 1 provides the prevalence of exposure of harbor seals to PhHV-1 by location, age, and sex. The proportion of samples that tested positive for each region was: Alaska 90% (27/30), the northwest 85.7% (245/286), the southwest 79.3% (325/410), and the northeast 52.9% (74/140). Within each region seropositivity increased significantly with age (P<0.001) but did not differ between males and females of similar ages. The proportion of seropositive pre-weaned pups did not differ significantly between the southwest and northeast regions, but it was substantially lower in both areas compared with the northwest region (P<0.001). Prevalence in Alaska for this age class was similar to all other regions. Few (4/47) newborn animals (classified as such due to the presence of an attached umbilicus and/or a lanugo coat; Cottrell et al., 2002) admitted for rehabilitation in the southwest were seropositive. Fewer weaned pups were seropositive in the northwest versus the southwest region (P=0.01). No animals were sampled from this age group in the Alaska region. Equal proportions of seals >1 yr of age were seropositive among all regions. Moreover, many of these older seals had substantially higher antibody levels (>100 units/ml) than those measured in animals from the other two age classes, 38.8% (164/420) of older seals compared with 1.5% (4/269) of the pre-weaned pups and 10.7% (19/177) of the weaned pups. All seals that were permanently housed in facilities were seropositive (n=27, Table 1), with 33% (9/27) having antibody levels >100 units/ml.

Overall, rehabilitating harbor seals were almost 28 times less likely to test positive than free-ranging animals (95% CI=18.2–41.7), with 33% (74/224) rehabilitating compared with 93% (598/642) free-ranging testing positive (χ^2=345.17, P<0.001). Prevalence increased with age in both groups (P<0.001). To evaluate the difference between the wild sampled and rehabilitating group the data were adjusted for age in the southwest region, the only region from which there were samples from all groups to perform this analysis. After adjustment for age, rehabilitating pre-weaned pups were still significantly less likely to test positive than wild pre-weaned pups (P<0.001), but no rehabilitation status differences were found for the weaned and >1 yr age classes.

DISCUSSION

We found that exposure to PhHV-1 is highly prevalent in harbor seals along the Atlantic and Pacific coasts of North Amer-

TABLE 1. Prevalence (number exposed/number tested) of exposure of North American harbor seals (*Phoca vitulina*) to phocine herpesvirus-1 by location, age, and sex.

Region	Location	Prevalence	Age			Sex	
			Pre-weaned pup	Weaned pup	>1 year	Male	Female
All regions combined		77.6% (672/866)	37.5% (101/269)	87.6% (155/177)	99.0% (416/420)	76.6% (344/449)[a]	79.1% (325/411)[a]
Alaska (free-ranging only)	Bristol Bay: Egegik Bay Ugashik Bay	90% (27/30)	25% (1/4)	—	100% (26/26)	78% (7/9)	95% (20/21)
Northwest (free-ranging and rehabilitation combined)		85.7% (245/286)	58% (42/73)	75% (24/32)	98.9% (179/181)	85.0% (125/147)	86.3% (120/139)
Free-ranging (all locations)		91.9% (239/260)	76% (38/50)	76% (22/29)	98.9% (179/181)	93.0% (120/129)	90.8% (119/131)
	Northern WA: Strait of Juan de Fuca Hood Canal Eastern Bays	90% (65/72)	72% (18/25)	100% (2/2)	100% (45/45)	90% (37/41)	90% (28/31)
	Central WA: Puget Sound	90.8% (99/109)	87% (13/15)	73% (16/22)	97% (70/72)	94% (48/51)	88% (51/58)
	Southern WA: Grays Harbor Willapa Bay Columbia River	93% (40/43)	67% (6/9)	—	100% (34/34)	91% (19/21)	96% (21/22)
	Oregon: Umpaqua River Alsea River	97% (35/36)	100% (1/1)	80% (4/5)	100% (30/30)	100% (16/16)	95% (19/20)
Rehabilitation (all locations)	Island Wildlife Care Center, BC Vancouver Aquarium, BC Sarvey Wildlife Center, Arlington, WA	23% (6/26)	17% (4/23)	66.7% (2/3)	—	28% (5/18)	13% (1/8)
Southwest (free-ranging and rehabilitation combined)		79.3% (325/410)	31.1% (32/103)	91.1% (123/135)	98.8% (170/172)	78.1% (164/210)	81.4% (158/194)
Free-ranging (all locations)		93.7% (284/303)	76% (19/25)	90.1% (100/111)	98.8% (165/167)	93.8% (137/146)	95.4% (144/151)
	South Humboldt Bay	92% (12/13)	—	83% (5/6)	100% (7/7)	100% (3/3)	90% (9/10)
	San Francisco Bay: Castro Rocks Point Reyes National Seashores	93% (52/56)	—	84% (21/25)	100% (31/31)	93% (25/27)	93% (27/29)
	Monterey Bay: San Lorenzo River Elkhorn Slough Pebble Beach	96.2% (178/185)	78% (18/23)	100% (51/51)	98.2% (109/111)	97% (92/95)	97% (85/88)
	San Nicolas Island, Channel Islands	86% (42/49)	50% (1/2)	79% (23/29)	100% (18/18)	81% (17/21)	96% (23/24)

TABLE 1. Continued.

Region	Location	Prevalence	Age			Sex	
			Pre-weaned pup	Weaned pup	>1 year	Male	Female
Rehabilitation (all locations)	Northcoast Marine Mammal Center, Crescent City The Marine Mammal Center, Sausalito Marine Mammal Care Center, San Pedro	38.3% (41/107)	17% (13/78)	96% (23/24)	100% (5/5)	42% (27/64)	33% (14/43)
Northeast (free-ranging and rehabilitation combined)		52.9% (74/140)	28% (25/89)	80% (8/10)	100% (41/41)	54% (42/78)	52% (32/62)
Free-ranging (all locations)		98% (48/49)	—	78% (7/9)	100% (41/41)	100% (26/26)	92% (22/24)
	St Lawrence Estuary: Metis Sur Mer Quebec	100% (35/35)	—	100% (1/1)	100% (34/34)	100% (18/18)	100% (17/17)
	Gulf of St Lawrence: New Brunswick Prince Edward Island	87% (13/15)	—	75% (6/8)	100% (7/7)	100% (8/8)	71% (5/7)
Rehabilitation (all locations)	Marine Animal Lifeline, ME New England Aquarium, NE Marine Mammal Care Center, NJ	30% (27/91)	28% (25/89)	100% (1/1)	100% (1/1)	32% (17/53)	26% (10/38)
Permanent facilities (all locations)	Sea World Adventure Parks, OH Brookfield Zoo, IL National Aquarium in Baltimore, MD Mystic Aquarium, CT	100% (27/27)	—	—	100% (27/27)	100% (11/11)	100% (16/16)

[a] Sex was not noted for six free-ranging seals (two Pebble Beach, four San Nicolas Island).

ica, approaching 100% in individuals >1 yr. No difference between males and females was found. Because samples were collected from multiple years and geographic locations, results indicated that PhHV-1 is endemic in these populations. The common pattern of increasing prevalence with age in each region suggested harbor seals are exposed to PhHV-1 early in life, possibly during weaning, and remain seropositive throughout life following this exposure. Similar results have been found in human seroprevalence surveys that measured antibodies against herpes simplex-1, another alphaherpesvirus. Whitley and Gnann (1993) reported that approximately one third of human children <5 yr of age were seropositive, followed by an increase to 70% or 80% in adolescents and at least 95% in adults.

Lower prevalence estimates were obtained for the pre-weaned pups in the northeast and southwest compared with the northwest region. All samples (100%, 89/89) for this age class from the northeast and most from the southwest (75.7%, 78/103) were obtained from young seals tested at rehabilitation centers, compared with the northwest where only 32% (23/73) of this age class were young seals tested in rehabilitation centers. Furthermore, an overall difference in seropositivity was found between wild and rehabilitated pre-weaned pups ($P<0.001$). We think these differences are due in part to the rehabilitating pups being younger at the time of sampling than the free-ranging pups in this age class. In the southwest some premature pre-weaned pups were admitted into rehabilitation as early as February; normal pupping season in this region is March through May (Bigg, 1969; Tempte et al., 1991). Conversely, sample collection from free-ranging harbor seal pups in this region did not begin until April. Additionally, harbor seal pups recovered by rehabilitation centers are often orphans and may have only been exposed to infected seals at breeding and haul out sites for brief periods, if at all. Moreover, many of the orphaned pups may not have suckled sufficiently to have acquired maternal antibodies against PhHV-1. Because the ELISA is not able to distinguish between pup-derived and maternal antibodies, the higher seroprevalence in free-ranging versus rehabilitating pups may also be related to differential representation of maternal antibodies in free-ranging pups.

The prevalence estimate for the weaned age class in the northwest was lower than in the southwest. Most of those samples for this age class from the northwest were obtained from recently weaned seals captured in Oregon in August and in Washington in October in multiple years. It is possible that if animals were also sampled later in the year, as occurred for the southwest region (samples from weaned seals were obtained June through December), they may have been more likely to test positive, resulting in an increased prevalence of exposure for this age class in the northwest.

Our findings differ slightly from the estimate of seroprevalence previously reported for harbor seals in Alaska (Zarnke et al., 1997). We found a higher overall prevalence (90%) compared with the earlier study (77%). As our results clearly indicate that prevalence increases with age, we think this difference may be due to the small proportion of young seals that we tested in Alaska (four pups versus 27 animals >1 yr of age). While the age distribution was not reported by Zarnke et al. (1997), it is probable that the prevalence estimates for Alaska would be similar if adjusted for age. Additionally, different assay methods were used to measure antibodies against PhHV-1 in the two studies. Virus neutralization assays measure the amount of neutralizing antibody in a serum sample, whereas ELISA detect antibodies specific for the viral proteins (Flint et al., 2000). Therefore, our assay may have been more sensitive, resulting in a higher prevalence estimate.

Phocine herpesvirus-1 can be an important cause of illness and mortality in young

harbor seals housed in close contact and in stressful situations (Gulland et al., 1997; Harder et al., 1997; King et al., 2001) and may pose a threat to free-ranging seals if the population becomes stressed. An understanding of the epidemiology of PhHV-1 and an ability to interpret serologic data, therefore, are useful for monitoring the health status of harbor seal populations. Results of our study provide greater insight into the distribution of PhHV-1 in harbor seal populations. We speculate that it is possible for some newborn seals to be exposed in utero or during parturition if the mother has a reactivated infection during pregnancy, as happens with other types of herpesviruses (Whitley, 1983; Carmichael and Greene, 1990; Gaskell and Willoughby, 1999). Such in utero exposure and detection of maternal antibodies may explain the low prevalence of PhHV-1 specific antibodies measured in pre-weaned pups. Nonetheless, we think that the majority of pups probably become exposed to the virus around weaning when they are more mobile and interactive with adults or other pups that may be shedding the virus. This may explain the increase in seroprevalence we observed in weaned pups followed by the even greater prevalence in yearlings, subadults, and adults. In conclusion, prevalence of antibodies to PhHV-1 increases with age, and exposure is clearly widespread in North American populations, though the disease has evidently not had substantive population effects in these free-ranging harbor seals.

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